# Conformational Comparability of Factor IX–Fc Fusion Protein, Factor IX, and Purified Fc Fragment in the Absence and Presence of Calcium

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**ABSTRACT:** A long lasting recombinant factor IX –Fc fusion protein (rFIX–Fc) is being developed for the treatment of hemophilia B and is currently in late stage clinical investigation. By limiting injection frequency and maintaining efficacy, rFIX–Fc shows promise as a new therapeutic option for hemophilia B patients. However, before gaining regulatory approval, rFIX–Fc must undergo rigorous analytical and biological testing, in addition to clinical trials. Included in this testing is the need to understand this protein's higher-order structure and dynamics. In this study, we investigated and compared the biophysical properties of rFIX–Fc, rFIX, and Fc using hydrogen/deuterium exchange mass spectrometry and differential scanning calorimetry. Within the limits of these techniques, our results show that structural comparability exists between rFIX and the FIX region of rFIX–Fc. In addition, changes in the structure and dynamics of both proteins, in response to calcium binding, a requirement for FIX function, are also highly comparable. In the case of Fc and Fc region of rFIX–Fc, conformational comparability is also established. These biophysical results further support the conclusion that fusing an immunoglobulin gamma 1 Fc to rFIX does not significantly alter the higher-order structure of FIX or Fc, Ca binding to FIX, or Fc functionality. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:1688–1700, 2012

**Keywords:** hydrogen/deuterium exchange; mass spectrometry; calorimetry (DSC); coagulation factors; calcium binding; fusion protein; biotechnology; protein binding; protein structure

## INTRODUCTION

Biological drugs produced from recombinant technologies have played essential roles in combating disease for the last several decades.[1,2] Although most biopharmaceutical products are recombinant derivatives of naturally occurring proteins, recombinant proteins with "nonnative" sequence configurations or chemical conjugates have also been implemented as successful biotherapeutic alternatives.[3,4] These "nonnative" proteins are predominantly humanized chimeric monoclonal antibodies, which combine sequences from rodent or other animals directly with human sequences. Other examples of "nonnative" protein biopharmaceuticals are the recombinant fusion of different gene products with diverse functions into a single protein. These are frequently referred to as fusion proteins.[5,6] Those fusion proteins that involve the coupling of an immunoglobulin IgG1 constant region from the hinge and below termed "fragment crystallizable" (Fc) to a native protein offer a specific advantage over the traditional biopharmaceutical drugs. The advantage offered in these cases includes longer circulation life (which may ultimately limit the need for reoccurring treatments) and/or provide additional functionality that further improves

**Abbreviations used:** H/DX-MS, hydrogen–deuterium exchange mass spectrometry; DSC, differential scanning calorimetry; WCX, weak cation exchange chromatography; SEC, size-exclusion chromatography; rFIX, recombinant factor IX; Fc, IgG1 constant region below the hinge termed "fragment crystallizable"; TIC, total ion current.

Additional Supporting Information may be found in the online version of this article. Supporting Information

In this work, we interchangeably refer to protein backbone dynamics (i.e., the rate and amount of peptide backbone amide H/DX) as dynamics, conformational dynamics, structural dynamics, conformational flexibility, and structural flexibility.

The ordering of each peptide is based on its midpoint, see Ref. 30 (Houde et al.) for a more detailed description on how midpoints are determined and used.

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their therapeutic value. A successful example of such engineering is Etanercept, a soluble tumor necrosis factor receptor 2 expressed with the Fc of a human immunoglobulin gamma 1 (IgG1), used in the treatment of rheumatoid arthritis.[5] In developing such a fusion protein, the impact of chemical complexion and the juxtapose positioning of these two individual proteins on their higher-order structure needs to be assessed. Work in this paper focuses on showing conformational and therefore functional comparability, using the concept of structure–function, of a commercial recombinant factor IX (rFIX) with the FIX part of an innovative and promising recombinant fusion protein, factor IX–Fc (rFIX–Fc), which is being evaluated clinically for the treatment of the blood clotting disorder hemophilia B.[7] In addition, we also show the conformational comparability of the purified Fc fragment from rFIX–Fc with the Fc part of rFIX–Fc.

Hemophilia is caused by lack of or mutations in protein components of the blood coagulation pathway.[8] Following trauma, the blood coagulation cascade involves specific activation of a series of serine proteases. These enzymes form complexes, activate, and subsequently catalyze additional reactions to ultimately activate factor X, prothrombin, and fibrinogen.[9,10] In these complex series of proteolytic activations and interactions between blood components, a lack of or mutations in any one component (e.g., FIX or FVIII) could decrease or completely prevent formation of a fibrin clot leading to prolonged bleeding, internal tissue damage, and eventually death.[11,12] Hemophilia B is caused when aberrations exist in the FIX gene. Although not very common (approximately 1 in 25,000 male births[13,14]), deficiencies in FIX have been shown to disrupt the normal intrinsic blood clotting cascade, essentially inhibiting hemostasis.[11,15,16] Currently, the main treatment for hemophilia B is FIX replacement therapy (using either recombinant or plasma-derived FIX), which may involve regular intravenous infusions to prevent serious bleeding episodes.[15] Although this treatment regime works, more effective FIX drug products that last longer, thus reducing the number injections and potential side effects, are highly desirable. One potential candidate is the fusion protein rFIX–Fc, which was shown to possess a longer duration of action in several animal models compared with rFIX. This longer life is facilitated by the binding of the Fc unit present in rFIX–Fc to the neonatal Fc receptor (FcRn).[7]

Although rFIX–Fc data indicate significant benefit over present therapeutic options for hemophilia B patients, before gaining regulatory approval, detailed testing and characterization of rFIX–Fc (in term of its chemistry, biology, and biophysical properties) must be carried out, in addition to proving clinical efficacy. A particularly critical area where testing and characterization work on rFIX–Fc is required concerns the analysis of its higher-order structure. As the structure of a protein dictates its function,[17,18] small changes in a protein's conformation can have disruptive consequences on its function and/or safety. Hence, investigating the higher-order structure of a protein biopharmaceutical is essential for understanding its function, in assessing reproducibility of manufacturing, and ensuring its safety.

Both human FIX and rFIX–Fc are glycoproteins composed of an N-terminal γ-carboxyglutamic acid (Gla) domain, two epidermal growth factor (EGF)-like domains, and a C-terminal catalytic or serine protease domain.[19] However, unlike rFIX, rFIX–Fc has an additional component, an IgG1 Fc recombinantly attached to the C-terminal end of the FIX catalytic domain. This recombinant fusion results in an asymmetric molecule containing a single FIX molecule attached to the $C_H2$ domain on one side of the intact IgG1 Fc homodimer (IgG1 Fc is composed of two $C_H2$ and two $C_H3$ domains). For activation of FIX and the FIX part of rFIX–Fc, both require the binding of calcium ($Ca^{2+}$) ions, which interacts (with differential affinities) to the Gla, EGF, and serine protease domains. [20–24] For rFIX, an array of different methods has been used to probe the structure of various rFIX domains and changes to the structure of these domains as a result of $Ca^{2+}$ binding. These methods include amino acid substitution,[25] X-ray crystallography,[26] nuclear magnetic resonance,[27] spectroscopy, and calorimetry.[23,28] However, to our knowledge, detailed characterization of the structure and structural dynamics of an intact rFIX–Fc fusion protein, or for that matter rFIX, in solution with or without $Ca^{2+}$ is limited. In addition, the structural comparison between rFIX and the FIX region of the fusion protein rFIX–Fc is scarce,[23] as is the comparison between Fc and the Fc region of rFIX–Fc.

In this work, we were interested in comparing and characterizing the solution conformation and protein backbone dynamics (see Footnote 1 on title page) of rFIX and Fc with rFIX–Fc. In doing so, we asked the following set of questions. (1) Does the coupling of Fc to FIX influence the conformation or structural dynamics of FIX? (2) Does $Ca^{2+}$ affect the solution conformation and/or conformational dynamics of the FIX region of rFIX–Fc as it does in rFIX? (3) Does the coupling of rFIX to Fc influence the Fc conformation or structural dynamics of Fc? To answer these questions, we studied and compared rFIX–Fc, rFIX, and Fc fragment (isolated from rFIX–Fc) with and without $Ca^{2+}$ using hydrogen/deuterium exchange mass spectrometry (H/DX-MS),[29] an emerging biophysical tool that is gaining popularity for investigating the conformation of protein biopharmaceuticals, [30–33] and differential scanning calorimetry (DSC), a robust and routinely used biophysical tool for characterizing the