ChemPubSoc Europe

DOI: 10.1002/cbic.201700350

CHEMBIOCHEM Full Papers

Intramolecular Thioether Crosslinking to Increase the Proteolytic Stability of Affibody Molecules

Anders Nilsson, Joel Lindgren, and Amelie Eriksson Karlström*[a]

Protein therapeutics suffer from low oral bioavailability, mainly due to poor membrane permeability and digestion by gastrointestinal proteases. To improve proteolytic stability, intramolecular thioether crosslinks were introduced into a three-helix affibody molecule binding the human epidermal growth factor receptor (EGFR). Solid-phase peptide synthesis was used to produce an unmodified control protein domain and three different crosslinked protein domain variants: one with a thioether crosslink between the N-terminal lysine residue and a cysteine residue in the second loop region (denoted K4), a second with a crosslink between the C-terminal lysine residue and a cysteine residue in the first loop region (denoted K58), and a third with crosslinks in both positions (denoted K4K58). Circular dichroism (CD) and surface-plasmon-resonance-based (SPR-based) biosensor studies of the protein domains showed that the three-helix structure and high-affinity binding to EGFR were preserved in the crosslinked protein domains. In vitro digestion by gastrointestinal proteases demonstrated that the crosslinked protein domains showed increased stability towards pepsin and towards a combination of trypsin and chymotrypsin.

Introduction

Protein-based drugs have been successfully developed for the treatment of autoimmune and inflammatory diseases, as well as different cancers.[1–3] An important aspect of protein drugs, contributing to their successful introduction in the clinic, is the large interaction area between the protein and its molecular target, leading to specific, high-affinity binding and to low levels of off-target effects. In contrast with small-molecule drugs, which can be accompanied by side effects due to their conversion into toxic metabolites, proteins are typically degraded by proteases and present low risk of toxicity.[4] However, a major disadvantage of protein drugs is poor absorption from the gastrointestinal tract. The passage through the epithelial cell layer is inefficient, because most proteins are too hydrophilic for transcytotic passage and too large for paracellular passage through the tight junctions.[5,6] Furthermore, the acidic conditions of the stomach and the secretion of proteolytic enzymes, such as pepsin, are unfavorable for proteins. In the intestine, a different panel of enzymes—in particular trypsin and chymotrypsin—further contributes to the digestive process.[7] Because of their poor oral availability, the majority of protein drugs are administered by injection or infusion at a hospital, which is both costly and inconvenient for the patient.

Most efforts directed towards the development of orally active protein drugs have been focused on their formulation.[5,8,9] Enteric coatings that protect the drug in the acidic environment of the stomach but dissolve in the higher pH of the intestine are commonly applied, but they do not prevent degradation by the intestinal enzymes or improve passage through the epithelium.[9] Another strategy is co-administration with protease inhibitors, but this will affect the normal digestion of food and potentially lead to side effects such as nephrotoxicity and insufficient absorption of nutrients.[10,11] A third approach is the application of permeation enhancers that target the epithelial cell layer to make it more permeable to the protein, but also this strategy is linked to the risk of side effects such as mucosal damage and inflammation.[12–14]

An alternative strategy is to find inspiration from the peptides and small proteins that are found in nature and are known to be orally active. One well-known example is the biologically active peptide kalata B1, isolated from the plant *Oldenlandia affinis*. This 29-residue peptide is a member of the cyclotide class of molecules, characterized by a cyclic backbone and a cystine-knot fold with three intramolecular disulfide bridges.[15] Kalata B1 has been extensively studied and has been shown to be exceptionally stable to proteases, high temperature, denaturing agents, and acidic conditions.[16] Thanks to its members' high stability and their potential for oral administration,[17] the cyclotide family represents a promising scaffold for drug design.[18] Peptide ligands have been grafted onto the cyclotide scaffold to produce biologically active peptides demonstrating oral activity, as exemplified by the orally active bradykinin B1 receptor antagonist generated by insertion of a 9 aa peptide analogue into one of the loops of kalata B1.[19]

In addition to the cyclotide family, there are other biologically active peptides derived from nature that are head-to-tail

[a] A. Nilsson, Dr. J. Lindgren, Prof. Dr. A. Eriksson Karlström
KTH Royal Institute of Technology, School of Biotechnology
Division of Protein Technology, AlbaNova University Center
106 91 Stockholm (Sweden)
E-mail: amelie@biotech.kth.se

Supporting information and the ORCID identification numbers for the authors of this article can be found under https://doi.org/10.1002/cbic.201700350.

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

cyclic and/or structurally constrained by intramolecular bridges; examples include the immunosuppressant cyclic peptide cyclosporine[20] and the defensin family of peptides with antimicrobial properties.[21]

The oral bioavailability demonstrated for these compounds, sufficiently high to give biological effects upon oral administration, can possibly be explained by their small sizes and conformational constraints, which facilitate passage through the tight junctions of the epithelial cells, and the intramolecular crosslinking, which protects the polypeptides from proteolytic degradation. The same principles can hypothetically be applied to improve the bioavailability of small proteins with therapeutic potential. Affibody molecules are a class of small (58 aa) proteins with great potential as alternatives to monoclonal antibodies as therapeutic targeting proteins.[22] Affibody molecules are so-called engineered scaffold proteins (ESPs), which typically are selected from combinatorial libraries based on a protein scaffold. The starting scaffold of the affibody molecules is the three-helical Z domain, derived from staphylococcal protein A, which is remarkably stable to heat, extreme pH, and denaturing conditions and can easily be engineered to incorporate new features or binding specificities. A vast number of affibody molecules binding targets of therapeutic relevance has been generated, and the first affibody-based drug candidates are currently in clinical trials.[23] Thanks to their small sizes, affibody molecules can be produced by total chemical synthesis, allowing for backbone modification and incorporation of nonribosomal amino acids or other synthetic building blocks.[24]

In this study, we have explored stabilization towards intestinal proteases through the introduction of intramolecular thioether bridges into an affibody molecule that binds the epidermal growth factor receptor (EGFR), which is known to be overexpressed in a number of different cancers.[25] This affibody was chosen for study because it binds a molecular target that is of therapeutic relevance for treatment of, for example, lung cancer, but it also serves as a prototype for the panel of available affibody molecules, binding targets such as human epidermal growth factor receptor 3 (HER3), insulin-like growth factor 1 receptor (IGF-1R), and interleukin-6 (IL-6).[22] The incorporation of thioether bridges instead of disulfide bridges, which are more commonly used in protein engineering, was motivated by their superior stability to reducing environments and by the reduced risk of thiol–disulfide exchange with endogenous proteins.[26] Several examples of thioether modification of peptides have previously been reported in the literature. A native disulfide bond can be replaced with a thioether bond to increase the metabolic stability of a peptide.[27] Peptide ligands lacking disulfides can be cyclized by the introduction of a thioether bridge, through reaction between, for example, a cysteine side chain and a haloacetylated N terminus or an amine-containing side chain.[28–30] Another strategy is to treat pairs of appropriately positioned cysteine residues with bifunctional reagents that link the amino acid side chains through thioether bridges.[26,31–34] "Peptide stapling" techniques based on such bifunctional thiol-reactive linkers have been used to introduce conformational constraints in α-helices with the aim of increasing proteolytic stability, binding affinity, and cellular uptake, as recently reviewed.[35]

Along these lines we have previously applied a thioether-based intramolecular crosslinking strategy to a small (46 aa) albumin-binding domain.[36] It was shown that the introduction of a single intramolecular thioether bridge in this small protein domain dramatically increased its resistance to degradation by the major digestive enzymes of the gastrointestinal tract—that is, pepsin, trypsin, and chymotrypsin—thus suggesting improved potential for oral uptake. However, in that study only one out of five tested crosslinked protein variants demonstrated improved proteolytic stability with retained secondary structure and binding activity. Here we explore this concept further by application of the same crosslinking chemistry to the versatile and therapeutically relevant class of affibody molecules, with drug candidates now in clinical development for tumor imaging and inflammation disorders.[22]

Results and Discussion

Synthesis and crosslinking

To stabilize the 58-aa EGFR-binding affibody molecule $Z_{EGFR:1907}$ against proteolytic degradation, intramolecular crosslinks were introduced between the loop regions connecting the helices and the N and C termini of the protein domain. Cysteine residues introduced at position 21 (substituting Asn21) and/or position 39 (substituting Ser39), together with the naturally occurring lysine residues at positions 4 and/or 58, were used for the chemoselective crosslinking reaction. The crystal structure of the parental protein Z domain (PDB ID: 1LP1)[37] was used for rational design of the crosslinked variants (Figure S1 in the Supporting Information). The three unstructured N-terminal amino acids Val1-Asp2-Asn3 were deleted, and Pro57 was deleted in the variants involving Lys58 for crosslinking, to locate the amino acids in more favorable positions for the crosslinking reaction.[38] The affibodies were produced by solid-phase peptide synthesis on a Rink amide resin and purified by RP-HPLC (Figure 1). The peptides were synthesized with 4-methyltrityl (Mtt)-protected lysine derivatives for the residues designed to be involved in the crosslinking reaction. Orthogonal removal of the Mtt groups under mildly acidic conditions was successfully performed for all the peptides.

The unprotected lysine residues were functionalized with chloroacetyl groups by DCC-mediated coupling with chloroacetic acid. Ninhydrin tests showed that the chloracetylation was not always efficient after the first reaction, and repeated coupling reactions were performed to reach satisfactory levels, especially in the cases of the K58 and K4K58 variants, in which the C-terminal lysine residue would be expected to be sterically hindered by its proximity to the resin polymer. MS analysis of the crude peptides showed minor impurities with molecular weights corresponding to multiple chloroacetylation, thus indicating that some N_ε-Boc groups had been accidentally deprotected along with the N_ε-Mtt groups. However, the correct product could readily be obtained by RP-HPLC purification (Figure S2, Table S1).

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

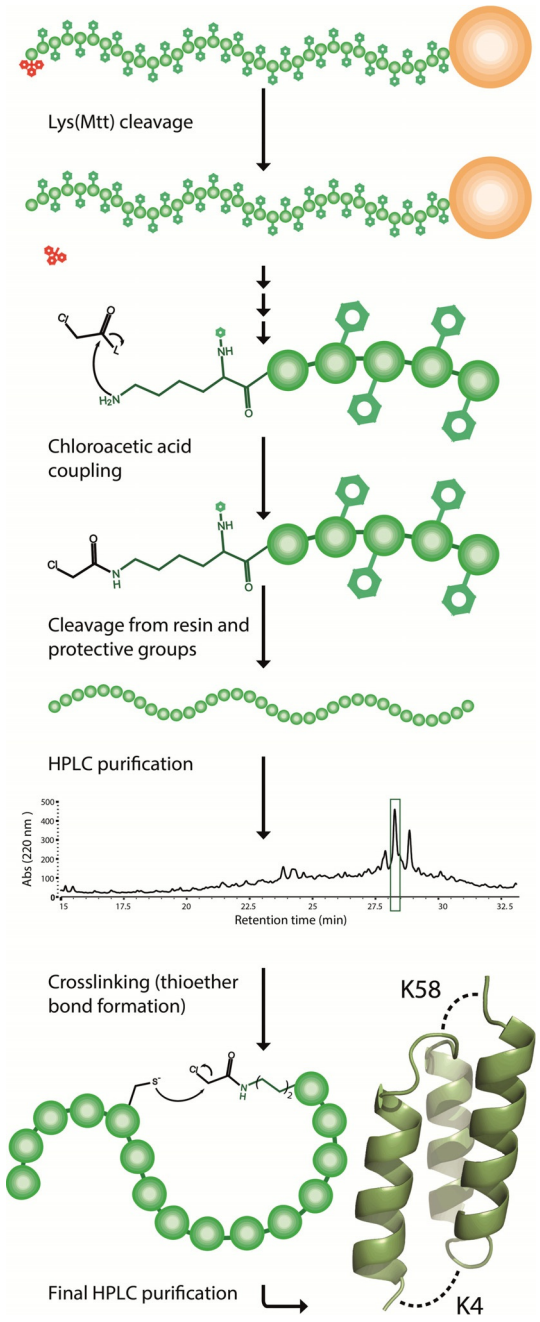

Figure 1. Overview of the synthesis and crosslinking strategy.

After purification and reconstitution in buffer, intramolecular crosslinks were formed through nucleophilic attacks by the thiol groups of the cysteine residues on the α-carbon atoms of the chloroacetyl groups. The amino acid positions were chosen in order to position the reactants in close proximity in the folded three-helix bundle protein domain (Figure S1), and MS analysis showed that the thioether crosslinks were formed in close to quantitative yield in all the protein variants. The correct positioning of the crosslinks was confirmed by a trypsin digestion assay with subsequent MALDI-MS analysis of the peptide fragments (Figures S3–S5, Tables S2–S4). The reaction produced stable, covalent thioether bonds, which would be expected to have stability superior to that of a regular disulfide bond under in vivo conditions.

Circular dichroism

CD measurements were performed to determine the secondary structures and melting temperatures of the crosslinked protein domains. All of the protein domains generated CD curves with local minima characteristic of a helical structure and perfect refolding after the variable-temperature measurements (Figure 2). The melting temperatures were determined by fitting the melting curves to a Boltzmann sigmoidal curve (Table 1). The K4 variant had a melting curve with a similar shape to that of the control protein domain, but with a slightly higher melting temperature, whereas the melting curves of the

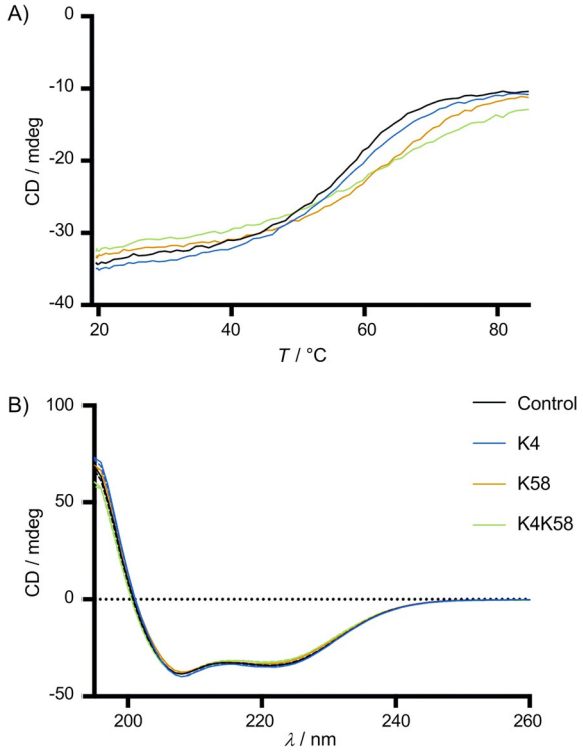

Figure 2. Circular dichroism studies of the protein domain variants. A) Variable-temperature measurements. B) Circular dichroism spectra before (solid lines) and after (dotted lines) variable-temperature measurements.

Table 1. Melting temperatures and kinetic parameters for binding to EGFR.

Protein domain	T_m [°C][a]	k_a [$M^{-1}s^{-1}$][b]	k_d [s^{-1}][b]	K_D [nM][b]
$Z_{EGFR:1907}$ (control)	56.4±0.4	1.7×10^5	6.8×10^{-14}	4.0
$Z_{EGFR:1907}$-K4	57.3±0.3	2.5×10^5	9.8×10^{-4}	3.9
$Z_{EGFR:1907}$-K58	62.7±0.5	2.6×10^5	5.8×10^{-4}	2.2
$Z_{EGFR:1907}$-K4K58	62.5±0.8	2.4×10^5	6.2×10^{-4}	2.9

[a] Melting temperatures determined from Boltzmann sigmoidal curve fit calculated with GraphPad Prism according to the following equation: $Y=$ bottom + (top−bottom)/[1 + exp((V50−X)/slope)]. [b] Kinetic parameters calculated from a 1:1 binding model.

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

K58 and K4K58 variants were flatter. The thermal melting curves of the K4 and K4K58 variants indicate that the protein domains are thermally stabilized by the thioether crosslink between residues 21 and 58. However, the discrepant shape of the melting curves suggests that the unfolding of the K58 and K4K58 variants is a gradual transition rather than a cooperative two-state transition. This result was not unexpected, because we had previously observed that intramolecular crosslinking of a different three-helix protein resulted in variants that showed non-cooperative unfolding in variable-temperature measurements, making it impossible to determine a melting temperature reliably.[36]

Surface-plasmon-resonance-based (SPR-based) biosensor analysis

The binding of the protein domains to the extracellular domain of EGFR was measured in real time by SPR-based biosensor analysis with a Biacore T200 instrument (Figure S6). The data were fitted to a 1:1 binding model, and all crosslinked variants showed equilibrium dissociation constant (K_D) values similar to that of the control protein domain, thus demonstrating that the function of the protein domains is not negatively affected by the intramolecular crosslinks (Table 1). The K4 and K4K58 variants had slightly improved K_D values, thus suggesting that the protein domains might even benefit from the increased structural rigidity. Reduction of conformational flexibility by cyclization has previously been linked to higher potency for peptide-based ligands.[26,39,40]

In vitro digestion assay

In vitro digestion assays were performed to test whether the proteolytic stability of the crosslinked protein domains was increased. The measurements were divided into two assays: one with pepsin, which is the main digestive enzymes present in the stomach, and one with trypsin and chymotrypsin, which are found in the small intestine. The digestion was followed over time by RP-HPLC analysis, and the peak corresponding to the intact protein domain was integrated (for a representative RP-HPLC elution profile, see Figure S7). All crosslinked protein domains showed increased proteolytic stability in the pepsin assay, with the greatest improvement for K4K58, followed by K4, and then K58 (Figure 3 A). In the trypsin-plus-chymotrypsin digestion assay, K4K58 and K58 were more stable than both the control protein domain and K4, and the K4 variant showed no significant improvement in stability relative to the control protein domain (Figure 3 B). The protease concentrations and assay time points were chosen to highlight the differences in stability between the crosslinked protein domains and the control. Under these experimental conditions, the half-life in the presence of pepsin was increased from 4 min for the unmodified protein domain to 102 min for K4K58, the most stable of the crosslinked variants (Table 2). In the presence of trypsin and chymotrypsin, the half-life was increased from 5 min for the unmodified protein domain to 19 min for the K4K58 variant.

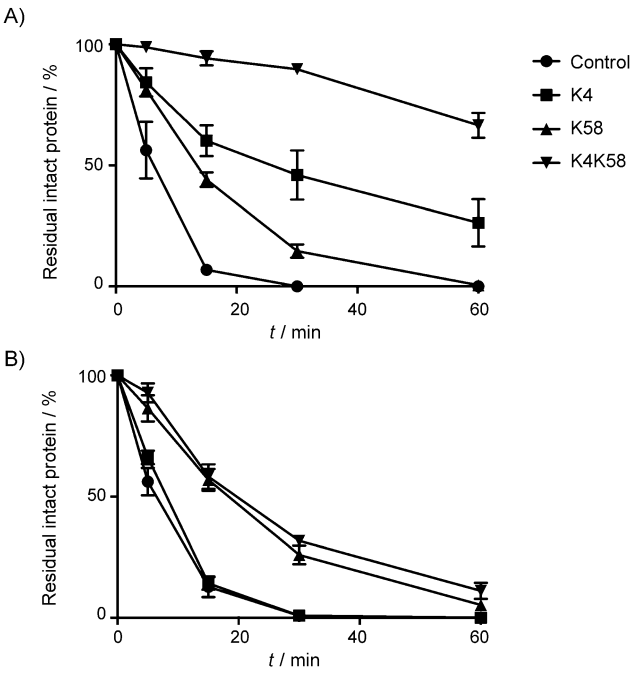

Figure 3. In vitro digestion of the crosslinked variants and the control protein domain. A) Digestion with pepsin (0.2 μg mL^{-1}). B) Digestion with trypsin (9 μg mL^{-1}) plus chymotrypsin (4 μg mL^{-1}).

Table 2. Proteolytic degradation.

Protease	Protein domain	$t_{1/2}$ [min]
pepsin	$Z_{EGFR:1907}$ (control)	4
pepsin	$Z_{EGFR:1907}$-K4	31
pepsin	$Z_{EGFR:1907}$-K58	11
pepsin	$Z_{EGFR:1907}$-K4K58	102
trypsin + chymotrypsin	$Z_{EGFR:1907}$ (control)	5
trypsin + chymotrypsin	$Z_{EGFR:1907}$-K4	5
trypsin + chymotrypsin	$Z_{EGFR:1907}$-K58	14
trypsin + chymotrypsin	$Z_{EGFR:1907}$-K4K58	19

The CD studies show that the protein domains K58 and K4K58, both containing crosslinks between positions 21 and 58, have higher thermal stability than the control protein domain at pH 7.4. Both K58 and K4K58 are also significantly more stable to trypsin and chymotrypsin than either the control protein domain or K4, and it can be speculated that the higher thermal stability is associated with lower accessibility for the action of proteases. In contrast, K4 shows the highest stability to pepsin assay, but this difference can be explained in terms of the protonation of the protein domains at pH 2.6, which is likely to affect the protein structure.

In a separate experiment, the control protein domain was digested with each protease separately and the molecular weights of the most abundant digestion products were determined by mass spectrometry. The measured molecular weights matched the theoretical cleavage sites with high accuracy (Figure 4).

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

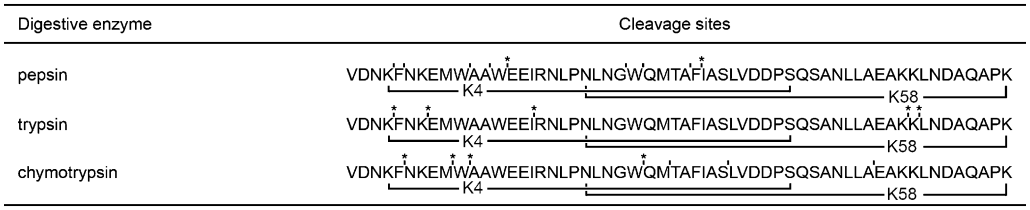

Figure 4. Amino acid sequence of the $Z_{EGFR:1907}$ affibody with the theoretically predicted (') and experimentally verified (*) cleavage sites for the gastrointestinal proteases pepsin, trypsin, and chymotrypsin. The positions used for intramolecular crosslinking in the K4, K58, and K4K58 variants are shown.

The results show that crosslinking between positions 21 and 58 in the K58 variant increases the stability in the trypsin-plus-chymotrypsin assay even though several of the most commonly observed cleavage sites are located in the N-terminal, non-crosslinked part of the protein domain. In the pepsin assay, the amino acids involved in the crosslinking were located seven residues, or even further, away from an experimentally confirmed cleavage site. These two observations indicate not only that the amino acid residues directly involved in the crosslinking reaction are protected from proteolytic cleavage, but also that the intramolecular bridges provide general stability to the protein domains, extending beyond the crosslinking sites. An explanation for this effect could be that the chemical crosslinking makes the protein domain structure less flexible, and thereby less accessible to the action of the proteases. Because of the long-range protective effect of the intramolecular crosslinks the strategy is more generally applicable than substitution of the amino acids in the predicted cleavage sites, which could be important for the function of the protein domain.

For the protein domain used in this study, several of the experimentally observed cleavage sites in the EGFR-binding affibody involve amino acids in positions of helices 1 and 2 that were randomized in the affibody library.[25] For example, the dipeptide Trp13–Glu14, which is cleaved by pepsin, is found in several of the selected clones that bind with high affinity to EGFR and can thus be expected to be involved in the binding interaction. Likewise, the protease sites Arg17–Asn18, which is cleaved by trypsin, and Met9–Trp10, which is cleaved by chymotrypsin, are composed of amino acids that were selected from randomized positions in the affibody library for binding to EGFR. Through the use of the chemical crosslinking strategy presented here the substitution of the amino acid residues that potentially are part of the binding site is avoided, and, as shown by the CD and SPR experiments, the intramolecular crosslinks do not affect the conformation of the protein domain or interfere with the binding. Together with our earlier results on stabilization of an albumin-binding domain,[36] this study shows that intramolecular chemical crosslinking can be used to increase the stability of small, helical proteins towards gastrointestinal proteases without negatively affecting their function. Even an oral bioavailability as low as 1% has been shown to result in oral activity for biologically active peptides,[17] and so we envision that the presented strategy could be a step towards orally available protein drugs. The crosslinking strategy can potentially be applied for stabilization of different proteins based on the affibody scaffold, many of which have great therapeutic potential. Several studies of affibody molecules have demonstrated in vivo function after subcutaneous administration, as exemplified by tumor targeting in molecular imaging applications based on radiolabeled HER2-binding affibody molecules[41] and by the anti-inflammatory effect demonstrated by an IL-6-binding affibody molecule fused to a TNF-binding antibody.[42] Furthermore, a Phase II clinical trial for treatment of psoriasis with an IL-17-binding affibody molecule has recently been initiated (www.affibody.se). These studies demonstrate that the affibody molecules can be biologically active if delivered to the blood and suggest that further investigation of opportunities for oral delivery are warranted.

Conclusion

We have explored a synthetic strategy based on intramolecular thioether crosslinking to stabilize an affibody molecule binding the cancer-associated target EGFR. Three different protein domain variants, containing single or double crosslinks, were synthesized and evaluated. It was shown that the intramolecular crosslinking increased the stability of the protein domains towards digestion by the gastrointestinal proteases pepsin, trypsin, and chymotrypsin, and that the crosslinked affibody molecules retained their high-affinity binding to EGFR. The results suggest that the crosslinking strategy could be used to stabilize therapeutic proteins based on the affibody protein scaffold for improved oral activity.

Experimental Section

Peptide synthesis: The EGFR-binding affibody molecule $Z_{EGFR:1907}$[25] and three variants thereof—$Z_{EGFR:1907}$-K4, $Z_{EGFR:1907}$-K58, and $Z_{EGFR:1907}$-K4K58, hereafter denoted K4, K58, and K4K58—were produced by solid-phase peptide synthesis with a fully automated synthesis instrument and microwave application (Liberty and Discovery, CEM Corporation) by use of Fmoc chemistry on a 0.1 mmol scale. A Rink amide resin was used as solid support (NovaBiochem), with 0.17 mmol g^{-1} substitution for K58 and 0.36 mmol g^{-1} substitution for the other peptides. *N*-Methylpyrrolidin-2-one (NMP, Carlo Erba) was used as the main solvent and during the washing steps of the synthesis. The amino acids were activated with DIEA/HBTU/HOBt/aa 2:1:1:1 [DIEA is diisopropylethylamine, HBTU is 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (AK Scientific), HOBt is 1-hydroxybenzotriazole (AK Scientific)]. The temporary Fmoc protecting group was cleaved with piperazine (Alfa Aesar, 5%) in NMP. Acetic anhydride [acetic anhydride (0.5 M), HOBt (0.015 M), DIEA 0.125 M, Merck)] was used as a capping agent after

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

each round of amino acid coupling. The protecting groups used for the amino acids were: 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) on Arg, *tert*-butyl (*t*Bu) on Thr and Ser, *tert*-butyl ester (O*t*Bu) on Glu and Asp, trityl (Trt) on Asn, Cys, and Gln, and *tert*-butyloxycarbonyl (Boc) on Lys and Trp. Mtt was used as an orthogonal protecting group for the lysine residues involved in the crosslinking reaction. After final Fmoc removal, the N-terminal lysine residues of K4 and K4K58 were Boc-protected by treatment of the peptide-resin with Boc_2O/DIEA/aa (10:5:1; Boc_2O is di-*tert*-butyl dicarbonate, Merck) dissolved in NMP for 2×1 h. The N-terminal lysine residue in K58 was introduced as a Boc-Lys(Boc) derivative. After completed assembly of the peptide sequence, Lys(Mtt) was deprotected with CH_2Cl_2/TIS/TFA 94:5:1 [CH_2Cl_2 (Carlo Erba), TIS is triisopropylsilane (Aldrich), TFA is trifluoroacetic acid (Merck)] for 1 min. The process was repeated either 15 times or until the reaction solution stopped turning yellow. The deprotected Lys was acylated with chloroacetic acid (AcCl, Aldrich) in the presence of 1,3-dicyclohexylcarbodiimide (DCC) as an activator. AcCl (40 equiv) and DCC (40 equiv) were dissolved separately in CH_2Cl_2, mixed, and added to the resin, and the solution was incubated with shaking for 45 min. A 20 times molar excess of DIEA was added, and the reaction mixture was incubated with shaking for an additional 90 min. The peptides were cleaved from the resin by treatment with TFA/H_2O/EDT/TIS 94:2.5:2.5:1 for 2 h at RT. The crude peptide was extracted three times in *tert*-butyl methyl ether (Merck)/H_2O 1:1. The water phase containing the peptide was mixed with acetonitrile (Merck) until the peptide was completely dissolved, and the solution was lyophilized.

Purification and crosslinking: The peptides were purified by reversed-phase HPLC (Agilent technologies) on an analytical column (Zorbax 300SB-C18, 3.5 μm particle size, 4.6×15 mm) and a gradient of 25–55% B (A is 0.1% TFA/H_2O, B is 0.1% TFA/CH_3CN) over 25 min at a flow rate of 1 mL min^{-1}. The peaks were collected and analyzed by MALDI mass spectrometry (LT3 Plus, Scientific Analysis Instruments, Manchester, UK). The fractions containing the correct product were pooled and lyophilized. For the intramolecular crosslinking reaction, the peptide was dissolved in 1×phosphate-buffered saline (PBS, pH 8.75) at a maximum concentration of 0.1 mg mL^{-1} and incubated overnight at RT on a shaker table. The 1×PBS buffer was exchanged for H_2O by using a centrifuge filter unit (Amicon Ultra-15, 3000 MWCO, Merck). Finally, the product was purified by RP-HPLC as described above and lyophilized. The lyophilized peptides were dissolved in a volatile buffer (ammonium acetate, 50 mM, pH 5.6) to provide the ability to aliquot and lyophilize the samples. The concentrations were determined by amino acid analysis (Alphalyse A/S, Odense, Denmark), and the amino acids involved in the crosslinking were excluded when calculating the concentrations. The crosslinked protein domains were digested with trypsin, and the cleavage products were analyzed to confirm that the crosslinks were at the desired locations. The affibody (5 μg) was dissolved in 1×PBS (pH 7.4) and mixed with trypsin (34 μL, 12443 U mg^{-1}, Sigma) to a final concentration of 5 μg mL^{-1}. The reaction was stopped after 2 h and the resulting peptide fragments were analyzed by MALDI mass spectrometry (4800 MALDI TOF-TOF, Sciex).

Circular dichroism: CD spectra of the protein domains were measured at a concentration of 32 μM in 1×PBS before and after variable-temperature measurements (VTMs). CD measurements were performed with a Chirascan qCD instrument (Applied Photophysics) at 260–195 mdeg, 0.5 s/point, at 20 °C, $N=5$ scans. The T_m was determined through a Boltzmann sigmoidal curve fit as the temperature at which 50% of the protein domain was denatured at 220 nm during a gradual increase in temperature from 20 to 85 °C.

SPR-based biosensor analysis: The binding of the crosslinked affibody molecules and the control protein domain to EGFR was analyzed by use of a Biacore T200 system (GE Healthcare) and a carboxylated dextran-coated CM5 sensor chip. The chip was activated with *N*′-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), followed by immobilization of recombinant human EGFR-Fc Chimera (R&D systems) to a level of 2450 RU. The samples were diluted in 1×PBST (pH 7.4) at five different approximate concentrations—50, 25, 12.5, 6.25, and 3.125 nM—and were allowed to flow over the chip at 50 μL min^{-1}. The association time was measured over 400 s and the dissociation time over 1000 s. The surface was regenerated with a pulse of HCl (10 mM) after each injection. The binding was replicated twice, and the K_D value was determined from fitting the data to a 1:1 binding model after adjustment of the concentrations according to the amino acid analysis.

In vitro digestion assays: To simulate the proteolytic degradation of proteins in the stomach the affibody variants were exposed to pepsin (3200–4500 U mg^{-1}, Sigma) from porcine gastric mucosa. To simulate the environment in the intestine the protein domains were exposed to a combination of trypsin (12443 U mg^{-1}, Sigma) and α-chymotrypsin (≥40 U mg^{-1}, Sigma), both from bovine pancreas. In all the assays the protein domains were diluted in 1×PBS (pH 7.4) to provide a final amount of 5 μg protein domain per time point. Cleavage was measured after $t=0$, 5, 15, 30, and 60 min. In the pepsin assay, pepsin (18 μL) in HCl (10 mM) was added to give a final concentration of 0.2 μg mL^{-1} pepsin, with a final volume of 52 μL and a pH of around 2.6. The reaction was stopped by addition of NaOH (0.1 M, 5 μL). In the pancreatin assay, trypsin and chymotrypsin (18 μL) in 1×PBS (pH 7.4) was added to the protein domain solution to give a final concentration of 0.9 μg mL^{-1} trypsin and 0.4 μg mL^{-1} chymotrypsin in a volume of 52 μL. The reaction was stopped by addition of TFA (10%, 3 μL). All samples were analyzed by RP-HPLC at 35 °C, with a flow rate of 1 mL min^{-1} and a gradient of 25–65% B over 25 min. The elution peaks from the different digestion time points were integrated and normalized to the undigested sample at $t=0$. The degradation experiments were performed in technical triplicates, and the mean and standard deviation for each time point were calculated.

Acknowledgements

This research was financially supported by grants from the Swedish Research Council (grants 621-2013-5135 and 2016-05207).

Conflict of Interest

The authors declare no conflict of interest.

Keywords: affibodies · crosslinking · protein engineering · protein modifications · thioethers

[1] A. C. Chan, P. J. Carter, *Nat. Rev. Immunol.* **2010**, *10*, 301.
[2] A. M. Scott, J. D. Wolchok, L. J. Old, *Nat. Rev. Cancer* **2012**, *12*, 278.
[3] B. Leader, Q. J. Baca, D. E. Golan, *Nat. Rev. Drug Discovery* **2008**, *7*, 21.
[4] D. J. Craik, D. P. Fairlie, S. Liras, D. Price, *Chem. Biol. Drug Des.* **2013**, *81*, 136.

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[5] J. Renukuntla, A. D. Vadlapudi, A. Patel, S. H. S. Boddu, A. K. Mitra, *Int. J. Pharm.* **2013**, *447*, 75.
[6] E. S. Swenson, W. J. Curatolo, *Adv. Drug Delivery Rev.* **1991**, *8*, 39.
[7] J. Woodley, *Crit. Rev. Ther. Drug Carr. Syst.* **1994**, *8*, 61.
[8] T. A. S. Aguirre, D. Teijeiro-Osorio, M. Rosa, I. S. Coulter, M. J. Alonso, D. J. Brayden, *Adv. Drug Delivery Rev.* **2016**, *106*, 223.
[9] E. Moroz, S. Matoori, J.-C. Leroux, *Adv. Drug Delivery Rev.* **2016**, *101*, 108.
[10] A. Bernkop-Schnürch, *J. Controlled Release* **1998**, *52*, 1.
[11] G. Drapeau, E. Petitclerc, A. Toulouse, F. Marceau, *Antimicrob. Agents Chemother.* **1992**, *36*, 955.
[12] B. J. Aungst, *AAPS J.* **2012**, *14*, 10.
[13] T. Lindmark, J. Söderholm, G. Olaison, G. Alván, G. Ocklind, P. Artursson, *Pharm. Res.* **1997**, *14*, 930.
[14] B. Chassaing, O. Koren, J. K. Goodrich, A. C. Poole, S. Srinivasan, R. E. Ley, A. T. Gewirtz, *Nature* **2015**, *519*, 92.
[15] D. J. Craik, N. L. Daly, T. Bond, C. Waine, *J. Mol. Biol.* **1999**, *294*, 1327.
[16] M. L. Colgrave, D. J. Craik, *Biochemistry* **2004**, *43*, 5965.
[17] K. Thell, R. Hellinger, E. Sahin, P. Michenthaler, M. Gold-Binder, T. Haider, M. Kuttke, Z. Liutkevičiūtė, U. Göransson, C. Gründemann, G. Schabbauer, C. W. Gruber, *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 3960.
[18] A. G. Poth, L. Y. Chan, D. J. Craik, *Biopolymers* **2013**, *100*, 480.
[19] C. T. T. Wong, D. K. Rowlands, C.-H. Wong, T. W. C. Lo, G. K. T. Nguyen, H.-Y. Li, J. P. Tam, *Angew. Chem. Int. Ed.* **2012**, *51*, 5620; *Angew. Chem.* **2012**, *124*, 5718.
[20] A. Fahr, *Clin. Pharmacokinet.* **1993**, *24*, 472.
[21] E. Kudryashova, S. M. Seveau, D. S. Kudryashov, *Biol. Chem.* **2017**, https://doi.org/10.1515/hsz-2017-0106.
[22] S. Ståhl, T. Gräslund, A. Eriksson Karlström, F. Y. Frejd, P.-Å. Nygren, J. Löfblom, *Trends Biotechnol.* **2017**, *35*, 691 – 712.
[23] R. Vazquez-Lombardi, T. G. Phan, C. Zimmermann, D. Lowe, L. Jermutus, D. Christ, *Drug Discovery Today* **2015**, *20*, 1271.
[24] T. Engfeldt, B. Renberg, H. Brumer, P. Å. Nygren, A. Eriksson Karlström, *ChemBioChem* **2005**, *6*, 1043.
[25] M. Friedman, A. Orlova, E. Johansson, T. L. J. Eriksson, I. Höidén-Guthenberg, V. Tolmachev, F. Y. Nilsson, S. Ståhl, *J. Mol. Biol.* **2008**, *376*, 1388.
[26] G. Gerona-Navarro, S. Mujtaba, A. Frasca, J. Patel, L. Zeng, A. N. Plotnikov, R. Osman, M.-M. Zhou, *J. Am. Chem. Soc.* **2011**, *133*, 2040.
[27] M. Muttenthaler, A. Andersson, A. D. de Araujo, Z. Dekan, R. J. Lewis, P. F. Alewood, *J. Med. Chem.* **2010**, *53*, 8585.
[28] M. Akamatsu, P. P. Roller, L. Chen, Z.-Y. Zhang, B. Ye, T. R. J. Burke, *Bioorg. Med. Chem.* **1997**, *5*, 157.
[29] M. Cai, M. Stankova, D. Muthu, A. Mayorov, Z. Yang, D. Trivedi, C. Cabello, V. J. Hruby, *Biochemistry* **2013**, *52*, 752.
[30] F. M. Brunel, P. E. Dawson, *Chem. Commun.* **2005**, 2552.
[31] A. M. Spokoyny, Y. Zou, J. J. Ling, H. Yu, Y.-S. Lin, B. L. Pentelute, *J. Am. Chem. Soc.* **2013**, *135*, 5946.
[32] T. Nishihara, H. Kitada, D. Fujiwara, I. Fujii, *Biopolymers* **2016**, *106*, 415.
[33] Y. Wang, D. H.-C. Chou, *Angew. Chem. Int. Ed.* **2015**, *54*, 10931; *Angew. Chem.* **2015**, *127*, 11081.
[34] Y. Wang, B. J. Bruno, S. Cornillie, J. M. Nogieira, D. Chen, T. E. Cheatham, C. S. Lim, D. H.-C. Chou, *Chem. Eur. J.* **2017**, *23*, 7087.
[35] D. P. Fairlie, A. Dantas de Araujo, *Biopolymers* **2016**, *106*, 843.
[36] J. Lindgren, A. Eriksson Karlström, *ChemBioChem* **2014**, *15*, 2132.
[37] M. Högbom, M. Eklund, P.-A. Nygren, P. Nordlund, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3191.
[38] T. Ekblad, V. Tolmachev, A. Orlova, C. Lendel, L. Abrahmsén, A. E. Karlström, *Biopolymers* **2009**, *92*, 116.
[39] H. Kumagai, M. Jajima, Y. Ueno, Y. Giga-Hama, M. Uhba, *Biochem. Biophys. Res. Commun.* **1991**, *177*, 74.
[40] Z. Zhang, Z. Lin, Z. Zhou, H. C. Shen, S. F. Yan, A. V. Mayweg, Z. Xu, N. Qin, J. C. Wong, Z. Zhang, Y. Rong, D. C. Fry, T. Hu, *ACS Med. Chem. Lett.* **2014**, *5*, 993.
[41] T. Ekblad, T. Tran, A. Orlova, C. Widström, J. Feldwisch, L. Abrahmsén, A. Wennborg, A. E. Karlström, V. Tolmachev, *Eur. J. Nucl. Med. Mol. Imaging* **2008**, *35*, 2245.
[42] F. Yu, L. Gudmundsdotter, A. Akal, E. Gunneriusson, F. Frejd, P.-Å. Nygren, *mAbs* **2014**, *6*, 1598.

Manuscript received: June 30, 2017
Accepted manuscript online: August 23, 2017
Version of record online: September 19, 2017

© 2017 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim