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Research paper

Selecting for antibody scFv fragments with improved stability using phage display with denaturation under reducing conditions

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Abstract

Stability of single-chain Fvs (scFvs) can be improved by mutagenesis followed by phage display selection where the unstable variants are first inactivated by, for example, denaturing treatment. Here we describe a modified strategy for the selection of stabilized antibody fragments by phage display, based on denaturation under reducing conditions. This strategy was applied to an anti-thyroid-stimulating hormone (TSH) scFv fragment which refolded remarkably during the selection if denaturation was carried out in conventionally used non-reducing conditions. Refolding was, however, efficiently prevented by combining denaturation with reduction of the intra-domain disulfide bridges, which created favourable conditions for selection of clones with improved stability. Using this strategy, scFv mutants with 8–9 °C improved thermal stability and 0.8–0.9 M improved stability for guanidinium chloride were found after 4–5 enrichment cycles. The most stable mutants selected contained either $Lys^{H}66Arg$ or $Asn^{H}52aSer$ mutations, which are known to stabilize other scFvs. Periplasmic expression level of the mutants was also improved.

Keywords: Antibody engineering; Disulfide bond; Phage display; Protein denaturation; Protein stability

Abbreviations: scFv, single-chain Fv antibody fragment; V_H, heavy chain of an antibody Fv fragment; V_L, light chain of an antibody Fv fragment; GdmCl, guanidinium chloride; TSH, thyroid-stimulating hormone; DTT, dithiotreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; MPB, *N*-(3-maleimidopropionyl)biocytin; BSA, bovine serum albumin; IPTG, isopropyl-β-D-galactopyranoside; EDTA, ethylenediaminetetraacetic acid.

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1. Introduction

Stability is an essential property for proteins of biotechnological interest. Among antibodies, single-chain Fv (scFv) fragments (Raag and Whitlow, 1995) may have limited stability and therefore scFvs have been targets of stability engineering (Wörn and Plückthun, 2001). The strategies described to improve scFv stability include both rational and evolutionary means. By rational design, stability can be improved by introducing known stabilizing mutations or mutations predicted by canonical immunoglobulin sequence (Steipe et al., 1994) or structure (Ewert et al., 2003). Grafting complementarity-determining region (CDR) loops to a stable framework (Jung and Plückthun, 1997) and introducing a disulfide bridge between the V_H and V_L domains (Young et al., 1995) also increase stability. As designing stabilizing mutations requires special know-how and testing different mutations and their combinations is labour-intensive, evolutionary methods are often preferred. Compared to rational design, these methods combine mutagenesis with cell-based screening (Kolmar et al., 1995; Auf der Maur et al., 2001; Philipps et al., 2003) or selection by some display technique, such as phage display (Hoogenboom et al., 1998) or ribosome display (Schaffitzel et al., 1999) and are extremely powerful. Random mutagenesis overcomes the need for rational design, although mutations known to have stabilizing effects can also be introduced into a library. Using display techniques, a single clone with improved stability can be enriched among millions of others by repeating selection cycles. If the selection threshold is correctly set, enrichment can be obtained within a few cycles.

In conjunction with phage display, stability selection can be based on irreversible inactivation of unstable proteins by heat or guanidinium chloride (GdmCl) or by protease digestion. Stable variants are then collected by antigen binding activity or phage infectivity (Sieber et al., 1998; Kristensen and Winter, 1998; Jung et al., 1999; Martin et al., 2001). If selection is based on GdmCl denaturation, the phage-displayed protein may refold during the collection step. Because of refolding, denaturation by GdmCl has been estimated to select less efficiently for stability than denaturation by temperature (Jung et al., 1999). Denaturation of scFv can, however, be made irreversible by reduction and the loss of disulfide bridges inside V_L and V_H domains which contribute to domain stability (Glockshuber et al., 1992). Only intrinsically stable scFvs or single domains are able to fold in reducing conditions (Frisch et al., 1996; Wörn and Plückthun, 1998a,b; Ohage and Steipe, 1999) and, accordingly, reduction has been used with ribosome display to improve scFv stability based on ability to fold under reducing conditions (Jermutus et al., 2001). Here we have used an immunoassay to study the efficiency of denaturation of an anti-thyroid-stimulating hormone (TSH) scFv fragment under different conditions and found that the selection efficiency could be improved by carrying out denaturation under reducing conditions. We have also successfully enriched stabilized variants of the scFv by phage display using denaturation in a reducing environment as a means to inactivate unstable scFv variants, thereby demonstrating the applicability of the strategy as a means to select for stability by phage display.

2. Materials and methods

2.1. Primers, buffers, culture media and reagents

HeavyFor (5′-GGA ATT CGG CCC CCG AGG CCG CAG AGA CAG TGA CCA GAG T-3′), HeavyRev (5′-GGC GGC GGC GGC TCC GGT GGT GGT GGA TCC SAG GTG CAG CTG CAG GAG-3′), scFOR (5′-GGA ATT CGG CCC CCG AGG CC-3′) and scREV (5′-TTA CTC GCG GCC CAG CCG GCC ATG GCG-3′) were from TAG Copenhagen (Copenhagen, Denmark); pAKfor (5′-TGAAATACCTATTGCCTACG-3′) and pAKrev (5′-CGCCATTTTTCACTTCACAG-3′) were from Alpha DNA (Montreal, Canada); WO267 (5′-CTA GAC TAG TAC AAT CCC TGG GCA CAA TTT TC-3′), MK24 (5′-GAT GGC AAA CGC TAA TAA G-3′), WO1236 (5′-GCC CAG CCG GCC ATG GCG CVW ATT GTK CTM ACY CAG TC-3′) and WO1244 (5′-GGA GCC GCC GCC GCC AGA ACC ACC ACC ACC AGA ACC ACC ACC ACC ACG TTT CAG CTC CAG CTT GG-3′) were synthesized in-house using standard phosphoramidite chemistry. *Sfi*I restriction sites are underlined.