Biochemical and Biophysical Research Communications xxx (2017) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel engineered interchain disulfide bond in the constant region enhances the thermostability of adalimumab Fab

Hitomi Nakamura [a], Naoko Oda-Ueda [a], Tadashi Ueda [b], Takatoshi Ohkuri [a, *]

[a] Faculty of Pharmaceutical Sciences, Sojo University, Japan
[b] Graduate School of Pharmaceutical Sciences, Kyushu University, Japan

ARTICLE INFO

Article history:
Received 19 October 2017
Accepted 26 October 2017
Available online xxx

Keywords:
Adalimumab
Antibody
Disulfide bond
Fab
Thermal stability

ABSTRACT

We constructed a system for expressing the Fab of the therapeutic human monoclonal antibody adalimumab at a yield of 20 mg/L in the methylotrophic yeast *Pichia pastoris*. To examine the contribution of interchain disulfide bonds to conformational stability, we prepared adalimumab Fab from which the interchain disulfide bond at the C-terminal region at both the CH_1 and CL domains was deleted by substitution of Cys with Ala ($Fab_{\Delta SS}$). DSC measurements showed that the Tm values of $Fab_{\Delta SS}$ were approximately 5 °C lower than those of wild-type Fab, suggesting that the interchain disulfide bond contributes to conformational thermostability. Using computer simulations, we designed a novel interchain disulfide bond outside the C-terminal region to increase the stability of $Fab_{\Delta SS}$. The resulting Fab (mutSS $Fab_{\Delta SS}$) had the mutations H:V177C and L:Q160C in $Fab_{\Delta SS}$, confirming the formation of the disulfide bond between CH_1 and CL. The thermostability of mutSS $Fab_{\Delta SS}$ was approximately 5 °C higher than that of $Fab_{\Delta SS}$. Therefore, the introduction of the designed interchain disulfide bond enhanced the thermostability of $Fab_{\Delta SS}$ and mitigated the destabilization caused by partial reduction of the interchain disulfide bond at the C-terminal region, which occurs in site-specific modification such as PEGylation.

1. Introduction

Monoclonal antibodies are widely used in diagnosis and therapy. Despite numerous advantages, the prohibitively high costs of the quantities needed in therapeutic doses are a serious problem. Small antibody fragments that can be rapidly produced on large scales in microbial systems, such as scFv or Fab, are emerging as credible alternatives for various applications in clinical diagnosis and therapy [1–3]. In particular, Fab would be the most suitable fragments for clinical applications, because they are more stable than scFv [4]. Additionally, Fab can penetrate tissues more rapidly than can whole antibodies [5]. Although Fab have considerably shorter serum half-lives than do whole antibodies in pharmacological therapy, conjugation of polyethylene glycol (PEG) to Fab has been shown to increase their circulating half-lives [6–10]. PEGylation has also been used to reduce the toxicity, immunogenicity, and antigenicity of proteins [5,11,12]. In general, cysteine residues are the best option for site-specific covalent labeling of antibodies. Certolizumab pegol (Cimzia®) is a PEGylated Fab' used in treatment of Crohn's disease and rheumatoid arthritis [13]. The PEGylation of Cimzia® has been engineered to retain a free cysteine in the hinge region of the Fab'. Targeted PEGylation through the hinge cysteine in the Fab' has proven a useful approach for site-specific modification. However, a free cysteine can result in disulfide scrambling and aggregation [14]. An alternative approach is to target the interchain disulfide bond between the accessible cysteine residue at the C-terminal region of CH_1 and the cysteine residue at the C-terminal region of CL. A mild reduction of interchain disulfide bonds to liberate the free cysteine residues can follow the treatment with PEG-bis-sulfone reagents, resulting in the bridging of the two cysteine thiols with PEG attached [14,15]. This method can be used on Fab containing accessible cysteine residues at the C-terminal region for therapeutic applications. On the other hand, interchain disulfide bonds are generically known to contribute to the conformational stability of proteins. Despite the success of inter-subunit disulfide engineering in enhancing the stability of some dimeric proteins [16–19], there have been no reported attempts to introduce engineered inter-subunit disulfide bonds into the constant region of Fab for stabilization.

Adalimumab is a therapeutic human monoclonal antibody

* Corresponding author. Faculty of Pharmaceutical Sciences, Sojo University, 4-22-1 Ikeda, Nishiku-ku, Kumamoto 860-0082, Japan.
E-mail address: ohkuri@ph.sojo-u.ac.jp (T. Ohkuri).

https://doi.org/10.1016/j.bbrc.2017.10.140
0006-291X/

against TNFα and is used to treat immune diseases, including rheumatoid arthritis and Crohn's disease [20]. In this study, the contribution of interchain disulfide bonds at the C-terminal region to conformational stability was examined using adalimumab Fab. Moreover, introduction of an engineered interchain disulfide bond into the constant region of the Fab was employed to mitigate destabilization due to the reduction of its interchain disulfide bonds.

2. Materials and methods

2.1. Materials

pPICZαA was employed as an expression vector (Invitrogen, Carlsbad, CA). Protein expression was performed in the *P. pastoris* X33 (Invitrogen). All other chemicals used were of the highest quality commercially available.

2.2. Construction of expression plasmids

VH gene and VL gene of adalimumab (GenBank accession no. JC497459.1 and JC497460.1) was amplified by PCR using the long oligo-nucleotides, and the CH_1 and CL gene were obtained by PCR from the human IgG_1 kappa gene, and then these genes were connected to the VH and VL genes by PCR, respectively [21]. Site-directed mutagenesis of the adalimumab gene was performed by PCR, according to previously described methods [22]. Mutations were confirmed by DNA sequence analysis. Adalimumab Fab expression vector, which contains the sequences encoding both chains of the Fab, was cloned in two steps according to the method of Lange *et al*. with slight modifications [23,24]. Adalimumab Fab-H chain gene was inserted into the *E. coli* − *P. pastoris* shuttle vector pPICZαA. The L chain gene was ligated into the modified pPICZαA−Δ*Pme*I vector, where the *Pme*I restriction site is destroyed by site-directed mutagenesis. Each gene was fused directly in frame with the α-factor secretion signal of pPICZαA and pPICZαA−Δ*Pme*I, and a nucleotide sequence encoding the Kex2 cleavage site was placed at upstream of the Fab gene. Each Fab-H chain and L chain was attached an extra Ser residue to the N-terminus for the cause of an efficient signal sequence processing [24]. Plasmids combining the expression cassettes for both the Fab-H chain and L chains on one vector were produced by double digestion of the L chain vectors with *Bgl*II and *Nco*I, and these constructs were sequentially inserted into the *Bam*HI and *Nco*I site of the vector already containing a single copy of the expression cassette of the Fab-H chain.

2.3. Expression of adalimumab Fab

P. pastoris X-33 was transformed with a linearized expression vector by digestion with *Pme*I. The transformation was performed using the electroporation method as described in the *P. pastoris* expression manual (Invitrogen). The transformant cells were plated on a yeast extract peptone dextrose medium with sorbitol (YPDS) plate containing 100 μg/ml of Zeocin. After incubation at 30 °C, some colonies appeared on the YPDS plate and then some of the larger colonies were selected.

The selected colonies were each grown in 5 ml of BMGY medium (1% yeast extract/2% peptone/100 mM potassium phosphate, pH 6.0/1.34% yeast nitrogen base/4×10^{-5}% biotin/1% glycerol) at 30 °C with shaking for 3 days. Each growth culture was centrifuged, and the pellet was suspended with 5 ml of BMMY medium (1% yeast extract/2% peptone/100 mM potassium phosphate, pH 6.0/1.34% yeast nitrogen base/4×10^{-5}% biotin/1% methanol) and incubated at 30 °C with shaking for 4 days. After 4 days, the secreted Fab in each culture medium was estimated by SDS-PAGE, and then the most efficiently secreting colony was selected. For large-scale expression of adalimumab Fab, *P. pastoris* transformant cells were grown in 1 L of BMGY medium at 30 °C with shaking for 3 days. The cells were centrifuged at 3000×*g* for 7 min and the pellets were suspended in 1 L of BMMY medium, and cultivated at 30 °C with fermenter (M-1000B, EYELA, Japan) for 4 days. The culture was supplemented daily with 5 ml of methanol. The culture supernatant was obtained by centrifugation at 8000×*g* for 7 min.

2.4. Purification of adalimumab Fab

The culture supernatant was brought to 60% ammonium sulfate saturation and precipitated protein was removed by centrifugation at 10,000×*g* for 15 min. To resuspend the precipitated protein, 50 mM acetate buffer, pH 4.0, was used. After dialysis against 50 mM acetate buffer, pH 4.0, the dialysate was applied to a cation-exchange column (2.2 × 12.0 cm) of SP-Toyopearl 650 M (Tosoh, Japan) equilibrated with 50 mM acetate buffer at pH 5.0. Then, this column was eluted with 50 mM acetate buffer, pH 4.0, containing 0.5 M NaCl. The elution fractions were dialyzed against 50 mM acetate buffer, pH 4.0. The dialysate was applied to affinity column (1.5 × 6.0 cm) of Blue sepharose 6 (GE Healthcare, USA) equilibrated with 50 mM acetate buffer, pH 5.0. Then, this column was eluted with equilibration buffer containing 1 M NaCl. Finally, the Fab fraction was isolated by cation exchange chromatography on a column (1.5 × 40 cm) of SP-Toyopearl with a gradient of 500 ml of 50 mM acetate buffer, pH 5.0 and 500 ml of the same buffer containing 0.5 M NaCl.

2.5. Preparation of human TNFα

The human TNFα gene was obtained by PCR using long primers and was inserted between the *Nde*I and *Eco*RI sites of the pET21c vector (Novagen, USA), just downstream of the T7 promoter. The human TNFα was overexpressed in *E. coli* BL21 cells harboring a recombinant plasmid constructed using a pET21c expression vector. The transformant cells were grown at 37 °C in 0.4 L of LB medium containing 100 μg/ml ampicillin. The culture was allowed to grow until mid-log phase ($OD_{600} = 0.5$—0.7) and the expression of human TNFα was induced by addition of 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and incubation for 4 h. The cultured cells were harvested by centrifugation at 8000×*g* for 7 min. The pellets were suspended in 20 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, and then sonicated for 5 min on ice, each pulse was 10 s in duration. The mixture was centrifuged at 39,000×*g* for 40 min. The supernatant was brought to 60% ammonium sulfate saturation and precipitated protein was removed by centrifugation at 10,000×*g* for 15 min. The precipitated protein was resuspended in 20 ml of 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA. After dialysis against 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA, the dialysate was applied to anion-exchange column (2.2 × 15.0 cm) of DEAE-Toyopearl 650 M (Tosoh, Japan) equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 1 mM EDTA. Then, this column was eluted with equilibration buffer containing 1 M NaCl. The elution fractions were analyzed by SDS-PAGE and dialyzed against 50 mM acetate buffer, pH 5.0. The TNFα fraction was isolated by cation exchange chromatography on a column (1.5 × 40 cm) of SP-Toyopearl with a gradient of 300 ml of 50 mM acetate buffer, pH 5.0 and 300 ml of the same buffer containing 0.5 M NaCl. The purity of eluted TNFα was confirmed by SDS-PAGE.

2.6. ELISA of adalimumab Fab

Ninety-six well ELISA plates were coated with human TNFα

Please cite this article in press as: H. Nakamura, et al., A novel engineered interchain disulfide bond in the constant region enhances the thermostability of adalimumab Fab, Biochemical and Biophysical Research Communications (2017), https://doi.org/10.1016/j.bbrc.2017.10.140