SHORTENED ENGINEERED HUMAN ANTIBODY CH2 DOMAINS: INCREASED STABILITY AND BINDING TO THE HUMAN NEONATAL RECEPTOR

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ABSTRACT

The immunoglobulin (Ig) constant CH2 domain is critical for antibody effector functions. Isolated CH2 domains are promising scaffolds for construction of libraries containing diverse binders that could also confer some effector functions. We have previously shown (Gong et al., JBC 2009) that an isolated human CH2 domain is relatively unstable to thermally induced unfolding but its stability can be improved by engineering an additional disulfide bond. We have hypothesized that the stability of this engineered antibody domain can be further increased by removing unstructured residues. To test our hypothesis we removed the seven N-terminal residues that are in a random coil as suggested by our analysis of the isolated CH2 crystal structure and NMR data. The resulting shortened engineered CH2 (m01s) was highly soluble, monomeric and remarkably stable with a melting temperature (Tm) of 82.6°C which is about 10°C and 30°C higher than that of the original stabilized CH2 (m01) and CH2, respectively. m01s and m01 were more resistant to protease digestion than CH2. A newly identified anti-CH2 antibody which recognizes a conformational epitope bound to m01s significantly better (>10-fold higher affinity) than to CH2 and slightly better than to m01. m01s bound to a recombinant soluble human Fc neonatal receptor at pH 6.0 more strongly than CH2. These data suggest that shortening of the m01 N-terminus significantly increases stability without disrupting its conformation, and that our approach for increasing stability and decreasing size by removing unstructured regions may also apply to other proteins.

INTRODUCTION

Monoclonal antibodies (mAbs) are now well established therapeutics and invaluable tools for biological research (1). A major problem for full-size mAbs is their poor penetration into some tissues (e.g. solid tumors) and poor or absent binding to regions on the surface of some molecules (e.g. on the HIV envelope glycoprotein) that are accessible by molecules of smaller size. Antibody fragments, e.g. Fabs (~60 kDa) or single chain Fv fragments (scFvs) (20~30 kDa), are significantly smaller than full-size antibodies (~150 kDa), and have been used as imaging reagents and candidate therapeutics. Therefore, discovery of even smaller scaffolds including engineered antibody domains (eAds), continues to be of major importance in the development of candidate therapeutics and imaging agents (2-4).

The second domain of the heavy chain constant regions, CH2, is unique among the other antibody domains in that it exhibits very weak carbohydrate-mediated interchain protein-protein
interactions in contrast to the extensive interchain interactions that occur between the other domains. The expression of mouse and human CH2 in bacteria which does not support glycosylation results in a monomeric domain (5;6). We have proposed that the CH2 domain (CH2 of IgG, IgA and IgD, and CH3 of IgE and IgM) could be used as a scaffold and could offer additional advantages compared to those of eAds based on other domains because it contains binding sites or portions of binding sites conferring effector and stability functions (7). Supporting this possibility is the finding that the half-time of human CH2 (about 70 hr) in rabbits is much longer than that of CH3 and Fab (about 15 hr) and CH2 might function to trigger the complement system (8;9).

The native CH2 domain has significantly lower thermal stability compared to other small scaffolds such as the 10th type III domain of human fibronectin (FN3) (5;6;10) which increases the probability of instability when engineering binding to antigens and enhanced effector functions. In the quest for a more stable CH2-based scaffold we have previously found that the stability of an isolated human IgG1 CH2 can be significantly increased by engineering an additional disulfide bond between the A and G strands (6). One of the newly developed mutants, denoted as m01, exhibited significantly higher stability than that of wild type CH2.

We have hypothesized that the stability of m01 can be further increased by removing unstructured terminal residues such as the seven N-terminal residues that are in a random coil as suggested by our analysis of the isolated CH2 crystal structure and NMR data (6;11). To test our hypothesis we removed these residues and characterized the resulting shortened engineered CH2 (m01s). m01s was remarkably stable with a melting temperature (Tm) of 82.6°C which is about 10°C and 30°C higher than that of the original stabilized CH2 (m01) and CH2, respectively. To detect possible conformational changes in m01s compared to CH2 and m01, a novel anti-CH2 antibody (m01m1) was identified which recognizes a conformational epitope. It bound to m01s significantly better (>10-fold higher affinity) than to CH2 and slightly better than to m01. We also expressed CH2 and m01s on yeast cell surface and compared their binding to a soluble human Fc neonatal receptor (shFcRn) (12). Interestingly, we found that the binding of m01s was stronger than that of CH2 in a pH-dependent manner.

Based on these data we suggest that m01s, could be used as a scaffold for development of eAds. These results also demonstrate for the first time that the stability of constant antibody domains can be further increased by decreasing their size. Importantly, while shortening of the m01 N-terminus significantly increases stability it retains or increases some other properties of CH2 (e.g. binding to shFcRn). The increase in stability of isolated domains may result in an increase in stability of larger antibody fragments, e.g. Fc, and therefore could have implications as a general method for increasing antibody stability. It may also apply to other proteins as a method to increase stability and decrease size.

**EXPERIMENTAL PROCEDURES**

*m01 mutant design and plasmid construction*—To design the m01 mutant we used the isolated CH2 crystal structure and NMR data (6;11). The truncated m01 (denoted m01s) with absence of seven residues in N-terminal was cloned into pComb3X (provided by Dennis Burton, Scripps Institute, La Jolla, CAL). The clone was verified by direct sequencing and used for transformation of the Escherichia coli (E. coli) strain HB2151. m01s was expressed and purified similarly to the wild type CH2 (6).

*Size exclusion chromatography (SEC)*—The purified m01s was loaded into the Hiload 26/60
Superdex 75 HR 10/30 column (GE Healthcare, NJ) running on ÄKTA BASIC pH/C chromatography system (GE Healthcare, NJ) to assess possible oligomer formation. Phosphate Buffered Saline (PBS), pH 7.4 was selected as mobile phase. A gel-filtration of standards consisting of Aldolase (158 kD), Bovine serum albumin (67 kDa), Ovalbumin (44 kDa), Chymotrypsinogen A (25 kD) and Ribonuclease A (13.7 kDa) was used to define the molecular weight.

Circular dichroism (CD) – The secondary structures of m01s was determined by CD spectroscopy. The purified proteins were diluted in PBS at the final concentration of 0.54 mg/ml, and the CD spectra were recorded on AVIV Model 202 CD Spectrometer (Aviv Biomedical, NJ). Wavelength spectra were recorded at 25°C using a 0.1-cm path-length cuvette for native structure measurements. In order to measure the Tm value, m01s was diluted in PBS, pH 7.4 with 0M, 3M, 3.5M, 4M and 5M Urea respectively. Thermal stabilities in different conditions with 0M, 3M, 3.5M, 4M and 5M Urea were measured at 216 nm (in the absence of Urea) and 225 nm (in the presence of Urea with different concentrations) by recording the CD signal in the temperature range of 25-90°C with heating rate 1°C/min. The temperature was recorded with an external probe sensor and the temperature inside the microcuvette was calculated by calibration – it was about 2-3°C lower that the one measured by the external sensor.

Limited proteolysis – The purified CH2, m01 and m01s proteins were subjected to trypsin digestion in the proportions: 1/5 ([wt/wt], trypsin/protein) for 0, 1.5 and 3h at 37°C. After digestion, the loading buffer was added into the samples immediately to terminate the digestion and all the samples were examined on SDS-PAGE.

Conformational changes of m01s detected by an anti-CH2 Fab m01m1– Enzyme-Linked Immunosorbent Assay (ELISA) was used for comparison of the structure of CH2, m01, m01s. Briefly, CH2, m01, m01s and HSA (negative control) were coated on 96-well plate with concentration of 2 µg/ml. An anti-CH2 Fab m01m1 identified from an human Fab naïve library (13) by panning against isolated CH2 expressed in E. coli, which recognized the conformational epitope of CH2 (data not shown), was added with concentrations from 0.064 to 200 µg/ml. HRP-anti-Fab (Sigma) was used as secondary antibody. To confirm the result, a biotin-conjugated commercial monoclonal mouse anti-human CH2 antibody (Abd sterotec) from immunized mouse by human polyclonal IgG was also used for ELISA with concentrations from 0 to 10 µg/ml. HRP-streptavidin (Sigma) was used for detection of biotinylated-mouse anti-human CH2 antibody.

Construction of CH2, m01s, Fc and CH3 for yeast surface expression – CH2, m01s, Fc and CH3 were cloned into pYD7 vector which was a modified version from pCTCON2 (14) with moving the agglutinin protein aga2p to the C-terminal of interest proteins. The clones were verified by direct sequencing. The constructs were transformed into EBY100 cells for surface expression according to the protocol described previously (14).

Flow cytometry assay – For measurement of the binding of CH2, m01s, Fc and CH3 to shFcRn, yeast cells containing pYD7-CH2, pYD7-m01s, pYD7-Fc and pYD7-CH3 were grown in SDCAA medium respectively and then the expression was induced in SGCAA medium according to published protocols (14). For shFcRn binding, 5 × 10^5 yeast cells were harvested, washed by PBSA (PBS + 0.1% bovine serum albumin, pH 6.0) and re-suspended in 50 µl PBSA (pH 6.0) containing 100 nM biotin-conjugated shFcRn.
The samples were kept on ice for 2 hours, then the cells were washed by PBSA (pH 6.0) again and still re-suspended in 50 μl PBSA (pH 6.0). 1 μl PE-streptavidin (Invitrogen) was added into the re-suspended cells. After 30 min incubation on ice, the cells were washed by PBSA (pH6.0) and re-suspend in 0.5 ml PBSA (pH6.0) for flow cytometry measurement. The mouse anti-human CH2 monoclonal antibody described above and Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) were used for testing the expression of CH2, m01s and Fc on the yeast while the FITC conjugated goat anti-human Fc polyclonal antibody (Sigma) was used for testing the expression of CH3 on the yeast. Same samples prepared at pH7.4 were used as controls.

**Competition flow cytometry assay**– In order to verify the specificity of binding of m01s to FcRn, A human IgG1 was used as competitor. 5×10^5 yeast cells expressing m01s were harvested, washed by PBSA (pH 6.0) and re-suspended in 50 μl PBSA (pH 6.0) containing 100 nM biotin–conjugated shFcRn and human IgG1 with serial concentrations (0, 125, 250, 500, 1000, 2000 and 4000 nM). The sample prepared at pH7.4 with 100nM biotin-conjugated shFcRn only was used as control. Then the binding was analyzed by the same method described above. The mean value was taken to calculate the inhibition efficiency.

### RESULTS

**Analysis of an isolated CH2 crystal structure and NMR data.** We have previously solved the crystal structure of an isolated γ1 CH2 domain (11). An analysis of the CH2 N-terminus suggested that the first seven residues form a random coil. Our previously reported NMR data (6) indicate that the very N-terminal residues exhibit increased flexibility. Therefore, we have hypothesized that the disordered N-terminus with increased dynamics may contribute to a decreased thermal stability and that its removal may result in increased stability.

**Design and generation of an N-terminal truncated engineered CH2 domain.** To test our hypothesis we used a previously developed engineered CH2 domain (m01) where an additional disulfide bond was introduced between the A and G strands resulting in an increased stability (6). The first seven N-terminal residues of m01 were deleted resulting in a shorter variant of m01 termed m01s (Fig. 1A). The expression of m01s in *E.coli.* was better than that of CH2 and m01 (Fig. 1B), m01s was completely monomeric in PBS at pH7.4 as determined by size exclusion chromatography (Fig. 1C).

**m01 is significantly more stable than CH2 and m01.** The secondary structure and thermodynamic stability of m01s was measured using circular dichroism. The CD spectra of m01s showed that it has high β-sheet content at 25°C (Fig. 2A). The β-sheet structure was gradually disrupted and the protein unfolded as the temperature increased (Fig. 2B). However, even at the highest temperature measured (90°C) part of the protein was still in a folded state and there was no a second transition point (Fig. 2B). Therefore, we used an indirect approach to estimate the melting temperature at which 50% of the protein is in folded state (Tm) by using increasing concentrations (3, 3.5, 4 and 5 M) of urea to decrease the Tm and extrapolate to 0 urea concentration (Fig. 2C). The sigmoidal curves were fitted by a two-state model which was also previously used (5). Tm was calculated from each urea concentrations and fitted to a linear equation according to a previously described method (15), Tm = 82.6 – 4.7 Uc, where Uc is the urea concentration (Fig. 2D). Therefore, at Uc=0 Tm equals 82.6°C. This value is significantly higher than those previously measured by the same method for CH2 (54.1°C) and m01 (73.4°C) (6).
m01s and m01 are more resistant to protease (trypsin) digestion than CH2 but are equally stable in human serum in vitro. To evaluate the stabilities of CH2, m01 and m01s against digestion by a typical protease trypsin was used as an example. Significantly larger amounts of CH2 were digested than m01 and m01s in 3 hours (Fig. 3). We also estimated their stability in human serum by incubation at 37°C for 9 days (see EXPERIMENTAL PROCEDURES in Supplemental Material). No significant degradation was observed (Figure S1). These data suggest that the stabilized variants of CH2 are more resistance to protease (trypsin) digestion than CH2 but are equally stable in human serum in vitro.

Conformational changes in m01s and m01 demonstrated by a newly identified anti-CH2 Fab, m01m1. To determine whether the CH2 engineering resulted in conformational changes we developed a human Fab, m01m1, specific for CH2. The Fab m01m1 was identified by panning and screening of an Fab library against purified CH2 as described in EXPERIMENTAL PROCEDURES. It was expressed in E. coli and purified. m01m1 bound to CH2 with relatively low affinity (EC50 > 1305 nM) (Fig. 4A). Interestingly, the addition of a disulfide bond in m01 and m01s resulted in a significant increase of binding - EC50 = 181 nM and 129 nM, respectively (Fig. 4A). The shortening of m01 led to slight increase in the m01m1 affinity (Fig. 4A). Similar results were obtained with a commercial mouse anti-human CH2 monoclonal antibody that binds a conformational epitope (Fig. 4B). These results indicate that some conformational epitopes are largely conserved in CH2, m01 and m01s but exposed better in the engineered CH2 domains than CH2.

m01s binds to shFcRn at pH 6.0 better than CH2. Recently, we developed a method to produce shFcRn in mammalian cells with high yield (12). We used this shFcRn to test its binding at pH 6.0 to CH2 and m01s expressed on yeast cells (Fig. 5). The fluorescence intensity shift for m01s was larger than that for CH2, indicating stronger binding of m01s to shFcRn than that of CH2. The largest fluorescence intensity shift in the case of Fc indicated that binding of Fc on yeast cells to shFcRn at pH 6.0 was better than m01s. No fluorescence intensity shift was observed for CH3 at pH 6.0 indicating that CH3 has minor contribution for the binding of Fc to shFcRn.

Binding of m01s to shFcRn is inhibited by human IgG1. In order to test the specificity of binding of m01s to shFcRn at pH 6.0, IgG1 was used as a competitor in a flow cytometry assay. With an increase of the IgG1 concentration, the fluorescence intensity shift decreased (Fig. 6A). Half of the binding inhibition (IC50) was at 804 nM, which is consistent with a previous report (Fig. 6B) (16). Therefore, as expected m01s shares the binding site of the IgG1 to shFcRn; this result also indicates that the binding of m01s to shFcRn at pH6.0 was specific.

DISCUSSION

The major findings of this study are that shortening of an isolated unglycosylated engineered human γ1 CH2 domain significantly increased its stability without affecting some other properties, and the identification of a new anti-CH2 Fab, m01m1, which is sensitive to CH2 conformational changes induced by an additional disulfide bond. The measured m01s Tm is the highest achieved for isolated CH2-based domains and comparable to that of the FN3 domain 10 (10). We have not investigated possible molecular mechanisms that determine the higher stability of m01s compared to that of CH2 and m01. One can speculate that decreased dynamics at the N-terminus is a major factor of the increased stability. The decreased size could also contribute. These results also indicate that the stability of other
proteins could be increased by removing unstructured regions and decreasing size. The size dependence of folding and stability is in agreement with a previous analysis (17). The increased thermal stability combined with high solubility and expression levels as well as resistance to proteases are highly desirable properties for a candidate protein therapeutic. Shortening of CH2 in intact Fcs and Igs could also result in increase of their stability and ongoing experiments are testing this possibility.

Naturally occurring additional disulfide bonds are found in camel single-domain antibody fragments (VHHs) (between CDR1 and CDR3) and in shark IgNAR V domain (between CDR3 and frameworks) (18;19). The thermo-stability of a single isolated antibody domain is typically increased by 10°C after introduction of an additional disulfide bond (20). The strategy is similar to that used in the design of m01. It would be interesting to find out whether shortening of such domains with a naturally occurring second disulfide bond and also in general other antibody domains could result in an increase of their stability.

We also found that shortening of m01 (m01s) does not perturb to any significant degree some other properties including binding to FcRn. Because of the lack of CH3, the binding of m01s to FeRn was relatively weak compared to whole Fc. However, interestingly there was an increase in binding compared to CH2. We are currently further improving binding of m01s to shFcRn, which may lead to extended half-life of m01s in vivo. Removal of N-terminal residues from CH2, however, may affect binding to Fcγ receptors and related effector functions. Further studies are in progress to elucidate this and other possible effects due to the N-terminal shortening including binding to complement.

These findings could have implications for exploration of the unfolding mechanisms of antibody domains and for the development of candidate therapeutic proteins with increased stability and extended half-life. Whether the observed increase of stability to temperature and chemical agents, and of binding to FcRn in vitro will result in increased stability and long half-life in vivo remains to be seen.

REFERENCES

FOOTNOTES

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The abbreviations used are: Ig, immunoglobulin; nAbs, nanoantibodies; eAds, engineered antibody domains; SEC, size exclusion chromatography; CD, circular dichroism; shFcRn, soluble human Fc neonatal receptor.
FIGURE LEGENDS

Figure 1 Design, expression and estimation of oligomer formation of m01s. A. Amino acid sequence alignment of wide-type CH2 (NCB Accession No. J00228), m01 and m01s. B. Comparison of the expression of CH2, m01 and m01s. C. Size exclusion chromatography of m01s. The insert is a standard curve.

Figure 2 Stability of m01s. A. CD spectra of m01s measured at 25°C. m01s displayed maximum negative peak between 210 nm and 225 nm, which indicated a typical β-helical secondary structure. B. Thermo-induced unfolding of m01s in PBS without Urea. The change of mean residue ellipticities was monitored at 216 nm. No second transition point was observed when temperature arrived at 90°C. C. Thermo-induced unfolding of m01s in PBS with different concentrations of Urea (3M, 3.5M, 4M and 5M). The fraction folded of the protein (ff) was calculated as ff = ([θ] - [θm])/(θT - [θm]). [θT] and [θm] were the mean residue ellipticities at 225 nm of folded state at 25°C and unfolded state of 90°C. The Tm values (68.9°C, 65.7°C 63.6°C and 59.3°C were corresponding to the existence of 3 M, 3.5 M, 4 M and 5 M Urea respectively) from CD were determined by the first derivative [d(Fraction folded)/dT] with respect to temperature (T). D. Calculation of m01s Tm (82.6°C) at 0 M Urea.

Figure 3 Limited proteolysis of CH2, m01 and m01s by trypsin. After 3h digestion, m01 and m01s were partially digested, while CH2 was almost completely digested.

Figure 4 Binding of CH2 (■), m01 (●), m01s (▲) and HSA (▽) to an anti-human CH2 Fab (m01m1) selected from a human naïve Fab library (A) and a commercial mouse anti-human CH2 IgG (B). The EC50s of Fab m01m1 to CH2, m01 and m01s were >1305 nM, 181 nM and 129 nM respectively, while the EC50s of the commercial mouse anti-human CH2 IgG to CH2 and m01s were 4.9 nM and 0.59 nM respectively. In both cases, m01s could be recognized by the antibodies (Fab and mouse IgG) better than CH2.

Figure 5 Binding of yeast-expressed CH2, m01s, Fc and CH3 to shFcRn at pH6 (red) and pH7.4 (blue). Very slight fluorescence intensity shift occurred in the case of CH2, which indicated very weak binding to shFcRn. Modest fluorescence intensity shift was observed in the case of m01s, which indicated modest binding to shFcRn. Largest fluorescence intensity shift was observed in the case of Fc, which indicated strong binding to shFcRn. CH3 did not bind to shFcRn in a pH-dependent manner – there was no observable fluorescence intensity shift. The expression of CH2, m01s, Fc and CH3 was tested by the corresponding antibodies. PE-streptavidin was used as negative control.

Figure 6 Inhibition of yeast-expressed m01s binding to shFcRn by IgG1. A. Fluorescence intensity shift in the presence of IgG1 at different concentrations. B. Inhibition curve. Percent of inhibition (%) = [(mean max at pH 6.0 –mean at pH 6.0)/(mean max at pH 6.0 – mean at pH7.4)] × 100, while mean max at pH 6.0 was the mean value of the fluorescence intensity measured at pH 6.0 in the absence of IgG1, mean at pH 7.4 was the mean value of the fluorescence intensity measured at pH 7.4 in the absence of IgG1 and mean at pH 6.0 was mean value of the fluorescence intensity measured at pH6.0 with different IgG1 concentrations. The binding decreased with an increase of the IgG1 concentration; IC50 = 804 nM.
Figure 1

A

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<thead>
<tr>
<th>CH2</th>
<th>APELLGGPSVFPLPGEKDTLMISRTPEVTCVVHDPEVFHYGDVEVHNAKTK</th>
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<td>m01</td>
<td>....................................................................</td>
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<tr>
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B

CH2 m01 m0ls M

17 kD 14 kD

C

Absorbance

mAU

0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0 11.0 12.0 13.0 14.0 15.0

Elution volume (ml)

Standard curve

Aldolase: 158 kDa

Albumin: 67 kDa

Ovalbumin: 44 kDa

Albumin: 25 kDa

Ribonuclease A: 12.7 kDa
Figure 6

A

B

IC50 = 804 nM
Shortened engineered human antibody CH2 domains: increased stability and binding to the human neonatal receptor
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