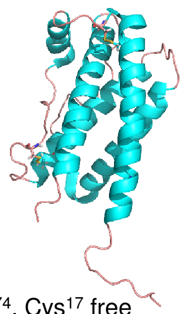
Kinetic Pathway Analysis of Aggregation of Therapeutic Proteins

ULRICH ROESSL[1,2], J. WIESBAUER[1,2], S. LEITGEB[1,2], B. NIDETZKY[2]
[1] Research Center Pharmaceutical Engineering GmbH, Graz, Austria
[2] Institute of Biotechnology and Biochemical Engineering, University of Technology Graz, Graz, Austria

Introduction

Protein aggregation represents probably the most common and troubling manifestation of protein instability. Shelf life of therapeutic proteins is particularly impaired by aggregation, which can occur almost in all phases of protein drug development. Administration of protein aggregates may lead to reduced pharmacological activity and adverse drug reactions. Thus, prediction of protein aggregation is of major interest for pharmaceutical industry. Existing sequence based bioinformatic tools are barely able to predict the aggregation behavior of therapeutic proteins. To implement new approaches for this, detailed analysis of protein aggregation, its underlying mechanisms and its kinetics is necessary.

Granulocyte Colony Stimulating Factor (G-CSF)



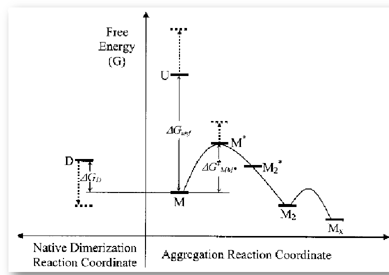
- Differentiation of precursor cells to neutrophils, proliferation of neutrophil colonies, stimulating activity of mature neutrophils
- IL-6 superfamily
- 19.6 kDa, 174 residues
- Two disulfide Bonds: Cys[36]-Cys[42] and Cys[64]-Cys[74], Cys[17] free

Typical four-α-helix bundle motif

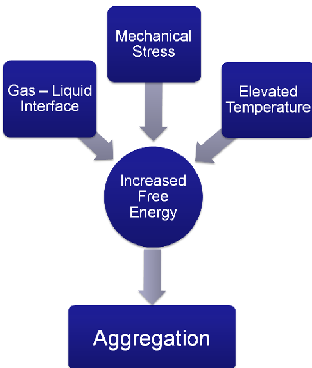
Hill et al. *PNAS* (1993) 90, 5167-5171
Zink et al. *Biochem* (1994) 33, 8453-8463

Aggregation

A hypothetical aggregation profile of our model protein G-CSF proposes aggregation via structural expanded transition state species (M*) to dimers (M_2) and multimeric aggregates (M_x)

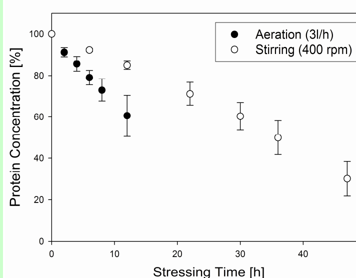


Chi et al. *Protein Sci* (2003) 12, 903-913



Free energy increases during processing and storage. In oder to mimic process conditions we exposed G-CSF to gas-liquid interfaces, mechanical stress and elevated temperature. Bubble aeration occurred with compressed air at a flow rate of 3 liters per hour. Stirring was performed with a magnetic stir bar (30x6mm) at 400 rpm. G-CSF was used in 10 mM glutamate buffer, pH 4.4, containing 5 % (w/v) sorbitol.

Loss of soluble G-CSF



The content of soluble G-CSF was determined with UV-spectroscopy at 280 nm. Despite the lack of visible precipitate bubble aerated samples showed accelerated protein loss compared to stirred ones.

Fig 4: Amount of soluble G-CSF in bubble aerated and stirred samples (Mean ± SD).

Soluble Aggregates and Precipitates

Exposure of G-CSF to 37 C did not lead to aggregation in any of our experiments. With SDS-PAGE no increased aggregation was found. However, stirred and aerated samples showed many distinct aggregate bands. Reduction with dithiothreitol (DTT) did only lead to a partial disintegration of aggregate bands. Formation of intermolecular disulfide bonds might be involved in the aggregation process.

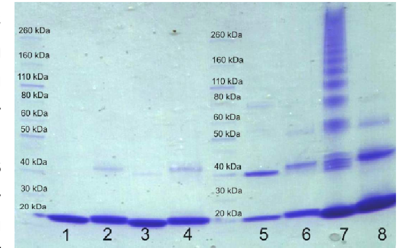


Fig 1: SDS-PAGE of stressed samples. Lane 1+2: unstressed G-CSF, Lane 3+4: 7 days at 37°C, Lane 5+6: 12 hours bubble aeration, Lane 7+8: 47 hours stirring; all samples in even lanes were reduced with DTT

The fraction of soluble aggregates compared to soluble monomer was determined using size exclusion HPLC (SEC). Bubble aeration caused drastic increase of soluble aggregates to more than 40 % after 12 hours. Stirring only led to max. 3 %, even after 47 hours.

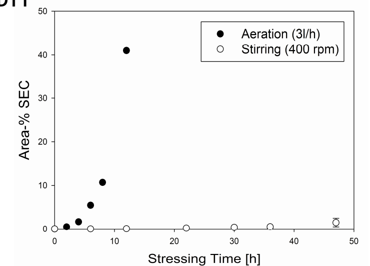
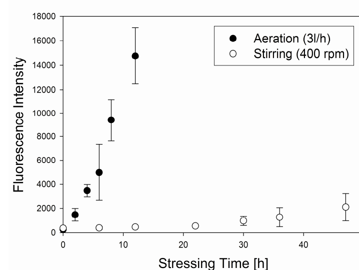


Fig 2: Fractions of soluble aggregates found with SEC in stirred samples (Mean ± SD) and bubble aerated samples (Single Experiment)

Exposure of Hydrophobic Domains



1-anilinonaphthalene-8-sulfonic acid (ANS) displays increased fluorescence in hydrophobic environment. Elevated fluorescence activity in aerated samples suggests more structural perturbance through air-liquid interface exposure than after stirring.

Fig 3: ANS fluorescence in bubble aerated and stirred samples (Mean ± SD).

Conclusions and Objectives

- G-CSF in glutamate buffer, pH 4.4 is stable at 37 C for at least one week
- Air-liquid interfaces lead to faster exposure of hydrophobic domains, decrease of soluble protein and formation of soluble aggregates, despite less precipitation than with stirring
  → **Process kinetic analysis**
- Mass spectrometry to elicit involvement of intermolecular disulfide bonds
- Monitoring of precipitation using Confocal Laser Scanning Microscopy

FFG   Das Land Steiermark   SFG Steirische Wirtschaftsförderung

K1 Competence Center - Initiated by the Federal Ministry of Economy, Family and Youth (BMWFJ) and the Federal Ministry of Transport, Innovation and Technology (BMVIT). Funded by FFG, Land Steiermark and Steirische Wirtschaftsförderung (SFG).

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