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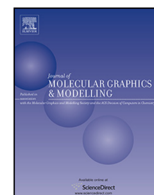

In silico designing of hyper-glycosylated analogs for the human coagulation factor IX

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ABSTRACT

N-glycosylation is a process during which a glycan moiety attaches to the asparagine residue in the N-glycosylation consensus sequence (Asn-Xxx-Ser/Thr), where Xxx can be any amino acid except proline. Introduction of a new N-glycosylation site into a protein backbone leads to its hyper-glycosylation, and may improve the protein properties such as solubility, folding, stability, and secretion. Glyco-engineering is an approach to facilitate the hyper-glycosylation of recombinant proteins by application of the site-directed mutagenesis methods. In this regard, selection of a suitable location on the surface of a protein for introduction of a new N-glycosylation site is a main concern. In this work, a computational approach was conducted to select suitable location(s) for introducing new N-glycosylation sites into the human coagulation factor IX (hFIX). With this aim, the first 45 residues of mature hFIX were explored to find out suitable positions for introducing either Asn or Ser/Thr residues, to create new N-glycosylation site(s). Our exploration lead to detection of five potential positions, for hyper-glycosylation. For each suggested position, an analog was defined and subjected for N-glycosylation efficiency prediction. After generation of three-dimensional structures, by homology-based modeling, the five designed analogs were examined by molecular dynamic (MD) simulations, to predict their stability levels and probable structural distortions caused by amino acid substitutions, relative to the native counterpart.

Three out of five suggested analogs, namely; E15T, K22N, and R37N, reached equilibration state with relatively constant Root Mean Square Deviation values. Additional analysis on the data obtained during MD simulations, lead us to conclude that, R37N is the only qualified analog with the most similar structure and dynamic behavior to that of the native counterpart, to be considered for further experimental investigations.

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1. Introduction

The human factor IX (hFIX or Christmas factor) is a vitamin K-dependent (VKD) plasma serine protease that plays a central role in the intrinsic pathway of blood coagulation [1]. It is synthesized by hepatocytes in the liver as a 461-amino acid (-aa) single-chain pre-pro-zymogen, containing a signal peptide (28 aa) and a pro-peptide (18 aa) on its N-terminal end [2]. During its biosynthesis, hFIX undergoes several post-translational modifications including; removal of the pre- and pro-peptides, VKD γ-carboxylation of 12 N-terminal glutamic acid residues, β-hydroxylation of Asp^{64}, and both N-linked and O-linked glycosylation [2,3], which are required for its normal activity. The resultant mature hFIX (M_r 57,000), comprises several functional and structural domains [2]. One of the major domains of hFIX is a γ-carboxyglutamic acid rich (Gla) domain (residues 1–45), which is a membrane-anchoring region with affinity to Ca^{2+} ions. In the presence of Ca^{+2} ions, Gla domain undergoes conformational changes and takes full and stable struc-

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ture and mediates an interaction between hFIX and phospholipid membrane [4,5]. The hFIX Gla domain is followed by two epidermal growth factor (EGF)–like domains (EGF1, residues 47–85 and EGF2, residues 86–127) and a 35-residue activation peptide region (residues 146–180). A serine protease (SP) domain (residues 181–415), consisting of two sub-domains, is located at the hFIX C-terminus [5,6]. Activation of hFIX is achieved by two proteolytic cleavages, mediated either by coagulation factor XIa or a complex of factor VII-tissue factor, during which an 11 KDa activation peptide is released and an active serine protease (FIXαβ, M_r 46,000) is generated. The activated hFIX (hFIXαβ) has two chains, an N-terminal light chain containing Gla, EGF1 and EGF2 domains and a C-terminal heavy chain, comprising SP domain, which are hold together via disulfide bond(s) [1,5,7].

Absence or malfunctioning of the hFIX results in an X-linked severe bleeding disorder, known as; hemophilia B or Christmas disease, the second most common type of hemophilia (after hemophilia A) with average incidence of one in 30,000 live male birth [8,9]. The hFIX-deficient patients are treated with infusion of purified human plasma-derived (pd) concentrate or recombinant hFIX (rhFIX) [8,10]. Although several high pure pd-hFIX are commercially available, there are still concerns over transmission of human blood borne pathogens, such as HIV and prions. Therefore, the pathogen-free rhFIX is preferable in this regard, compared to the pd-hFIX [8,10].

Since 1982, when the hFIX gene was first cloned, production of rhFIX in genetically engineered cells has been subjected for investigations in several laboratories throughout the world [8,11]. With several post-translational modifications, mostly necessary for its biological activity, this protein preferably requires to be expressed in mammalian cells [8,11]. Different mammalian expression systems, including Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells, which provide post-translational modifications similar to that of human cells, have been reported to be used for production of functional rhFIX [8,11,12].

N-glycosylation is a process that occurs on a majority of secretory and membrane proteins in eukaryotic cells. It is a co- or post-translational modification in which an N-glycan is added to Asn residue in the Asn-Xxx-Ser/Thr sequence where Xxx can be any amino acid except proline [13–15]. This process, carried out by an oligosaccharyl transferase (OST) complex within the lumen of endoplasmic reticulum, has major impacts on folding, stability, and secretion of the proteins [16,17]. Accordingly, N-glycosylation of recombinant proteins, in particular those with pharmaceutical applications, is among major concerns, when they are subjected for over-production.

Hyper-glycosylation is an aspect of Glyco-engineering approach for introducing new N-glycosylation site(s) into the protein of interest, for attachment of extra glycan(s) [17,18]. In addition to its impact on pharmacokinetics of the proteins, hype-glycosylation may enhance the protein secretion, via facilitating proper folding of the proteins, when occurs in either N-terminus or C-terminus of the proteins [17,19,20].

For proteins with known three-dimensional (3D) structure, selection of a site for hyper-glycosylation can be done in a rational manner. Whereas, for proteins with no X-ray crystallographic structure, it can be done randomly [17]. When hyper-glycosylation of a protein is aimed, the new N-glycosylation site should be introduced into both non-functional and non-structural regions within the solvent accessible surface areas of the folded protein. Besides, it should not interfere with receptor-ligand binding properties of the protein [20,21].

Previously the calcium-stabilized hFIX Gla domain has been crystalized [4]. The hFIX Gla domain comprises an N-terminal ω-loop followed by three short α-helixes (helix A: residues 14–19; helix B: residue 24–32 and helix C: residue 35–45) [4] (Fig. 1). The main objective of this study is to predict novel N-glycosylation sites on the surface of the 3D structure of the hFIX Gla domain using computational methodologies. This study was performed based on the available X-ray crystallographic structure of the hFIX Gla domain as template to predict suitable positions for introducing new N-glycosylation sites in a rational manner, for further hyper-glycosylation studies.

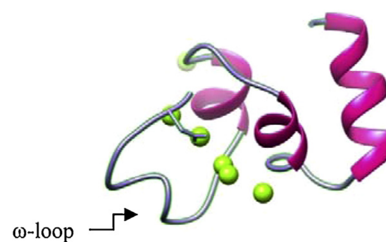

Fig. 1. Crystallographic structure of the hFIX Gla domain, (1NL0, chain G) visualized by Chimera [4].

2. Methods

2.1. Template selection

X-ray crystallographic structure of calcium–stabilized hFIX Gla domain, bound to a conformation-specific anti-hFIX with resolution of 2.2 A° [4], was retrieved from Protein Data Bank (PDB ID code: 1NL0) (http://www.rcsb.org/pdb/home/home.do) and used as template for comparative-based modeling of N-glycosylation analogs of the hFIX Gla domain.

2.2. Prediction of potential N-glycosylation sites

Selection of suitable locations for introduction of new N-glycosylation sites were carried out based on the 3D structural analysis of the hFIX Gla domain and available data concerning the non-structural and non-functional residues of hFIX [20]. The primary amino acid sequence of the hFIX was obtained from UniProt (UniProtKB: P00740) and the first 45 residues from its mature form, corresponding to the Gla domain was chosen for further analysis. In order to design alternative N-glycosylation sites, single amino acid substitutions were carried out to introduce either Asn or Ser/Thr. All the analogs were evaluated for N-glycosylation efficiency using NetNGlyc 1.0 server software (http://www.cbs.dtu.dk/services/NetNGlyc/) [22] and then subjected to 3D structure generation using homology modeling.

2.3. Homology (comparative)-based modeling and validations

3D structures of N-glycosylation analogs were generated using MODELLER 9v10 software [23]. X-ray crystallographic structure of the hFIX Gla domain (1NL0, chain G) was used as template. Align2d command in the MODELLER program was used to align the target sequence with the template structure [23]. The template contains non-protein residues includes; γ-carboxyglutamic acids (CGU) and Ca^{2+} ions, which are considered as heteroatoms (HETATMs) in the PDB file. So, the align2d output file was modified and BLK ('.') residues were used in both the template and target sequence in order to copy the HETATMs as a rigid body into the model. Then the output file was used as an input data at the model generation step. At this step, *env.io.hetatm* was set to True (http://salilab.org/modeller/manual/node18.html).

About 10,000 models were generated for each target sequence and a model with the lowest value of the Probability Density Function (PDF) was taken as the final comparative protein model.

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