Selective Domain Stabilization as a Strategy to Reduce Human Serum Albumin–Human Granulocyte Colony Stimulating Factor Aggregation Rate

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Received 11 December 2011; revised 5 February 2012; accepted 14 February 2012

Published online 15 March 2012 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23118

ABSTRACT: Therapeutic proteins must be generally formulated to reduce unwanted aggregation. Fusion proteins, which comprise domains assembled from separate proteins, may require unique formulation strategies in order to maximize their stability. A fusion protein of human serum albumin (HSA) and human granulocyte colony stimulating factor (GCSF; HSA–GCSF) was used as a model to test the hypothesis that formulations that increase the thermodynamic conformational stability of the least stable domain of a fusion protein will stabilize the entire fusion protein against aggregation. Conformational stability of HSA–GCSF was modulated by addition of octanoic acid, which was previously shown to increase the conformational stability of HSA, the least stable domain. Contrary to our hypothesis, increased conformational stability of the HSA domain did not result in increased resistance to aggregation of HSA–GCSF. These results for HSA–GCSF were also compared with similar studies conducted previously on a therapeutic protein formed by the fusion of HSA and human growth hormone (hGH; HSA–hGH). © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:2009–2016, 2012

Keywords: fusion protein; HSA; GCSF; aggregation; formulation; octanoic acid; conformational stability; colloidal stability

INTRODUCTION

The development of recombinant therapeutic proteins has provided new treatments for many serious conditions, including endogenous protein deficiencies, cancer, and autoimmune disorders.[1] In order to be successful drug candidates, these complex molecules require stabilization by formulation excipients so that degradation rates are minimized from manufacturing through transportation to administration to patients.[2,3] Both chemical and physical instabilities of therapeutic proteins have the potential to negatively impact product quality[3]; this work focuses on the aggregation, the most commonly observed physical instability. Much work has been carried out to investigate the stability and aggregation behaviors of protein therapeutics.[3,4,5,6] Aggregation of protein products is a concern for several reasons. Aggregation can lead to loss of product during manufacture, storage and, shipping.[7,8] Moreover, if aggregates are administered to patients, they may trigger patient immune responses.[9,10,11] These potential adverse immune responses include anaphylactic shock and the production of anti-drug antibodies, which can increase drug clearance and potentially cross-react with endogenous protein.[12,13]

Aggregation rates can be modulated by both a protein's conformational stability and its colloidal stability in solution.[8] Conformational stability refers to the thermodynamic stability of the protein's proper three-dimensional folded structure, whereas colloidal stability refers to the energetics of protein–protein self-interactions between molecules. Protein–protein interactions are repulsive in colloidally stable systems. For the purposes of this report, we use the free

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Journal of Pharmaceutical Sciences, Vol. 101, 2009–2016 (2012)

energy of unfolding, $\Delta G_{NU,}$ for a fusion protein of human serum albumin and human granulocyte colony stimulating factor (HSA–GCSF) as a measure of its conformational stability, and the osmotic second virial coefficient, B_{22}, for HSA–GCSF as a measure of its colloidal stability.[8]

Granulocyte colony stimulating factor is an important therapeutic and is used to increase the production of white blood cells in patients undergoing chemotherapy.[14] Its propensity to aggregate has been extensively studied and characterized. Previous studies[4] showed that the rate of aggregation of 1.5 mg/mL GCSF in solution at near-physiological conditions [pH 6.9 in phosphate-buffered saline (PBS), 37°C] is rapid, with a reaction rate of 7.3 ± 0.6 μmol/(L day) and an apparent reaction order that is second order in protein concentration. However, an apparent first-order dependency on protein concentration has also been observed at GCSF concentrations greater than 2.5 mg/mL [pH 7.0, 0.1 M 3-(N-morpholino)propanesulfonic acid],[15] with aggregation under these conditions involving a conformationally altered monomer state.[14] Both conformational and colloidal instabilities play a role in the aggregation of GCSF under varying solution conditions.[16] Addition of sucrose, a molecule that is preferentially excluded from the surface of proteins,[17] increases the conformational stability of GCSF and reduces its rate of aggregation.[4] Furthermore, because of this stabilizing effect, sucrose partially counteracts the acceleration of GCSF aggregation caused by benzyl alcohol.[18,19] Protein–protein interactions that impact the colloidal stability of proteins can be modified by the choice of solution pH. GCSF is more colloidally stable at pH 3.5 than at pH 7.0 (i.e., protein–protein interactions are more repulsive), and aggregates much less rapidly at pH 3.5,[4] even when the tertiary structure of protein is perturbed by benzyl alcohol.[19] Increasing the formulation ionic strength, which screens repulsive protein–protein electrostatic interactions at pH 3.5, results in an increased aggregation rate.[16]

Human granulocyte colony stimulating factor aggregation is of interest, given a current trend for development of biopharmaceutical products with increased patient convenience and compliance. These efforts include the development of strategies to increase the circulation half-life of the drug product and thus reduce the administration frequency.[20,21] One method of increasing the circulation half-life is to create a fusion protein, coexpressing the drug molecule with another protein such as the Fc domain of an antibody[22] or HSA.[21,23] HSA–GCSF has been developed to increase the circulation half-life of GCSF,[24] and this fusion protein is the focus of the current research.

There are added stability challenges involved in the formulation of fusion proteins. Because the individual fusion domains did not coevolve, they may lack built-in interdomain interactions that contribute favorably to native-state stability. Also, solution conditions that stabilize one domain may not adequately stabilize the other domain(s) of the fusion protein. However, because aggregation of proteins generally thought to result from their (partial) unfolding,[8,25] we hypothesized that the aggregation rate of fusion proteins can be reduced by increasing the conformational stability of the least stable domain.[26] In the case of HSA fusion proteins, the addition of octanoic acid, an HSA ligand, is one potential strategy to selectively stabilize the HSA domain.[8,17,27] HSA was chosen as the target for selective domain stabilization because it has a lower ΔG_{NU} value than GCSF (ΔG_{NU} for HSA is approximately 22.0 ± 0.5 kJ/mol, compared with 39.7 ± 2.1 kJ/mol for GCSF),[16,28] and is thus presumed to be the least conformationally stable domain in HSA–GCSF. In addition, we hypothesized that the addition of the less thermodynamically stable HSA domain will increase the aggregation rate for the resulting fusion protein as compared with that for GCSF alone. To test these hypotheses, the aggregation rates, aggregation reaction orders, and stability behavior of HSA–GCSF with and without selective domain stabilization were investigated and compared with the aggregation rates of both GCSF[4,16] and another HSA fusion, HSA–human growth hormone (hGH).[29] Conformational stability was investigated using chaotrope-induced and thermally induced denaturation, whereas colloidal stability was determined by static light scattering and zeta potential measurements. Octanoic acid was used as a small molecule ligand for the stabilization of HSA.[30,31]

MATERIALS AND METHODS

Stock Protein Preparation

Human serum albumin–GCSF was donated by Teva Biopharmaceuticals (Rockville, Maryland) and stored frozen at–80°C. For experimentation, HSA–GCSF was thawed and dialyzed into 10 mM sodium phosphate buffer (pH 7.0). For experiments where sodium chloride was added, HSA–GCSF was dialyzed into 10 mM phosphate, 150 mM NaCl buffer (pH 7.0; PBS). The concentration of HSA–GCSF after dialysis was determined by absorbance at 280 nm, using a theoretical extinction coefficient of 0.6 cm^2/mg. For experiments wherein the stabilizing effect of a binding ligand was tested, octanoic acid was added to the protein sample in 10 mM sodium phosphate buffer, pH 7.0, to a final concentration of 0.5 mM octanoic acid.