Pathway to Insoluble Aggregates on the Refolding of a Single-chain Fv Antibody: Morphological Changes of Aggregated Protein on Refolding

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In vitro aggregated single-chain Fv antibody (scFv) was analyzed by attenuated total reflectance Fourier transform infrared spectra and scanning electron microscope images. The spectroscopic results demonstrate that the formation of β-strands is a first trigger to the off-pathway toward insoluble aggregates, which is independent of redox condition; however, the morphology of the insoluble aggregates is strongly influenced by the environment in which scFv is aggregated.

Appropriate steric structure of protein is essential to its activity, but the formation process is so complicated that the folding to the appropriate form is hard in vitro and even in vivo.

Single-chain antibodies (scFvs) are a key protein used for diagnostics in medicine and in various studies in the field of proteomics.[1] Expression of scFv in *Escherichia (E.) coli*, however, is usually limited to the periplasm and often leads to insoluble aggregate formation. Studies on folding of the variable fragment of antibody molecules have suggested that formation of a disulfide bond linking two β sheets in the lightchain and heavy-chain domains is critical to stabilizing the immunoglobulin fold structure;[2] that is, reducing conditions in the bacterial cytoplasm may interfere with correct folding by preventing the formation of disulfide linkages. In this study, we show that the primary amino acid sequence of the singlechain antibody strongly drives the formation of an amount of β-strand structure comparable to that in the native form, regardless of redox conditions, but also that the gross morphology of aggregated single-chain antibody depends on the refolding environment. Here, we investigated the aggregation of scFv during refolding in vitro and in vivo in terms of a nucleation mechanism.

Figure 1 shows the attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectra in the amide I region for the scFv aggregates formed in vitro from a denatured, reduced form by a dialysis from 6 M guanidine hydrochloride (GdnHCl) to a denaturant-free solution at 4 °C (solid line) and 80 °C (dotted line). The IR spectra for the in-vitro scFv aggregates formed at 4 and 80 °C showed a maximum at 1625 and 1622 cm^{-1}, respectively, implying, according to previous reports,[3,4] that its secondary structure is rich in β structure. The deconvolution analysis by Gaussian–Lorentzian functions (GRAMS/32 V5.0, Galactic, USA) revealed that the relative amount of the band around 1625 cm^{-1} was comparable to that of β-strand in native form determined by X-ray crystallography (Table 1).[5] Considering that scFv formed the β-strands even at 80 °C, these results indicate that the amino acid sequence of the scFv results in the comparable amount of β-strands in the aggregated and native forms.

Further, we formed aggregated scFv in vitro from the denatured, reduced form by the dialysis to a denaturant-free solution with a 50-fold molar excess of oxidized form of glutathione (GSSG) or β-mercaptoethanol (β-ME) relative to the protein solution at 4 °C (dashed and chained lines in Figure 1, respectively). However, the FT-IR spectra were similar to those of the in-vitro aggregates formed in the denaturant-free solution without redox reagents (Figure 1). This suggests that the formation of β-strand is independent of disulfide linkages. Recently, we attained a highly efficient refolding yield for scFv in a stepwise dialysis where disulfide exchange and suppression of protein aggregates were controlled by the addition of GSSG and L-arginine at appropriate stages.[6] Interestingly, use of the additives-introduced stepwise dialysis to the refolding of scFv at 80 °C resulted in no insoluble aggregates; instead, the circular dichroism (CD) spectrum of soluble scFv showed a random structure (dotted line in Figure 2). The soluble random scFv was not precipitated for a

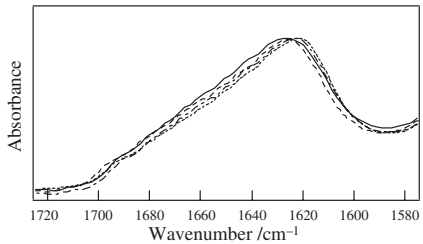

Figure 1. FT-IR spectrum for the scFv aggregates formed from a denatured, reduced form. ScFv was aggregated in vitro by dialysis from 6 M GdnHCl to a denaturant-free solution with no redox reagents at 4 °C (solid line) and 80 °C (dotted line), or to a denaturant-free solution with GSSG (dashed line) or β-Me (chained line) at 50-fold molar excess relative to the scFv concentration (7.5 μM) at 4 °C.

Table 1. Peak positions and relative areas[a] of the fitting components in the FT-IR spectra for the in vitro and in vivo aggregated scFv

Secondary Structure	Turn	α Helix/ Irregular	Random Coil	β Structure
in vitro agg.	1676 cm^{-1}	1662 cm^{-1}	1649 cm^{-1}	1625 cm^{-1}
formed at 4 °C[b]	18%	2%	31%	49%
in vitro agg.	1672 cm^{-1}	1662 cm^{-1}	1650 cm^{-1}	1622 cm^{-1}
formed at 80 °C[b]	15%	4%	26%	55%
Inclusion bodies	1680 cm^{-1}	1661 cm^{-1}	1647 cm^{-1}	1625 cm^{-1}
	18%	15%	16%	51%

[a]The relative areas were estimated with only the bands from a backbone structure.
[b]The aggregates were formed by a direct dialysis from 6 M GdnHCl solution to a denaturant- and redox-reagent free solution (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA).

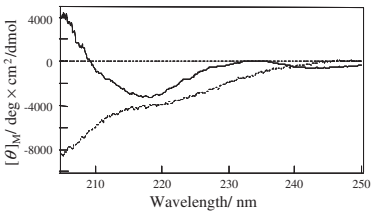

Figure 2. CD spectra for the scFv refolded by the additive-induced stepwise dialysis procedure at 4 °C (solid line) and 80 °C (dotted line).

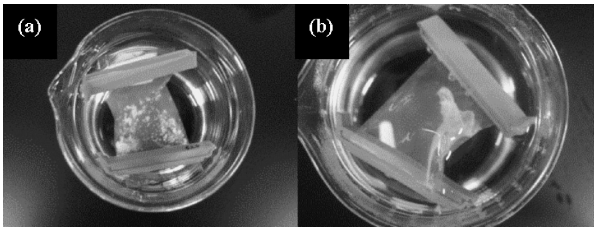

Figure 3. Overall appearance of in vitro aggregated scFv formed by direct dialysis from 6 M GdnHCl to a denaturant-free solution at 4 °C (a) and 80 °C (b). No redox reagents are in the denaturant-free solution.

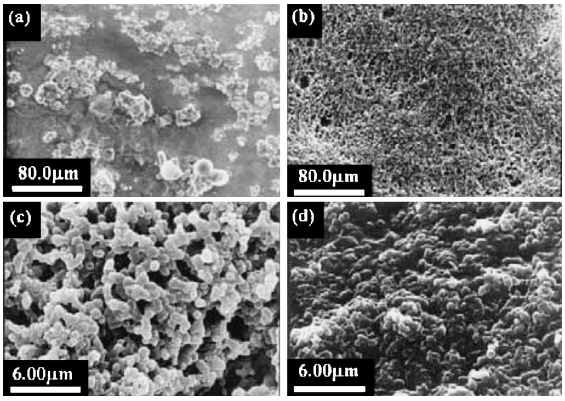

Figure 4. SEM images for the in vitro aggregated scFv formed from a denatured and reduced form by a dialysis from 6 M GdnHCl to a denaturant-free solution at 4 °C (a) and 80 °C (b,c), and scFv inclusion bodies (d).

week. L-Arginine suppresses the formation of insoluble aggregates by inhibition of hydrophobic interaction but without causing protein denaturation.[6,7] Considering that soluble scFv lacking β-strand formed no insoluble aggregates even after transfer to a L-arginine-free solution, the formation of β-strand might trigger the nucleation that leads to insoluble aggregates.

Internal disulfide linkages promotes the refolding of the immunoglobulin-fold,[2,8] and the formation of native-like β-strand leads to a correct disulfide bond formation because it enables a free thiol group to approach to the appropriate cysteinyl residue.[6] Here, we observed that β-strands begin to be formed independently of disulfide linkage, suggesting that a rapid formation of β-strand makes it hard to bring native pair of thiol groups close to each other.

The amount of β-strand in the insoluble scFv aggregates was not changed by temperature and redox condition (Table 1); however, the morphology was affected by temperature. During dialysis from 6 M GdnHCl to a denaturant-free solution, scFv formed dispersed particles at 4 °C (Figure 3a), and a large sheet was formed at 80 °C (Figure 3b). The morphology was not affected by the presence of redox reagent in the outer dialysis solution. Figure 4 shows the SEM images for in vitro aggregated scFv. The scFv aggregated at 4 °C formed dispersing large clumps with heterogeneous diameter of 10–700 μm (Figure 4a), whereas, the aggregates formed at 80 °C formed conjugating small spheres with a relatively homogeneous diameter of 0.7–1 μm (Figure 4b). In general, the particle size of precipitated organic molecules is related to the nucleation process.[9] An increased number of nuclei results in smaller particles, because a given amount of mass is distributed over a greater number of centers. The formation of the smaller particles also leads to a decrease in the average separation distance, which increases interparticle interaction. Therefore, the difference in size between the aggregated scFv formed at 4 and 80 °C leads us to the following suggestion: during aggregation of scFv at 4 °C, a few nuclei leading to insoluble aggregates are heterogeneously formed through relatively rare nonnative intermolecular interactions after β-strands are formed, and then the growth of the nuclei happens more rapidly than new nucleation. In contrast, many nuclei were rapidly formed before they had time to grow large at 80 °C.

We also analyzed the in vivo aggregates of scFv formed in the cytoplasm during the expression in *E. coli*. The FT-IR spectrum had the identical properties to the in vitro aggregates (data not shown), but SEM image showed spheres smaller than those in scFv aggregates formed at 80 °C (the diameter is 200–600 nm, Figure 4d); the size was comparable than inclusion bodies reported previously.[10] Therefore, the in vivo aggregates of scFv might be formed after the formation of β-strands, and the nuclei leading to insoluble aggregates may be more rapidly formed than in in vitro aggregation at 80 °C. The formation of small insoluble particles with β strands in vivo suggests rapid formation of β-strands during expression of scFv in *E. coli*.

In conclusion, the primary amino acid sequence of the immunoglobulin strongly favors the formation of β-strands independently of redox conditions, and aggregation due to inappropriate interaction between β-strands causes nucleation, leading to insoluble aggregates in vitro. However, nucleation and nucleus growth are influenced by the conditions under which scFv is aggregated. Our results suggest that the nucleation occurs rapidly in vivo.

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