Molecular Immunology 90 (2017) 143–149

Contents lists available at ScienceDirect

Molecular Immunology

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

An unusual cysteine V_L87 affects the antibody fragment conformations without interfering with the disulfide bond formation

Carolina Attallah[a], María Fernanda Aguilar[a], A. Sergio Garay[b], Fernando E. Herrera[b], Marina Etcheverrigaray[a], Marcos Oggero[a], Daniel E. Rodrigues[b,c,*]

[a] UNL, CONICET, FBCB, Cell Culture Laboratory, Ciudad Universitaria UNL, Pje. "El Pozo" - C.C. 242, S3000ZAA Santa Fe, Argentina
[b] UNL, CONICET, FBCB, Departamento de Física, Ciudad Universitaria UNL, Pje. "El Pozo" - C.C. 242, S3000ZAA Santa Fe, Argentina
[c] INTEC, CONICET-UNL, Predio CONICET Santa Fe, Pje. "El Pozo", S3000 Santa Fe, Argentina

ARTICLE INFO

Keywords:
Single-chain variable fragment
Cysteine contiguous residues
Molecular modeling
Mutating cysteine

ABSTRACT

The Cys residues are almost perfectly conserved in all antibodies. They contribute significantly to the antibody fragment stability. The relevance of two natural contiguous Cys residues of an anti-recombinant human-follicle stimulation hormone (rhFSH) in a format of single-chain variable fragment (scFv) was studied. This scFv contains 5 Cys residues: V_H22 and V_H92 in the variable heavy chain (V_H) and V_L23, V_L87 and V_L88 in the variable light chain (V_L). The influence of two unusual contiguous Cys at positions V_L87 and V_L88 was studied by considering the wild type fragment and mutant variants: V_L-C88S, V_L-C87S, V_L-C87Y. The analysis was carried out using antigen-binding ability measurement by indirect specific ELISA and a detailed molecular modeling that comprises homology methods, long molecular dynamics simulations and docking. We found that V_L-C87 affected the antibody fragment stability without interfering with the disulfide bond formation. The effect of mutating the V_L-C87 by a usual residue at this position like Tyr caused distant structural changes at the V_H region that confers a higher mobility to the V_H-CDR2 and V_H-CDR3 loops improving the scFv binding to the antigen.

1. Introduction

scFvs are recombinant antibody fragments consisting of the variable light chain (V_L) and the variable heavy chain (V_H) domains connected via a short flexible peptide (linker) (Huston et al., 1988). Each variable domain has a characteristic tertiary structure consisting of two β-sheets that contain three hypervariable loops, known as complementary determining regions (CDRs), evenly distributed between four less variable framework regions (FRs). The antigen binding site is primarily composed by the six CDRs located in the variable domains that are close to each other (Morea et al., 2000).

The Cys residues at positions V_H22 and V_H92 in V_H, and V_L23 and V_L88 in V_L (numbering according to Kabat et al. (1991)) are almost perfectly conserved in all antibodies. They form buried intra-domain disulfide bridges which contribute significantly to the antibody fragment stability (Langedijk et al., 1998). Only 0.3% of the 6042 mouse V_L sequences listed in the Kabat database (http://www.bioinf.org.uk/abysis2.7/), contains a residue different from Cys at position V_L88. The conserved Cys residue at position V_L88 locates as the flanking residue of V_L-CDR3. The V_L87 position contains the Phe residue in 28% of V_L sequences of the database and Tyr in 69% of the instances. Less than 1% of sequences contain a Cys at position V_L87, including some which present a residue different from Cys at V_L88. Although these unusual sequences are available in the Kabat database, there is no information concerning the function of the corresponding antibodies. Some studies demonstrated that only few antibody molecules were able to tolerate the loss of the referred disulfide bonds. Nevertheless for some sequences it has been proven that additional mutations stabilized the antibody fragment structures (Frisch et al., 1996; Langedijk et al., 1998; Proba et al., 1998; Rudikoff and Pumphrey, 1986). The instability of these antibodies with missing disulfide bonds might be the reason that limits the occurrence of Cys at unusual positions. (Duan et al., 2012) reported the presence of 5 Cys residues in a scFv anti-rabies virus G protein. The unusual Cys was found at the first position after the Cys that flanks the V_L-CDR3 (i.e., inside the CDR3). Despite of the presence of the free thiol residue, this scFv bound to the target molecule. These authors also proposed that the free thiol might form a disulfide bond mismatch in the scFv structure based on the evidence that mutating the unusual Cys to Ser improved the antibody fragment performance (Duan et al., 2014).

In this work we study the influence of contiguous Cys residues at positions V_L87 and V_L88 present in an anti-recombinant human follicle

* Corresponding author at: Ciudad Universitaria UNL. Pje. "El Pozo" - C.C. 242, S3000ZAA Santa Fe, Argentina.
E-mail address: danielr@fbcb.unl.edu.ar (D.E. Rodrigues).

http://dx.doi.org/10.1016/j.molimm.2017.07.008
Received 26 April 2017; Received in revised form 10 July 2017; Accepted 15 July 2017

stimulating hormone (rhFSH) scFv, derived from a monoclonal antibody (mAb) that naturally contains 5 Cys residues. For the present sequence, the unusual Cys residue was found at the position immediately before the V_L-C88 that flanks the V_L-CDR3 (i.e., inside the FR3), at variant with the case studied by Duan et al. (2014, 2012). The aim of the present work is to analyze the role of the unusual Cys residue at position V_L87 and the changes that produce the mutation of this amino acid. Different mutants: V_L-C88S, V_L-C87S and V_L-C87Y were experimentally developed and their binding ability to rhFSH were evaluated. 3D structures of the wild type, mutants V_L-C87S and V_L-C87Y of the scFv were obtained by comparative modeling and long molecular dynamics simulations (MD) were run to evaluate the equilibrium properties of the fragment. In silico docking analysis of the scFv over rhFSH structure were conducted to identify the interacting residues. We found that the Cys residue at V_L87 is not involved in the disulfide bond formation and therefore a disulfide bridge mismatch is not feasible as it was described by Duan et al. (2014) for their scFv. The unusual Cys residue in the scFv anti-rhFSH affects the antibody fragment stability and its ability to bind the antigen.

2. Materials and methods

2.1. scFv expression vectors

The wild type scFv anti-rhFSH gene was amplified by overlap extension PCR (SOE-PCR). The V_H and V_L cDNAs templates were previously obtained from hybridoma cells producing the anti-rhFSH mAb. A flexible peptide linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$ coding DNA was used to connect V_H and V_L. The reactions were catalyzed by Platinum™ Pfx DNA Polymerase (Invitrogen, USA). V_H, linker and V_L products were amplified with primers 1-2, 3-4 and 5-6 (listed in Table 1-SI, see supplementary information), respectively. The wild type scFv final product was amplified with primers 1 and 6. Site-directed mutagenesis was employed to obtain the mutants V_L-C87Y, V_L-C88S and V_L-C87S by SOE-PCR using the following sets of primer pairs 1-8 and 7-6, 1-10 and 9-6, and 1-12 and 11-6, listed in Table 1-SI (supplementary information), where the UGC, UGU and UGC codons were changed for UAC, UCU and UCC, respectively. The final mutated scFv products were amplified with primers 1 and 6. All constructs were clone into modified pET22b(+) vector (Novagen®, USA) through the removal of the N-terminal pelB signal peptide (pET22b-pelB) to express the scFv variants in the cell cytoplasm of E. coli SHuffle® T7 strain (NEB Inc., USA). This strain possesses a chromosomal copy of the disulfide bond isomerase DsbC (de Marco, 2009) to assure a proper folding of disulfide bridges. All constructs were confirmed by sequencing (Macrogen Inc, Korea).

2.2. scFv expression and extraction

The plasmids pET22b-pelB containing the wild type scFv or each of the mutated scFvs (V_L-C87Y, V_L-C88S and V_L-C87S) were used to transform *E. coli* SHuffle® T7 competent cells (NEB Inc., USA). To express the wild type scFv and the mutated ones, single colonies were used to inoculate 10 ml LB medium supplemented with 0.1 mg/ml ampicillin and grown at 30 °C and 200 rpm. After overnight (ON) incubation, the cultures were inoculated into 200 ml LB medium supplemented with 0.1 mg/ml ampicillin and 1% (w/v) glucose, and grown at 30 °C and 200 rpm until they reached an OD_{600} of 0.8. Protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cells were allowed to grow at 20 °C at 150 rpm for another 16 h. Cells were harvested by centrifugation at 5000 rpm for 15 min and then resuspended in 20 mM phosphate-buffered saline (PBS: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 14 mM NaCl, 2.7 mM KCl, pH 7.4), 500 mM NaCl and 0.1 mM EDTA, and lysed using sonication 8 × 30 s. The cell lysates were centrifuged at 10,000 rpm for 30 min and the supernatants containing the soluble scFv were stored at −20 °C.

2.3. scFv quantification by competitive ELISA

A competitive ELISA was performed in 96-well plates (Greiner, Austria) coated with 50 ng per well of rabbit anti-6xHisTag polyclonal antibodies (ab125265, Abcam, UK) prepared in 50 mM carbonate/bicarbonate buffer (pH 9.6). Plates were incubated for 1 h at 37 °C and then ON at 4 °C. After blocking 1 h at 37 °C with 1% (w/v) bovine serum albumin in PBS (PBS-BSA), plates were incubated with 2-fold serial dilutions of the test samples, followed by the addition of equal volume of 50 ng/ml of a biotinylated C-terminal 6xHis-tagged irrelevant protein. This protein was trivial for the system and it was only used as a His-fused protein to compete with the His-tagged scFv to bind to the coated anti-6xHisTag antibody. After incubating for 1 h at 37 °C, horseradish peroxidase (HRP)-conjugated streptavidin (Amdex™, GE Healthcare) at a dilution of 1:10,000 was added to each well. After 1 h at 37 °C, plates were incubated for 10 min with substrate solution (3 mg/ml *o*-phenylenediamine, 0.12% (v/v) H_2O_2 in 50 mM phosphate-citrate buffer). The reaction was stopped by the addition of 2 N H_2SO_4. Absorbance was measured at 492 nm with a microtiter plate reader (Labsystems Multiskan MCC/340, Finland). Between every step, plates were washed 6 times with PBS containing 0.05% (v/v) Tween 20 (PBS-T). Dilutions of tested samples and antibodies were prepared in PBS-T containing 0.1% (w/v) BSA. The C-terminal 6xHis-tagged irrelevant protein was previously purified by immobilized-metal affinity chromatography (IMAC) and used as standard to prepare a 2-fold serial dilution curve from 400 ng/ml to 3.125 ng/ml. Also, its biotinylated form was used as tracer for the competition step. The assay was reproduced in triplicate.

2.4. scFv-rhFSH binding determination by indirect specific ELISA

Indirect ELISA was performed in 96-well plates. Coating was achieved by incubating the plates with 50 ng per well of rhFSH prepared in 50 mM carbonate/bicarbonate buffer (pH 9.6). Plates were incubated for 1 h at 37 °C and then ON at 4 °C. After blocking 1 h at 37 °C with 1% (w/v) bovine serum albumin in PBS (PBS-BSA), plates were incubated with 2-fold serial dilutions of the test samples for 1 h at 37 °C. Then, plates were incubated with appropriately diluted rabbit anti-6XHisTag polyclonal antibodies for 1 h at 37 °C. Finally, an horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (DAKO, Denmark) at a dilution 1/2000 was added to the wells. After 1 h at 37 °C, plates were incubated for 10 min with substrate solution and then, the reaction was stopped by the addition of 2 N H_2SO_4. Absorbance was measured at 492 nm with a microtiter plate reader. Washing between steps and dilutions were carried out using the buffers mentioned in 2.3.

2.5. Molecular modeling

2.5.1. Homology modeling

Template identification was performed using Psi-Blast (Jones and Ward, 2003) and HHblits (Remmert et al., 2012), which were able to find very good templates to build the wild type scFv model from Protein Data Bank (http://www.rcsb.org/pdb/)(Berman et al., 2000). The best of them, 4h0g (Tapryal et al., 2013), had 71.5% and 80.2% of identical and similar residues, respectively with 4% of gaps; these values guarantee the quality of the model. The alignment was obtained running HHblits and it was used to build the model using Modeller software (Eswar et al., 2001) after visual inspection of the alignment to eliminate any possible mistake. The initial model quality was verified using Qmean6 composite score (Benkert et al., 2008) and the stereochemical quality of the model was monitored with PROCHECK (Laskowski et al., 1993). The Qmean6 Z-score of −1.20 indicates that our model fell at −1.20 standard deviation from a distribution of scores calculated for reference structures solved by X-ray crystallography with the same number of residues. Taking into account that the model was going to be