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Engineered staphylococcal protein A's IgG-binding domain with cathepsin L inhibitory activity

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Abstract

Inhibitory peptide of papain-like cysteine proteases, affinity selected from a random disulfide constrained phage-displayed peptide library, was grafted to staphylococcal protein A's B domain. Scaffold protein was additionally modified in order to allow solvent exposed display of peptide loop. Correct folding of fusion proteins was confirmed by CD-spectroscopy and by the ability to bind the Fc-region of rabbit IgG, a characteristic of parent domain. The recombinant constructs inhibited cathepsin L with inhibitory constants in the low-micromolar range.

Keywords: Cysteine proteases; Inhibitory peptide; Constrained loop; Staphylococcal protein A; Protein scaffold; Dual functionality

Peptides are often looked upon as prototype modulators of biological function, since they are found in all living beings performing functions of hormones, antigens, biochemical inhibitors, growth factors, transmembrane carriers, etc. [1]. They have also attracted attention of medicinal chemists ever since it became evident they generally form highly specific interactions with (protein) targets. Linear peptides, however, are usually hampered by relatively low affinity for receptor proteins due to their pronounced flexibility and—as a direct consequence of that—considerable entropy change on receptor binding. Cyclization of linear peptides is thus frequently used to provide conformationally restricted analogues [1].

We have recently reported on small peptide inhibitors of cysteine cathepsins' affinity selected through phage display [2]. Conformational freedom of these peptides was restricted by an intramolecular disulfide bond, resulting in looped peptides possessing higher inhibitory activity compared to opened linear peptides. Unfortunately, cysteine proteases require reductive media for optimal enzymatic activity, preventing oxidation of the active site thiol group. The incompatibility of our peptides' structural features (unstable cyclic form) was overcome by preparing head-to-tail cyclized peptide *G*NWTLGGYK*GG*, an analogue of the relatively broadly acting inhibitor *C*NWTLGGYK*C*. The former peptide turned out to have increased affinity for cathepsins L and K (26- and 2.7-fold, respectively), but was also more selective (it no longer inhibited cathepsin H and had diminished activity towards papain).

In this paper we describe the grafting of inhibitory peptide sequence NWTLGGYK to a small three helical protein, domain B from staphylococcal protein A (SpA). B domain was chosen as a scaffold for numerous reasons: it is composed of only 58 AA and is cysteine free (thus resistant to reductants) but nevertheless possesses a stable fold, it is highly soluble, easily expressed in *Escherichia coli*, fairly resistant to proteases, its 3D structure is well-known, and, last but not least, its IgG-Fc-binding property is an attractive feature for isolation purposes or use as Fc-affinity marker.

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Z domain, a recombinant analogue of B domain, has been extensively used as a scaffold protein to (in combination with site-directed mutagenesis and screening techniques such as phagemid display) select new ligands to virtually any target [3–5]. In contrast to ''affibodies'', where amino acids in the first two helices, responsible for Fc-binding, were mutated, we have prepared B domain analogues with exchanged single loop between the second and the third helix.

Materials and methods

Protein engineering, expression, and purification. Molecular cloning was essentially performed as described in [6]. Oligonucleotide primers (Table 1) were synthesized by Invitrogen (Carlsbad, CA, USA). Enzymes for molecular cloning were supplied by Fermentas (Burlington, Ont., Canada; *Nco*I and *Hin*dIII), New England Biolabs (Ipswich, MA, USA; *Xho*I), and Promega (Madison, WI, USA; T4 DNA ligase). Cloning vector pGEM-T Easy was procured from Promega and expression vector pET28a(+) was from Novagen (San Diego, CA, USA). For isolation of high-copy plasmids (pGEM-T Easy) FastPlasmid Mini from Eppendorf (Hamburg, Germany) was used, while low-copy plasmids (pET28a(+)) were isolated by Wizard Plus Minipreps DNA Purification System from Promega.

Two types of fusion proteins were constructed (Table 2): both types (I and II) had an exchanged loop between the second and the third helix while type II was additionally N-terminally abridged. All the recombinant proteins produced had C-terminal hexahistidine tag to facilitate isolation.

Colony PCR with primers F-Bd and R-Bd was used to obtain the gene coding for B domain of SpA from genomic DNA of *Staphylococcus aureus*. This was further amplified with primers F-*Nco*I and R-*Xho*I to introduce appropriate restriction sites to 5′- and 3′-end of the gene (pB, basis for all successive constructs). Gene for pB- was PCR-amplified from gene coding for pB using primers F-*Nco*I_2 and R-*Xho*I. Gene fragments coding for loops in pB_I10, $pB_{II}10$, and $pB_{II}12$ were exchanged through endogenously present *Hin*dIII restriction site within the B domain gene (Fig. 1). 5′- and 3′-gene segments were separately PCR-amplified from appropriate templates using the following combinations of primers: F-*Nco*I/R-*Hin*dIII and F-*Hin*dIII/R-*Xho*I for pB_I10 (gene coding for pB was used as the template), F-*Nco*I_2/R-*Hin*dIII and F-*Hin*dIII/R-*Xho*I for $pB_{II}10$, and *Nco*I_2/R-*Hin*dIII and F-*Hin*dIII_2/R-*Xho*I for $pB_{II}12$ (both times gene coding for pB- was used as the template). Proper 5′- and 3′-segments were ligated to produce genes coding for pB_I10, $pB_{II}10$, or $pB_{II}12$. All gene constructs were finally subcloned into pET28a(+) in a manner enabling fusions to hexahistidine tag.

Proteins were produced in *E. coli* BL21 (DE3)/pLysS expression host at 37 or 25 °C following induction with 1 mM IPTG. Bacterial cells were sonicated, cellular debris was pelleted by centrifugation, and proteins from supernatant fraction were isolated on Talon resin (BD Biosciences, Bedford, MA, USA). Proteins' purity was checked by performing SDS–PAGE followed by Coomassie staining.

Determination of protein concentration. Concentration of purified proteins was estimated by the Bradford assay using commercial Coomassie Plus kit from Pierce (Rockford, IL, USA) and BSA as protein standard.

CD spectroscopy. Far-UV CD-spectra of recombinant proteins were taken on a 62A DS Aviv CD-spectrophotometer (Lakewood, NJ, USA) in a 1 mm quartz cell at 25 °C (scan window 260–200 nm, intervals of 1 nm). Samples were diluted to 30–80 μM with 50 mM NaOAc, pH 5.0, 300 mM NaCl. Buffer baseline was subtracted from the protein CD data.

ELISA. Each protein was diluted to concentrations of 10, 20, 50, and 100 μg/ml in PBS, pH 7.4. Two-hundred microliters of each dilution was pipetted into preblocked HIS-Select High Capacity Nickel Coated microtiter plate wells (Sigma–Aldrich) and agitated gently for 4 h at room temperature. Additionally, PBS alone was used as negative control. Wells were washed 3 times with PBS containing 0.05% Tween 20 (PBST). Two-hundred microliters of horseradish peroxidase-labeled polyclonal rabbit anti-goat IgG (Jackson ImmunoResearch Europe, Soham, UK) (1:1000) in PBS containing 0.05% casein was added and incubation was continued for 2 h at room temperature. Wells were washed again 3 times with PBST. Finally, 200 μl of substrate solution (0.22 mg/ml diammonium 2,2′-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) in 50 mM citric acid, 0.05% H_2O_2, pH 4.0) was added. Absorbance was determined after 10-min incubation at 37 °C on a Safire^2 plate reader (Tecan, Salzburg, Austria) at 405 nm.

Determination of inhibitory activity of recombinant proteins. The inhibitory activities of recombinant proteins were determined by an *in vitro* assay for measuring cysteine protease activity as previously described [2], except that reductant concentration was kept high throughout the assay. Briefly, the enzymes, papain and cathepsins L and K, were activated with 1 mM DTT in an optimal pH buffer, containing 1 mM EDTA (0.1 M phosphate buffer, pH 6.8, for papain; 0.1 M acetate buffer, pH 5.5, for cathepsins L and K). Constant amount of activated enzyme was preincubated with increasing amounts of recombinant proteins. Then, solution of Z-Phe-Arg-MCA (Sigma–Aldrich, Steinheim, Germany) was added and incubation was continued for 10 min at 37 °C. Addition of 1 mM iodoacetic acid stopped the reaction and released methylcoumarylamide was measured fluorimetrically at 460 nm (370 nm excitation wavelength). In parallel, control assay of enzyme activity in absence of recombinant proteins was performed. Non-linear dose–response curve of residual activity was obtained and graphically determined apparent inhibition constant [7] was finally corrected for substrate influence using equation

$$K_i = K_{i(app)}/(1 + [S]/K_m), \quad (1)$$

where [S] is the substrate concentration and K_m is the Michaelis constant.

Results

Protein engineering, expression, and purification

Due to high homology between extracellular domains of SpA on genetic level, gene fragments coding for different domains were PCR-amplified from genomic DNA of

Table 1
Nucleotide sequences of the primers

Primer	Nucleotide sequence (5′ → 3′)
F-Bd	GCTGATAACAAATTCAACAAAGAAC
R-Bd	TTTTGGTGCTTGTGCATCATTTAGC
F-*Nco*I	ACCTCCATGGCTGATAACAAATTCAAC
F-*Nco*I_2	CTTGCCATGGAACAACAAAATGCTTTC
R-*Xho*I	TCACCTCGAGTTTTGGTGCTTGTGCATC
F-*Hin*dIII	TCCAAAGCTTAAAAGATGCTAATTGGACTCTGGGTGGTTATAAGGCTAGCGCTAACCTTTTAG
F-*Hin*dIII_2	TCCAAAGCTTAAAAGATGGTGCTAATTGGACTCTGGGTGGTTATAAGGCTGGTAGCGCTAACCTTTTAG
R-*Hin*dIII	TTTTAAGCTTTGGATGAAACC

Restriction sites are underlined.

Table 2
Primary structures of native B domain and recombinant proteins

Protein	Type[a]	Amino acid sequence
B domain	Native	ADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKD----*DPSQ*----SANLLAEAKKLNDAQAPK
pB	–	MADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKD----*DPSQ*----SANLLAEAKKLNDAQAPKLEHHHHHH
pB_I10	I	MADNKFNKEQQNAFYEILHLPNLNEEQRNGFIQSLKD-*ANWTLGGYKA*-SANLLAEAKKLNDAQAPKLEHHHHHH
pB-	–	MEQQNAFYEILHLPNLNEEQRNGFIQSLKD----*DPSQ*----SANLLAEAKKLNDAQAPKLEHHHHHH
$pB_{II}10$	II	MEQQNAFYEILHLPNLNEEQRNGFIQSLKD-*ANWTLGGYKA*-SANLLAEAKKLNDAQAPKLEHHHHHH
$pB_{II}12$	II	MEQQNAFYEILHLPNLNEEQRNGFIQSLKD*GANWTLGGYKAG*SANLLAEAKKLNDAQAPKLEHHHHHH

Helical amino acids are shown in gray and the loop between the second and the third helix is shown in italics.
[a] See text for details.

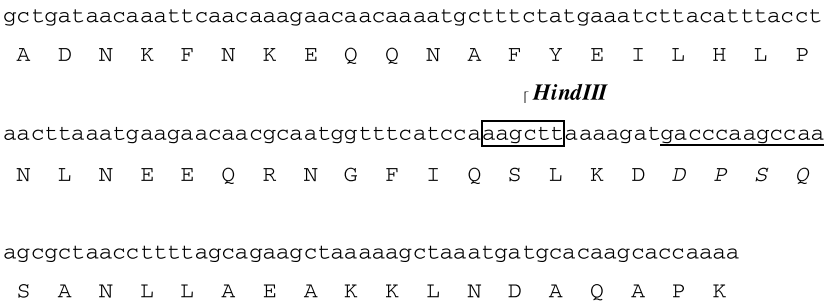

Fig. 1. Nucleotide and encoded amino acid sequence of native B domain. Endogenously present restriction site recognized by endonuclease *Hin*dIII is boxed. Segment encoding second loop is underlined.

S. aureus with the combination of primers F-Bd and R-Bd. Some of them were sequenced and the gene encoding B domain was used as the starting point for further molecular cloning.

Proteins were purified from sonically lysed bacteria under native conditions. Expression of soluble proteins was found to be optimal at 37 °C for pB and pB_I10 or 25 °C for pB-, $pB_{II}10$, and $pB_{II}12$. Purified proteins were visualized as a single ~7–8 kDa band on SDS–PAGE (data not shown).

CD-spectroscopy

For each protein three CD-spectroscopic measurements were performed. In Fig. 2 averaged data are given with buffer baseline subtracted.

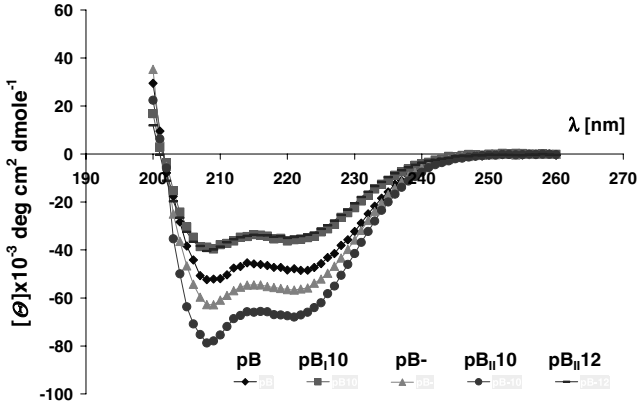

Fig. 2. CD-spectra of recombinant B domain analogues.

ELISA

All five recombinant constructs retained the ability to bind IgG. Affinities were, however, not identical, as pB and pB_I10 bound strongest, binding of pB- was somewhat weaker, and abridged fusion proteins $pB_{II}10$ and $pB_{II}12$ interacted weaker still with rabbit polyclonal IgG (Fig. 3).

Determination of inhibitory activity of recombinant proteins

All five proteins were tested for potential inhibitory activity against papain and cathepsins L and K. $pB_{II}10$ and $pB_{II}12$ inhibited cathepsin L with K_i of 14 and 5.8 μM, respectively. None of the proteins inhibited papain or cathepsin K.

Discussion

In order for our fusion proteins to exhibit inhibitory action towards cysteine cathepsins two conditions had to be met. First, the exchanged inhibitory loop should be able to adopt the active conformation. For this reason peptide sequence (G)ANWTLGGYKA(G) was inserted in place

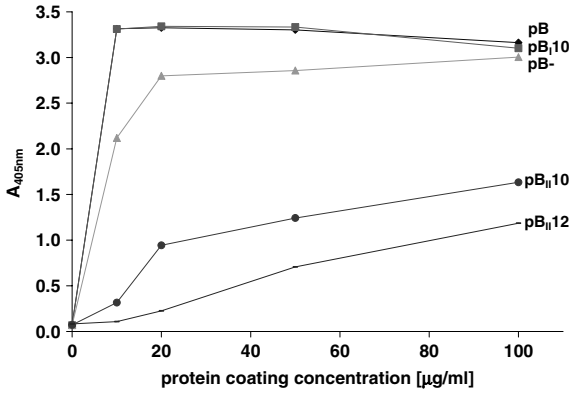

Fig. 3. Binding of B domain analogues to rabbit IgG in ELISA. High affinity of pB and pB_I10 to IgG is confirmed by reaching absorbance plateau at protein coating concentrations as low as 10 μg/ml. Affinity of pB- to IgG is somewhat lower, while Fc-binding of $pB_{II}10$ and $pB_{II}12$ is the weakest.

of the shorter of B domain's loops (the second loop, see Table 2 and Fig. 4C), where the two helices come closer together. Tighter clamping is more similar to the situation in parent cyclic peptide CNWTLGGYKC. Second, the loop has to be solvent exposed. In native B domain the second loop is partially occluded by the protein's N-terminal (Fig. 4A). We have therefore constructed an abridged version of B domain (Fig. 4B) to serve as a presentation scaffold, excising amino acids A1-K7 (numbering according to native B domain).

Altogether, five recombinant B domain analogues were prepared. Proteins pB, pB_I10, and pB- were initially prepared as controls to examine whether either the N-terminal shortening or the loop swap would alter the fold of the parent domain. Far-UV CD-spectra of all three proteins contain extrema in proximity of 222 and 209 nm (typical for α-helices [8]) and correspond to previously published spectra for native B domain [9], as do the spectra of subsequent constructs $pB_{II}10$ and $pB_{II}12$. Thus, the scaffold proved to be extremely robust, even more so considering that mainly amino acids not primarily involved in IgG-binding (and therefore potentially serving as a framework for those) were exchanged or excised. Remarkable scaffold stability was further confirmed by the fact that all engineered domains retained the ability to bind (rabbit) IgG. Huston et al. [10] have previously shown that affinity of A1-K7-truncated B domain to human IgG is reduced 10-fold compared to the parent domain. This can be attributed to the loss of K7, a non-helical residue which is involved in binding to the Fc part of IgG according to X-ray crystallographic data [11], and seems also to be true for our protein pB-. Binding of the two fusion proteins most different from the parent domain ($pB_{II}10$ and $pB_{II}12$) to rabbit IgG is weaker still as suggested by ELISA results (Fig. 3). We hypothesize that the grafted peptide loop might slightly distort the packing of the three helices relative one to another. While this proposed effect seems to be negligible in case of pB_I10, its influence on $pB_{II}10$ and $pB_{II}12$ IgG-binding could be intensified by N-terminal shortening.

Of the five constructs that were tested for potential inhibitory activity against three cysteine proteases only $pB_{II}10$ and $pB_{II}12$ inhibited cathepsin L with K_i of 14 and 5.8 μM, respectively, which is roughly comparable to the activity of the parent peptide CNWTLGGYKC ($K_i = 1.3$ μM). Despite longer loop insertion, $pB_{II}12$ was a slightly more potent inhibitor as compared to $pB_{II}10$. Greater conformational freedom, although unfavorable on binding cathepsin L from the entropic point of view, in this case obviously benefits inhibitory activity as it allows the peptide loop to occupy a conformation (or a subset of conformations) more closely resembling the optimal active conformation. On the other hand, glycine spacers added to the peptide insert in $pB_{II}12$ might be responsible for easier accessing of the loop to enzyme's active site. Protein pB_I10 differing from $pB_{II}10$ only in presence of amino acids A1-K7 showed no activity to cathepsin L, indicating the importance of removal of the N-terminal for solvent accessibility of the second loop. The observed inactivity of $pB_{II}10$

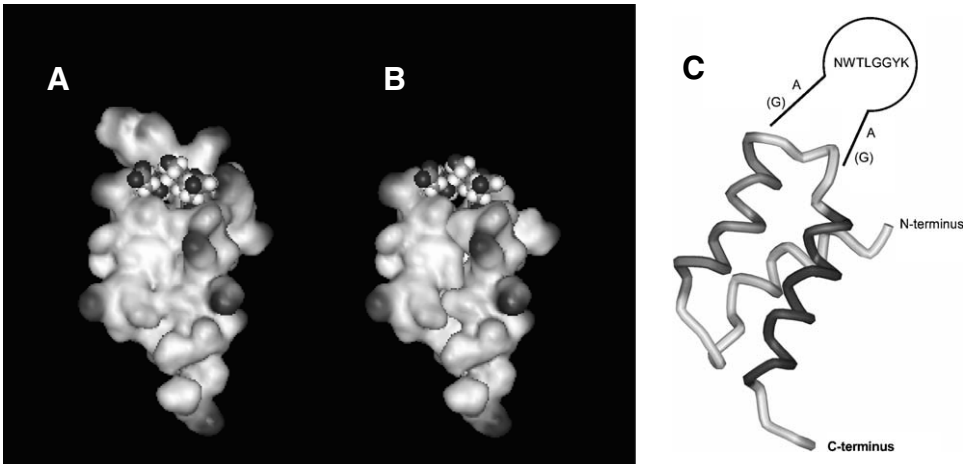

Fig. 4. 3D-models of native B domain (A) and recombinant protein pB- (B). The second loop D37-Q40 is presented as a CPK model, whereas for other parts of molecules molecular surface is shown. C-terminal his-tag in pB- is not shown. (C) Structure of $pB_{II}10$ and $pB_{II}12$ is schematically depicted. Images were drawn with ViewerLite (Accelerys) based on PDB coordinates 1BDC.

and $pB_{II}12$ towards papain and cathepsin K can be explained by the inability of the peptide loop to convey to a conformation complementary to enzymes' active sites.

In conclusion, analogues of IgG-binding domain B of SpA were constructed by transferring a peptide, previously selected by phage display against papain, to one of the domain's loops. In the original peptide a covalent bond (disulfide or head-to-tail peptide bond) was required for strong inhibitory activity, but this was not the case when the peptide was grafted in a robust scaffold protein as a tight loop. Two fusion proteins exhibited inhibitory activity towards cathepsin L comparable to the parent peptide as well as maintained the Fc-binding ability. Finally, SpA IgG-binding domains with engineered additional functionalities, such as the ones reported here, might find use in *in vitro* diagnostic immunological assays.

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