Selective Domain Stabilization as a Strategy to Reduce Fusion Protein Aggregation

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ABSTRACT: A human serum albumin–human growth hormone (HSA–hGH) fusion protein was used as a model to understand the contributions of individual domains to the aggregation behavior of the overall fusion protein. Aggregation of HSA–hGH was studied at two different pH conditions, pH 5 and pH 7. Conformational stability of the HSA domain was modulated by addition of octanoic acid, a binding ligand. Conformational stability of the fusion protein and the HSA domain were determined from experimentally measured values for free energies of unfolding (ΔG_{unf}) with midpoint of apparent unfolding temperatures (T_m) used as surrogate in some cases. Apparent T_ms of both HSA and HSA–hGH were increased by octanoic acid binding. Osmotic second virial coefficients were measured to monitor protein–protein interactions in solution. Reductions in rates of aggregation were observed under solution conditions that increased protein–protein repulsive interactions even when no changes in conformational stability were detected. The results indicate that colloidal instabilities are responsible for HSA–hGH aggregation and that conformational stability of the HSA domain does not play a dominant role in the aggregation of HSA–hGH. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:1400–1409, 2012

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INTRODUCTION

Fusion proteins are a growing class of protein therapeutics.[1] These are molecules that combine unrelated proteins, or domains from unrelated proteins, to create a new therapeutic protein. Etanercept, romiplostim, abatacept, and rilonacept are all examples of current US Food and Drug Administration-approved fusion proteins, and there are others in late-stage clinical trials.[2] Compared with other protein therapeutics, there can be several benefits of fusion proteins, such as extended serum half-life[3,4] or added functionality.[5] Despite these benefits, there are also inherent challenges in creating stable formulations of fusion proteins. The increasing numbers of fusion proteins in development make it desirable to understand and improve fusion protein formulations.

As with other therapeutic proteins, fusion proteins are susceptible to instabilities such as a propensity to aggregate, which can negatively impact production and product quality.[6] Aggregation has been implicated in causing adverse immune responses in patients.[7–9] Aggregation can also cause loss of protein during manufacture, transportation, and storage,[10,11] leading to decreased product yields and profits.[12]

The Lumry–Eyring model has been extensively used as the basis for the understanding of protein aggregation.[11,13,14] Roberts et al.[15,16] have described an extended model of non-native protein aggregation composed of six steps, although any specific protein need not go through all steps in order to form aggregates. The initial step involves a conformational change to form an aggregation-competent species and the second step involves the association of aggregation-competent monomers to form a reversible aggregate. Later steps describe further conformational changes that result in irreversibility of

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

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the initial aggregate and then growth to larger aggregates. The two initial steps are essentially the Lumry–Eyring framework of protein aggregation, which may be depicted schematically as[11,13,15]

1 $N \leftrightarrow N^*$
2 $2N^* \leftrightarrow A_2$

where N^* is an aggregation-competent conformation of the native protein and A is the initial aggregate. The rate of the initial step is impacted by the conformational stability of the protein, which is measured experimentally as the free energy of unfolding (ΔG_{unf}); larger ΔG_{unf} values indicate proteins or protein domains with increased conformational stability and thus lower equilibrium populations[11] of aggregation-competent species N^*.

The rate constant for the association step is affected by the energetics of protein–protein interactions, that is, colloidal stability. Experimentally, osmotic second virial coefficient (SVC) values (B_{22}) are used to reflect the net contribution of all protein–protein interactions (e.g., hard sphere, electrostatic, van der Waals). Positive B_{22} values indicate that protein–protein self-interactions are repulsive, whereas negative B_{22} values indicate that self-interactions are attractive. Either the unfolding or association step can be rate limiting in the formation of initial aggregates,[17] depending on the solution conditions. Both steps are potential targets for strategies to reduce protein aggregation. For example, excipients that increase ΔG_{unf} may be added and solution conditions such as pH may be adjusted to increase repulsive protein–protein interactions.[17]

Fusion proteins face an additional set of unique stability challenges that can contribute to their propensity to aggregate. Unlike naturally occurring multidomain proteins, the individual domains in fusion proteins have not coevolved for stability and may lack stabilizing intradomain interactions, thus reducing ΔG_{unf}. Formulation conditions that favor conformational stability of one domain may not adequately stabilize other domains.[18] In addition, under solution conditions where the domains have different net charges, large dipoles may be created, adding additional attractive protein–protein interactions and colloidally destabilizing the protein solution.[18]

Previous work by our group on an Fc fusion protein showed increases in aggregation rates that correlated with decreased domain conformational stability.[18] The model protein for those studies was Fc–human cytotoxic T-lymphocyte associated factor (CTLA-4), an immunoglobulin G Fc domain fused with the extracellular domain of CTLA-4. During accelerated stability studies, Fc–CTLA-4 exhibited markedly different aggregation rates with only a small shift in pH. Conditions that increased aggregation also reduced the conformational stability of the CTLA-4 domain and the C_H2 region of the Fc domain. Thermally and chaotrope-induced denaturation studies showed that these two domains were the least conformationally stable of the protein's domains, leading to the conclusion that domain conformational instability was the primary driving force for Fc–CTLA-4 aggregation.

These previous findings now lead us to develop two hypotheses regarding fusion protein behavior. We hypothesize that the overall stability and aggregation behavior of multi-domain proteins can be controlled by choosing formulation conditions that favor the stability of the least conformationally stable domain and that selective stabilization of this domain will reduce overall aggregation rates of the entire fusion protein. In this work, we use a human serum albumin–human growth hormone (HSA–hGH) fusion protein as a model system to determine the impact of domain stability on overall protein stability.

To test our hypotheses, we measured the conformational stabilities of the least conformationally stable domain and the complete fusion protein. In addition, we measured B_{22} values for the fusion protein. During these studies, cosolute addition was investigated as a way to achieve selective domain conformational stabilization through the preferential binding of the cosolutes to the native state.[11] HSA is the least conformationally stable domain in the thermodynamic sense because it has a lower free energy of unfolding than hGH. The free energy of unfolding for hGH has been reported as 60.7 ± 4.2 and 62.3 kJ/mol at pH 7.5[19,20] and 62.3 kJ/mol at pH 6.[21] These relatively high stabilities can be compared with 17.2 ± 4.2 kJ/mol at pH 7.4 and 14.6 ± 1.3 kJ/mol at pH 5.3 for HSA, as reported by Farruggia and Pico.[22] On the basis of these data, the HSA domain was chosen as the target for selective stabilization. Additionally, because there are other HSA fusion proteins that have either been patented or commercially developed to varying degrees,[25,26] this approach has the potential to be useful on a platform level. Conformational and colloidal stability of the fusion protein with the cosolute were measured to determine cosolute influence on overall protein stability.

Octanoic acid was used as a cosolute to selectively stabilize the HSA domain. Although HSA binds long-chain fatty acids with a higher affinity than octanoic acid,[27,28] octanoic acid was chosen for its historical role as a stabilizer during heat treatment of HSA[29,30] and for its higher solubility as compared with long-chain fatty acids.[30] Most of the association constants[28,29,31] that have been measured under solution conditions similar to those used in the present study are of the order of 10^6 M^{-1}, although a binding constant of 2.6×10^4 M^{-1} has also been reported.[27] Binding of octanoic acid to HSA is consistent with a single binding site in the HSA subdomain IIIA,[28,29]