Switching an anti-IgG binding site between archaeal extremophilic proteins results in Affitins with enhanced pH stability

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ABSTRACT

As a useful reagent for biotechnological applications, a scaffold protein needs to be as stable as possible to ensure longer lifetimes. We have developed archaeal extremophilic proteins from the "7 kDa DNA-binding" family as scaffolds to derive affinity proteins (Affitins). In this study, we evaluated a rational structure/sequence-guided approach to stabilize an Affitin derived from Sac7d by transferring its human IgG binding site onto the framework of the more thermally stable Sso7d homolog. The chimera obtained was functional, well expressed in *E. coli*, but less thermally stable than the original Affitin (T_m = 74.2°C *vs.* 80.4°C). Two single mutations described as thermally stabilizing wild type Sso7d were introduced into chimeras. Only the double mutation nearly restored thermal stability (T_m = 76.9°C). Interestingly, the chimera and its double mutant were stable from pH = 0 up to at least pH = 13. Our results show that it is possible to increase further the stability of Affitins towards alkaline conditions (+2 pH units) while conserving their advantageous properties. As Affitins are based on a growing family of homologs from archaeal extremophiles, we conclude that this approach offers new potential for their improvement, which will be useful in demanding biotechnological applications.

Keywords: Sac7d, Sso7d, molecular grafting, thermostability, pH stability, Affitin

1. INTRODUCTION

During the last two decades, a class of proteins called “scaffold proteins” has emerged as an alternative to classic antibodies for deriving artificial affinity reagents. These proteins are useful for numerous biotechnological and clinical applications (for a review, see (Gebauer and Skerra, 2009)). Depending on the targeted application, a given scaffold protein might be considered the best candidate due to its properties such as thermal stability, immunogenicity, size or high yield. However, it is technologically and economically more interesting to combine as many favorable properties as possible in one protein scaffold to provide a versatile solution for different applications.

A scaffold protein can be improved *via* either rational design or directed evolution to gain stability (temperature, pH, proteases) (Getz et al., 2011) or to decrease its immunogenicity (Baker and Jones, 2007) for example. The method of improving a scaffold for binding is often driven by structure/function relationships with other proteins. For instance, the binding mode of antibodies involving CDR loops has inspired the engineering of new generations of Monobodies, DARPins, and Affitins with artificially extended loop(s) (Correa et al., 2014; Koide et al., 2012; Schilling et al., 2014).

Nevertheless, improving the biophysical properties of scaffold proteins, e.g. thermal and chemical stabilities, remains challenging and laborious, as our understanding of the effects of sequence modifications is incomplete. Thus, the combinatorial approach, which consists of generating random variants coupled to a screening for stability, and methods based on sequence/structure homology have proved to be some of the most successful strategies to improve the stability of

numerous proteins (for reviews, see (Bommarius et al., 2006; Romero and Arnold, 2009)).

Affitins are artificial affinity proteins that we have developed which are derived from extremophilic proteins from the "7 kDa DNA-binding" family found in Archaea, such as Sac7d (Béhar et al., 2013; Buddelmeijer et al., 2009; Krehenbrink et al., 2008; Mouratou et al., 2007). Sac7d is a hyperthermostable protein (T_m = 90.4°C) (Edmondson and Shriver, 2001; McCrary et al., 1996; Mouratou et al., 2007) and is chemically resistant from pH = 0 up to pH = 12 (Béhar et al., 2013). Binders with high pH and thermal stabilities have been obtained by coupling the generation of combinatorial libraries of Sac7d variants corresponding to the randomization of 10 to 14 residues of the β-sheet surface originally involved in the binding of DNA and selections against different targets. We recently reported that natural (Béhar et al., 2013) or artificially extended loops (Correa et al., 2014) of Sac7d can be advantageously recruited to generate Affitins, some of them showing potent inhibition properties by penetrating deep into a glycosidase active site.

Taken together, these results demonstrate the ability of this scaffold to evolve, an advantage for the design of improved Affitins that will necessarily require their sequence modification. Proteins with sequences homologous to that of Sac7d exist in various Archaea, including *Sulfolobus*, *Acidianus*, and *Metallospharea* genera, but no mesophilic equivalents are known (Razvi and Scholtz, 2006). Their three-dimensional structure is available for only two of them: Sac7d and Sso7d, from *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*, respectively. Although initially mistakenly identified as OB-fold proteins (Gao et al., 1998; Robinson et al., 1998), they both fold as an SH3-like five-stranded incomplete β-barrel capped by a C-terminal α-helix (Agback et al., 1998; Kahsai et al., 2005), and have closely related

sequences (~ 79% identity). Interestingly, Sso7d is about 10°C more stable than Sac7d with a T_m = 100.2°C. While it is stable under acidic conditions as low as pH = 0, the upper limit of its stability in extreme alkaline conditions is not known (Catanzano et al., 1998; Clark et al., 2004; Edmondson and Shriver, 2001; Shehi et al., 2003).

In this study, our aim was to evaluate an approach to stabilize thermally an Affitin derived from a scaffold protein of the "7 kDa DNA-binding" family by using sequence element(s) from another more stable protein of the same family. To this end, we grafted the binding site of the Affitin D1 derived from Sac7d, specific for human IgG and with a thermal stability of 80.7°C (Béhar et al., 2013), into the thermally more stable Sso7d protein. We also produced this chimera with two single mutations, described as thermally stabilizing wild type Sso7d (Consonni et al., 2007), and with the corresponding double mutation. We then studied the function and the thermal and pH stability properties of the resulting proteins.

2. MATERIALS AND METHODS

2.1 Molecular biology

The DNA sequences corresponding to the protein D1Sso7d (5'-GGATCCGCCACTGTGAAATTCAAATATAAAGGCGAAGAAAAAGAAGTGGACATCAGTAAGATCAAGAAAGTTTGGCGTGACCGCTTGGCCGCGGTGTTTACCTACGACGAAGGCGGAGGCAAGACCGGCTACGGCTGGGTGTTCACGAAGGATGCCCCGAAAGAGTTATTACAGATGTTAGAGAAACAGAAAAAGCTT-3'), and wild type Sso7d (5'-GGATCCGCAACGGTGAAATTCAAATACAAGGGCGAAGAGAAAGAAGTAGACATCTCTAAAATCAAAAAGGTTTGGCGCGTCGGCAAAATGATTTCCTTTA

CCTATGATGAAGGCGGTGGTAAAACTGGTCGTGGTGCGGTTAGCGAAAAA GATGCTCCGAAAGAGCTGCTGCAGATGCTGGAAAAGCAAAAAAAAAAGC TT-3') were obtained by gene synthesis (GeneCust) and contained BamHI and HindIII restriction sites (underlined sequences). The DNA corresponding to these sequences was cloned *via* BamHI/HindIII restriction enzymes into the pFP1001 expression vector that enables the fusion of RGS-His6 tag at the N-terminal (Mouratou et al., 2012).

Changes in residues were introduced by mutagenesis of the plasmids pFP1001 encoding Affitins with the Quickchange II site-directed mutagenesis kit (Agilent Technologies). The following oligonucleotides were used to produce the K13L and E36L mutants (numbering of Sso7d amino acids is according to Gao *et al*. and Robinson *et al*. (Gao et al., 1998; Robinson et al., 1998)): D1s_K13L_F (5'-CAAATATAAAGGCGAAGAATTAGAAGTGGACATCAGTAAG-3'), D1s_K13L_R (5'-CTTACTGATGTCCACTTCTAATTCTTCGCCTTTATATTTG-3'), D1s_E36L_F (5'-GCGGTGTTTACCTACGACTTAGGCGGAGGCAAGACCGGC-3'), D1s_E36L_R (5'-GCCGGTCTTGCCTCCGCCTAAGTCGTAGGTAAACACCGC-3'). The double mutant was obtained starting with the K13L mutant using D1s_E36L_F and D1s_E36L_R oligonucleotides. All sequences were checked by standard sequencing techniques.

2.2 Production and purification of proteins

Proteins were expressed in the *E. coli* strain DH5αF'IQ on a 1-liter scale and purified by immobilized metal ion affinity chromatography (IMAC) and gel exclusion chromatography, as described previously (Béhar et al., 2013). Monomeric purified

proteins were in 130 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4 (PBS) and quantified spectrophotometrically at 280 nm using an extinction coefficient of 8250 M^{-1} cm^{-1} (WT Sso7d) and 15220 M^{-1} cm^{-1} (D1Sso7d and its mutants).

2.3 Size-exclusion chromatography analysis of proteins

The proteins were injected at a concentration of 500 µM (50 µl) into a Superdex75 10/300 GL column (GE Healthcare) equilibrated with PBS using a Bio-Rad BioLogic DuoFlow 10 System with a flow rate of 1.0 ml/min. The following proteins were used as molecular mass standards: bovine serum albumin (66 kDa) ovalbumin (44.3 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa).

2.4 Immunoglobulins

IgGs used in this study were purchased from Fluka: hIgG (i.e. IgG pool from human serum containing hIgG1, hIgG2, hIgG3, and hIgG4); and from Sigma-Aldrich: hIgG1, hIgG2, hIgG3, hIgG4, IgG from mouse, rat, sheep, goat, rabbit, and pig.

2.5 Detection of anti-IgG activity by ELISA

IgGs (5 µg/ml) in 20 mM Tris–HCl, 150 mM NaCl, pH 7.4 (TBS), and BSA as a negative control, were coated on a Maxisorp plate (overnight, 4°C). One hundred microliters of 1 µM purified Affitins was then added to wells to test their binding. The detection was performed using the RGS-His6 tag antibody horseradish peroxidase (HRP) conjugate (Qiagen), which detects the RGS-His6 tag from Affitins, and a solution buffer (0.05 M citric acid, 0.05% hydrogen peroxide, 1 mg/ml o-phenylenediamine substrate (Sigma)) was added and absorbance at 450 nm was

measured with a Tecan NanoQuant 200 Pro plate reader. ELISA was performed at 25°C with 1 h of incubation for each step. All incubation steps were carried out in TBS with 0.1% Tween 20.

2.6 Affinity determination of anti-hIgG1 activity by surface plasmon resonance

Surface plasmon resonance (SPR) was measured using a BIAcore 3000 instrument at 25°C. hIgG1 (1500 RU) was immobilized on flow cells of a CM5-chip. The running buffer was HBSEP pH 7.4 (20 mM Hepes, 150 mM NaCl, and 0.005% P20). Regeneration was performed with 10 mM glycine, pH 3.0. Measurements for affinity determinations of hIgG binders were performed with size-exclusion purified proteins injected at concentrations ranging from 1.95 nM to 5 μM at a flow rate of 60 μl/min. Association and dissociation times were controlled at 3 and 10 min, respectively. Data were evaluated using Scrubber2 (Biologic software) and BIAeval software (BIAcore) using the steady state model and a global fitting procedure (Karlsson and Falt, 1997).

2.7 Thermal stability of proteins

Thermally-induced unfolding of proteins (0.02 mg/ml) in 1 mM potassium acetate buffer (pH 5.5) was studied by circular dichroism as described previously (Béhar et al., 2013).

2.8 pH stability of proteins monitored by circular dichroism

To study their pH stability, proteins were diluted to 0.33 mg/ml in a buffered solution or a strong acid or base corresponding to each pH unit from 0 to 14 containing 300 mM NaCl. Except for HCl and NaOH, the buffered solutions were adjusted to the required pH with HCl or NaOH: pH 0 = 1 M HCl, pH 1 = 0.1 M HCl,

pH 2 = 50 mM NaH_2PO_4, pH 3 = 50 mM NaH_2PO_4, pH 4 = 50 mM acetic acid, pH 5 = 50 mM acetic acid, pH 6 = 50 mM MES, pH 7 = 50 mM phosphoric acid, pH 8 = 50 mM NaH_2PO_4, pH 9 = 50 mM Tris base, pH 10 = 50 mM methylamine, pH 11 = 50 mM methylamine, pH 12 = 50 mM NaH_2PO_4, pH 13 = 0.1 M NaOH and pH 14 = 1 M NaOH. Protein samples were incubated overnight in these solutions at room temperature and CD spectra were recorded at 20°C as described above, using a quartz cell with a path length of 0.2 cm (Hellma).

2.9 pH stability of proteins monitored by SPR

Changes in the binding capacities of Affitins for hIgG1 following exposure to NaOH at various concentrations were monitored by SPR, essentially as described by Palmer *et al.* (Palmer et al., 2008) and Béhar *et al.* (Béhar et al., 2013). Briefly, hIgG1 (1600 RU) was immobilized by amine coupling chemistry on flow cells of a CM5-chip. Affitin proteins (250 µM) were independently incubated with 90 µl of buffer solution at pH 1, 2, 3, 11, 12 or 13. The kinetics of deactivation by pH were stopped by neutralizing 10 µl of the protein/buffer mixture in 490 µl of the SPR running buffer HBSEP, pH 7.4. The residual functional protein able to bind hIgG1 was measured by SPR by injecting Affitins at a concentration of 500 nM. Regeneration was performed with 10 mM glycine, pH 2.5.

3. RESULTS

To evaluate the possibility of further stabilizing an Affitin, we started from the previously described anti-hIgG Affitin D1 derived from Sac7d (Béhar et al., 2013), hereafter called D1Sac7d. This Affitin has already been shown to be thermally (T_m =

80.7°C) and chemically (up to pH 11) stable and is thus a good candidate to assess stabilization approaches involving molecular grafting and site-directed mutagenesis.

3.1 Construction and production of the proteins

Sac7d and Sso7d proteins show a high degree of structural and sequence similarity (Fig. 1A-B). The set of residues selected upon generation of D1Sac7d Affitin was transposed into the sequence of WT Sso7d, assuming it composes the IgG binding site, to give D1Sso7d (Fig. 1C-D). Two single mutants of D1Sso7d (K13L and E36L) were constructed, as previous studies with wild type Sso7d have described their role in thermal stabilization (Consonni et al., 2007). The double mutant K13L and E36L of D1Sso7d, hereafter called D1Sso7d-DM, was also produced to investigate a potential additive effect on stability. In addition, WT Sso7d was produced to determine its stability at extreme alkaline pH.

All proteins were expressed in the cytoplasm of *E. coli* strain DH5αF'IQ and purified to homogeneity in two steps by immobilized metal ion affinity chromatography and gel filtration as described previously (Mouratou et al., 2007). The proteins ran on a 15% SDS/PAGE gel at the positions expected for their calculated molecular masses (data not shown). Chimeras and WT proteins eluted from the gel filtration column as sharp and symmetric peaks. At a concentration of 500 μM, WT Sso7d eluted at a volume corresponding to the size expected for a monomer, while chimeras eluted with volumes smaller than expected, suggesting that they interacted with the column matrix (Fig. 2A). Nevertheless, they showed a single peak, consistent with the view that they are monomeric. The yields for chimeras ranged from 13 to 40 mg per liter of shake-flask culture (Table 1).

3.2 Characterization of proteins by circular dichroism

The circular dichroism spectra of the WT and chimera proteins were measured in the far-UV spectral region (Fig. 2B). All CD spectra were characteristic of mostly β-stranded proteins with an α-helix contribution as previously reported for WT Sso7d protein (Edmondson and Shriver, 2001).

3.3 Anti-IgG binding activity

The interaction of chimeras with different IgGs was investigated by ELISA and SPR to determine whether the recognition specificities were transferred by grafting the interaction surface between the scaffold proteins (Fig. 3). All chimeras behaved similarly, and the profile of recognition was unchanged compared to D1Sac7d (Béhar et al., 2013). They were able to interact with hIgG1, hIgG2, and hIgG4. No significant cross-reactivity was detected with hIgG3 or with IgGs from several other organisms tested. SPR experiments showed that the affinities of chimeras were in the micromolar range compared to the low nanomolar range observed for the parental D1Sac7d protein (Table 1). D1Sso7d-DM was the most affected, while the single mutants showed a slightly higher affinity.

3.4 Thermal stability of chimeras

The thermal stability of chimeras was determined by circular dichroism measurements (Fig. 4, Table 1). D1Sso7d was 6.5°C less stable than the previously characterized D1Sac7d (Béhar et al., 2013). The single mutations were either neutral (E36L, -0.1°C) or destabilizing (K13L, -4.7°C) compared to D1Sso7d, suggesting that the effects of single mutations identified as stabilizing WT Sso7d are context-dependent and cannot be transposed directly into a drastically modified protein such

as D1Sso7d. By contrast, D1Sso7d-DM was 2.7°C more stable than D1Sso7d and was studied further, together with D1Sso7d.

3.5 pH stability of proteins

To investigate whether the grafting onto Sso7d and the double mutation had an effect on pH stability, proteins were incubated overnight at pH from 0 to 14. Circular dichroism measurements indicated that secondary structures of D1Sso7d and D1Sso7d-DM remained largely stable under alkaline conditions up to pH 13, as for WT Sso7d (Table 1 and Fig. 5). This corresponded to a pH stabilization of +2 units compared to D1Sac7d (Béhar et al., 2013). Interestingly, we observed that WT Sso7d was more stable than WT Sac7d under alkaline pH, up to pH 13 and pH 12, respectively (Béhar et al., 2013). To investigate whether the effect of extreme pH was reversible, the fraction of active chimeras that remained active after denaturation and renaturation was quantified by SPR. D1Sso7d and D1Sso7d-DM remained fully active after incubation at pH 1, 2 and 3 followed by renaturation (Fig. 6A). Under alkaline conditions, both proteins remained equally fully functional after incubation at pH 11, 12 and 13 followed by renaturation (Fig. 6B-C). These results confirmed the circular dichroism measurements (Fig. 5).

4. DISCUSSION

We have published several studies showing that Affitins are chemically and thermally stable, two key points for their usefulness in demanding biotechnological applications. However, no attempt has yet been reported to improve their stabilities further.

We have demonstrated in this study that an Affitin derived from the scaffold of Sac7d can be stabilized by using sequence elements from another protein belonging to the same "7 kDa DNA-binding" protein family. This was exemplified by grafting the binding surface of D1Sac7d onto the more stable Sso7d, and by introducing point mutations previously identified as stabilizing for WT Sso7d.

All chimeras showed circular dichroism spectra similar to that of WT Sso7d, suggesting that mutagenesis had not altered the secondary structures. While all chimeras were still able to bind hIgG, their affinities dropped by a factor ranging from 9 to 117. This suggests that performing affinity transfer by grafting the 10 selected residues that were initially randomized to generate D1Sac7d was not sufficient to retain fully the binding strength, although WT Sac7d and Sso7d have highly similar sequences and three-dimensional structures. The drop in activities could be due to conformational changes induced by mutations, which could alter binding. Indeed, even a single mutation introduced in WT Sso7d was reported to lead to a reorientation of neighboring lateral chains while preserving the overall fold of the protein (Consonni et al., 2007). Furthermore, we have observed with the three-dimensional structure of the C3 anti-IgG Affitin that, while the fold of Sac7d was maintained upon mutagenesis necessary for its generation, a significant backbone deviation occurred in a β-turn-β region with respect to the crystal structures of WT Sac7d (Béhar et al., 2013). The deletion of three residues from the α-helix end of D1Sac7d could also explain the decrease in activity observed for D1Sso7d. In fact, we observed in the three-dimensional structure of a lysozyme/anti-lysozyme Affitin complex that this part of the Affitin was in contact with lysozyme (Correa et al., 2014). This work illustrates the difficulty to anticipate possible effects on binding activity of a mutational/grafting approach, even when applied to a rigid region (Kahsai et al.,

2005) of the protein. Nevertheless, the resulting proteins of this study inherited the fine specificity of their parental protein.

We hypothesized that grafting a binding site from Sac7d onto the more thermally stable Sso7d protein would result in a more thermally stable protein. This was not observed as the T_m of D1Sso7d was decreased by 6.5°C, indicating that some determinants for the higher thermal stability of Sso7d were altered by the grafting of the hIgG binding site. Similarly, the single mutations K13L and E36L identified as thermally stabilizing WT Sso7d (Consonni et al., 2007) did not stabilize D1Sso7d. However, the combination of both mutations was able to produce a stabilization effect (+2.7°C) and the final D1Sso7d-DM was nearly as stable as the original D1Sac7d (76.9°C *vs.* 80.7°C). According to the three-dimensional structure of Sso7d, positions 13 and 36 are in close proximity and face to face on β-strands 2 and 4, respectively (Fig. 1). Thus, a possible explanation for a synergistic effect of the mutations could be that amino acids Leu13 and Leu36 make a new stabilizing hydrophobic interaction. A similar result was observed for the stabilization of glutamate decarboxylase B, in which several single hydrophobic mutations decreased the thermostability while their combination induced a stabilizing effect (Jun et al., 2014).

One approach to improve the alkaline stability of proteins is to replace the alkali-susceptible residues, such as Asn, with other residues. This has been well described for several proteins (Gulich et al., 2000; Gulich et al., 2002; Linhult et al., 2004). However, our previous attempts to improve the alkaline stability of Affitins by this method were unsuccessful (unpublished results). Another approach to improve alkaline stability is to identify potential electrostatic repulsions at the surface of proteins that could occur at elevated pH between neighboring amino acids (Palmer et al., 2008). Although WT Sac7d and Sso7d are proteins with a high density of charged

surface residues and potential ion pairs, with acidic and basic residues representing about 44% of their sequences, it has been shown that their potential ion pairs do not contribute to stability (Clark et al., 2007). Interestingly, WT Sso7d was shown to be stable under extreme alkaline conditions, up to pH 13, indicating that this acidophilic protein can also be considered alkalinophilic as we showed previously for WT Sac7d (up to pH 12) (Béhar et al., 2013). Thus, we considered WT Sso7d as an attractive scaffold from which to derive more pH stable proteins. Noteworthy, D1Sso7d-DM was found as stable as WT Sso7d although about 19% of the sequence was mutated. After incubation for 25 h at pH 13, D1Sso7d and D1Sso7d-DM fully conserved their binding capacities, while D1Sac7d was found only about 30% active (Béhar et al., 2013). Additionally, their remarkable acidic stabilities were conserved. All together, these results highlight the interest of our grafting/mutational approach as a straightforward and efficient way to obtain proteins with longer lifetimes under a wide range of conditions.

Our initial choice of the archaeal "7 kDa DNA-binding" protein family as the molecular basis for the development of Affitins is now of further interest following our demonstration here of the additional benefit of natural diversity. In fact, this family is expanding regularly with whole genome sequences becoming available for more and more Archaea. This family of proteins is present in *Sulfolobus*, *Acidianus*, and *Metallospharea* genera, for example, and we anticipate that the discovery of new family members, potentially even more stable, will provide a new basis and/or information (e.g. sequences, structures) helpful for the design of improved artificial affinity proteins. This study also provides useful hints for the design of new libraries of Affitins, including for example the double mutation.

Although they decrease during the process, we think that the affinities of anti-IgG Affitins can probably be further improved by a diversification/selection step. This is not useful for all biotechnological applications, such as affinity chromatography. Indeed, dissociation constants in the range of 10^{-3} - 10^{-7} M are sufficient and a too high affinity would need a very stringent buffer for elution of the target (Firer, 2001), which could affect its biological activity. The robustness of Affitins combined with cost-efficient production processes might play a crucial role in their use in separomics. For these anti-IgG Affitins in particular, their alkaline resistance would clearly be an asset in designing reusable affinity columns for antibody purification able to resist harsh cleaning-in-place procedures involving steps with concentrated sodium hydroxide.

5. CONCLUSIONS

The stability enhancement of proteins for biotechnological applications is difficult to achieve since their function is often altered by mutations. Generally, these improvements are helpful as it is important that the affinity reagents used are stable, which is associated with a longer lifetime and constant binding capacities. Here, we have shown that suboptimal Affitins can be further engineered for improved pH stability under extreme conditions by switching a binding surface while preserving other favorable properties, including high expression yield in *E. coli*. This work highlights the interest of a grafting/mutational approach as a simple and effective way to obtain improved proteins.

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FIGURE LEGENDS

Fig. 1. Structures and sequences of Sac7d, and of Sso7d and its chimeras. (A) Schematic representations of the crystal structure of WT Sso7d (pdb code 1BNZ) superimposed on WT Sac7d (pdb code 1AZP). Residues substituted during the generation of D1Sac7d, and mutated residues at positions 16 and 36 are depicted as spheres. Molecular graphics were generated using PyMOL software (www.pymol.org). (B) Sequence alignment of WT Sac7d and WT Sso7d. Dots correspond to identical residues and dashes correspond to deletions. (C) Sequence alignment of WT Sac7d and D1Sac7d. (D) Sequences of D1Sso7d chimeras studied in this work. The sequence of the parent Affitin is shown at the top.

Fig. 2. Characterization of chimeras. (A) Size-exclusion chromatography of chimeras. The arrows indicate the elution volumes of the marker proteins (MW in kDa given above the arrow) and of blue dextran (V_0). The molecular mass standards, BSA (66 kDa), ovalbumin (44.3 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) are indicated. (B) Circular dichroism spectra of chimeras and WT Sso7d.

Fig. 3. Study of protein specificities by ELISA. Tested IgGs, and BSA as a negative control, were immobilized by adsorption in ELISA wells. Affitin binding was detected *via* its RGS-His6 tag using an anti- RGS-His6 tag antibody conjugated to HRP. Affitins were used at 1 µM.

Fig. 4. Thermal denaturation of chimeras monitored by circular dichroism. The unfolded fraction is plotted as a function of temperature. Protein solutions at a concentration of 0.02 mg/ml in a 1 mM potassium acetate buffer, pH 5.5, were used for this study. The fits for T_m calculations are indicated by bold lines.

Fig. 5. Study of the effect of pH on the structure of chimeras and WT proteins. Proteins were incubated at room temperature overnight in a solution adjusted to each pH unit from pH = 0 to pH = 14 and the residual ellipticity measured by circular dichroism. The continuous curves are drawn for clarity only.

Fig. 6. Comparative study of the kinetics of deactivation for chimeras at different pH. (A) Typical profile obtained at acidic pH, here for D1Sso7d-DM. (B) Stability profile obtained for D1Sso7d and for (C) D1Sso7d-DM at alkaline pH. Residual activities of binding proteins were monitored by SPR after incubation in a neutralizing buffer. The continuous curves are drawn for clarity only.

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535
536

TABLES

Table 1. Properties of proteins.

	Clone name						
Parameter	WT Sac7d	WT Sso7d	D1Sac7d[a]	D1Sso7d	D1Sso7d K13L	D1Sso7d E36L	D1Sso7d DM
Production yield (mg/L culture)	15[a]	6	30	40	13	22	26
K_D for hIgG (M)	-	-	$3.4\ 10^{-8}$	$1.5\ 10^{-6}$	$0.3\ 10^{-6}$	$0.3\ 10^{-6}$	$4\ 10^{-6}$
Thermal stability at pH 5.5 (°C)	90.4[b]	100.2[b]	80.7 ± 0.9	74.2 ± 0.1	70.9 ± 0.2	74.1 ± 0.1	76.9 ± 0.2
pH stability at equilibrium (up to)[c]	12[a]	13	11	12-13	n.d.	n.d.	13
pH stability after renaturation (up to)[d]	-	-	~11-12	≥ 13	n.d.	n.d.	≥ 13

a: According to Béhar *et al.* (Béhar et al., 2013)

b: According to Edmondson & Shriver (Edmondson and Shriver, 2001)

c: As determined by circular dichroism experiments

d: As determined by SPR experiments

-: not relevant

n.d.: not determined

545

Figure 1
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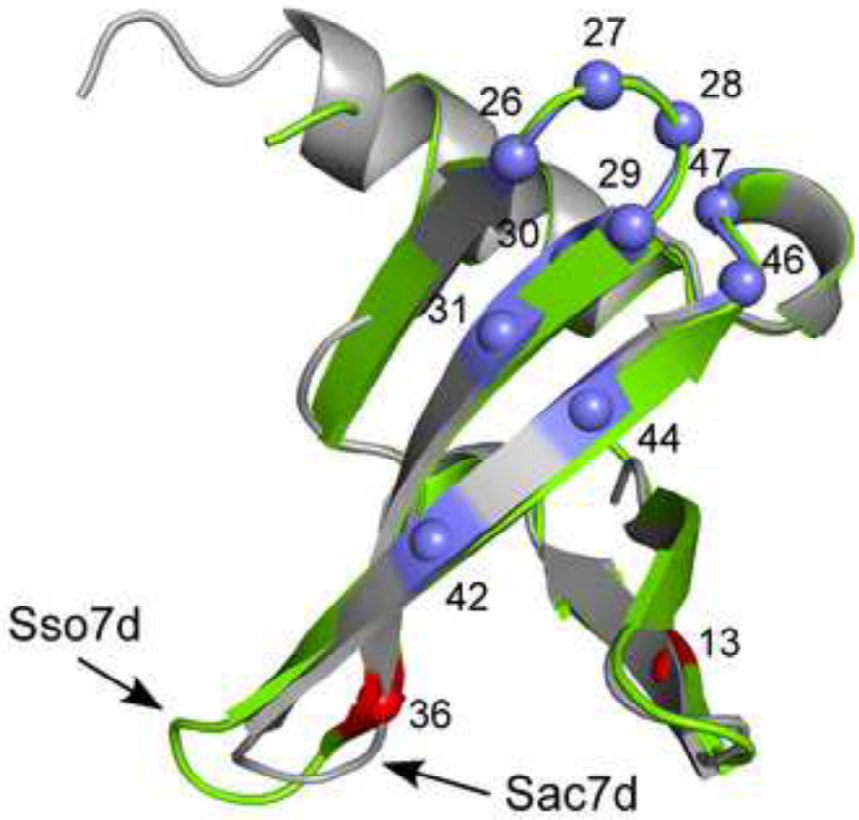
A



B

```
                  1         10        20        30        40        50        60
WT Sac7d          MVKVKFKYKGEEKEVDTSKIKKVWRVGKMVSFTYDDNG-KTGRGAVSEKDAPKELLDMLARAEREKK
WT Sso7d          .AT.............I............I.....EG.G.................Q..---.KQ..
```

C

```
                  1         10        20        30        40        50        60
WT Sac7d          MVKVKFKYKGEEKEVDTSKIKKVWRVGKMVSFTYDDNGKTGRGAVSEKDAPKELLDMLARAEREKK
D1 Sac7d          ........................DRLAAV..........Y.W.FT............C......
```

D

```
                  1         10        20        30        40        50        60
D1 Sac7d          MVKVKFKYKGEEKEVDTSKIKKVWRDRLAAVFTYDDNG-KTGYGWVFTKDAPKELLDMLACAEREKK
D1 Sso7d          .AT.............I..................EG.G.................Q..---.KQ..
D1 Sso7d-K13L     .AT.........L...I..................EG.G.................Q..---.KQ..
D1 Sso7d-E36L     .AT.............I..................LG.G.................Q..---.KQ..
D1 Sso7d-DM       .AT.........L...I..................LG.G.................Q..---.KQ..
```

Figure 2

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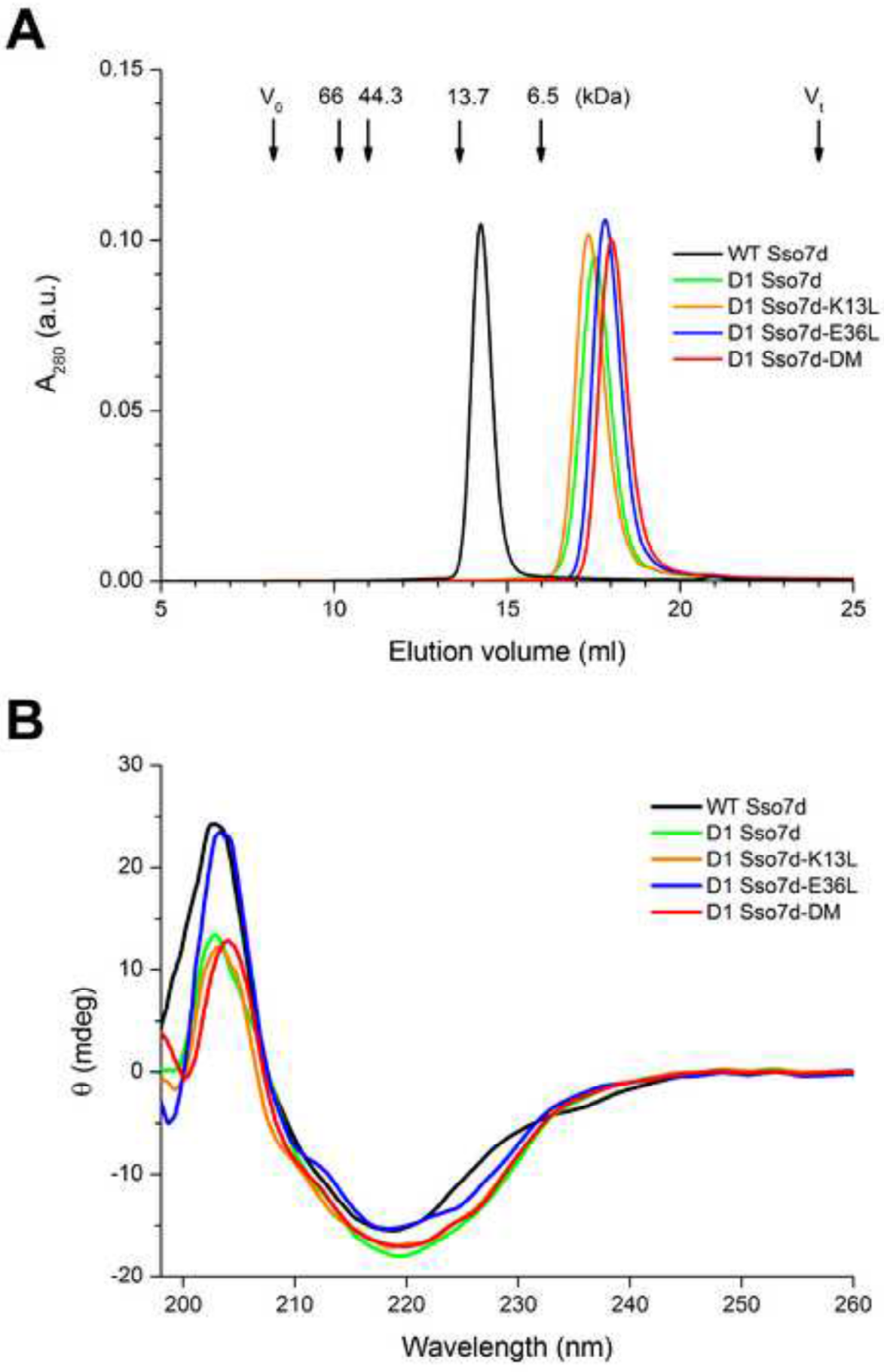

Figure 3

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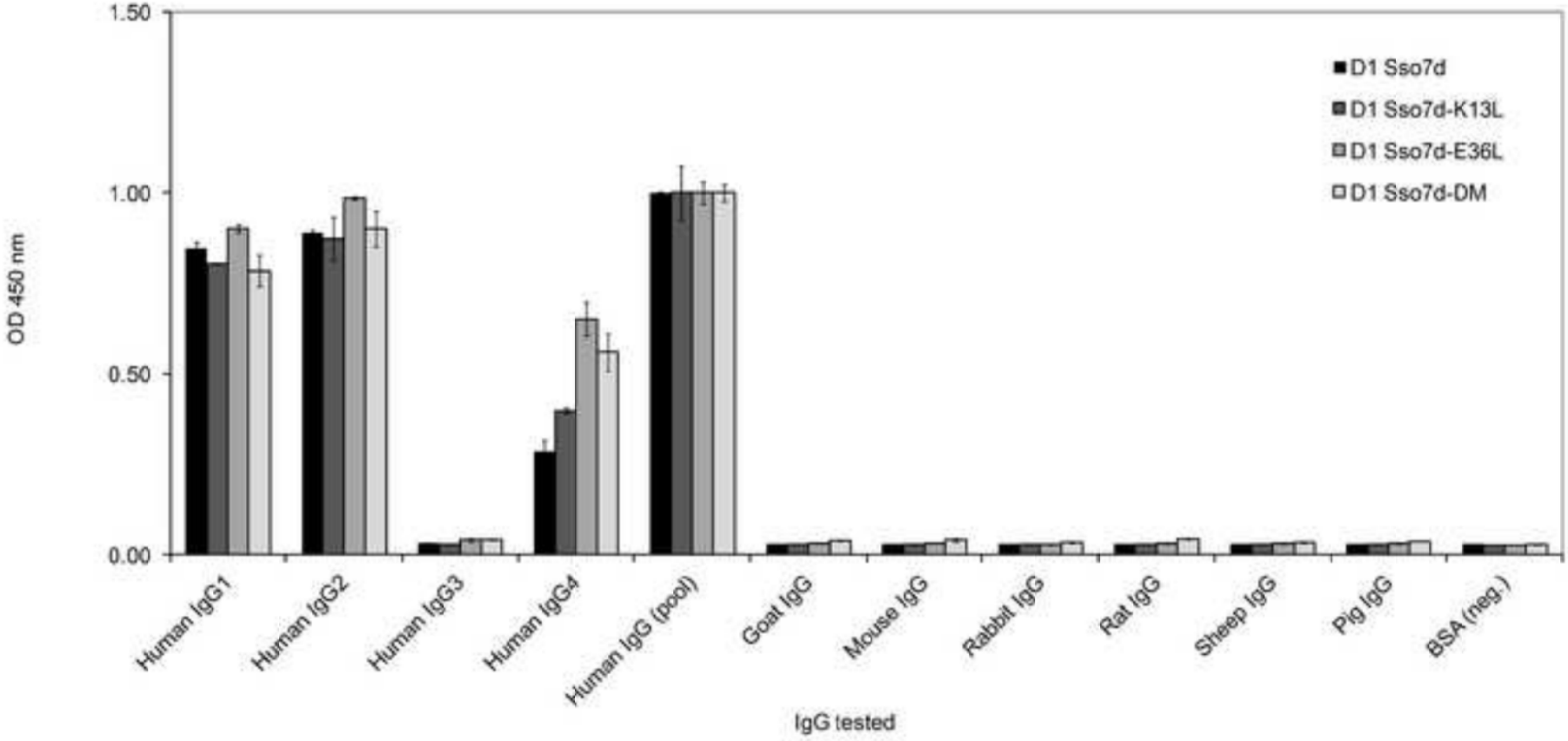

Figure 4

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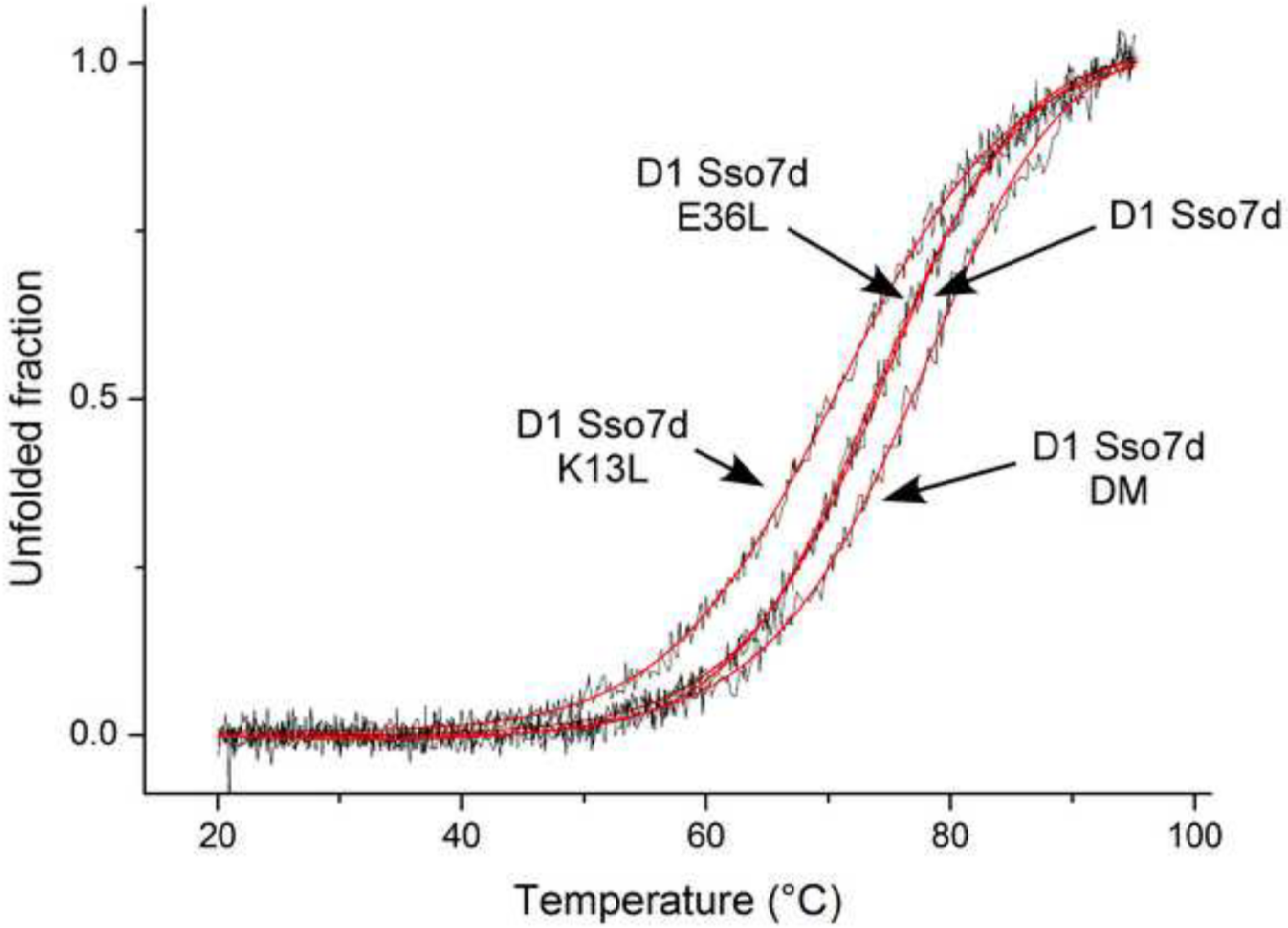

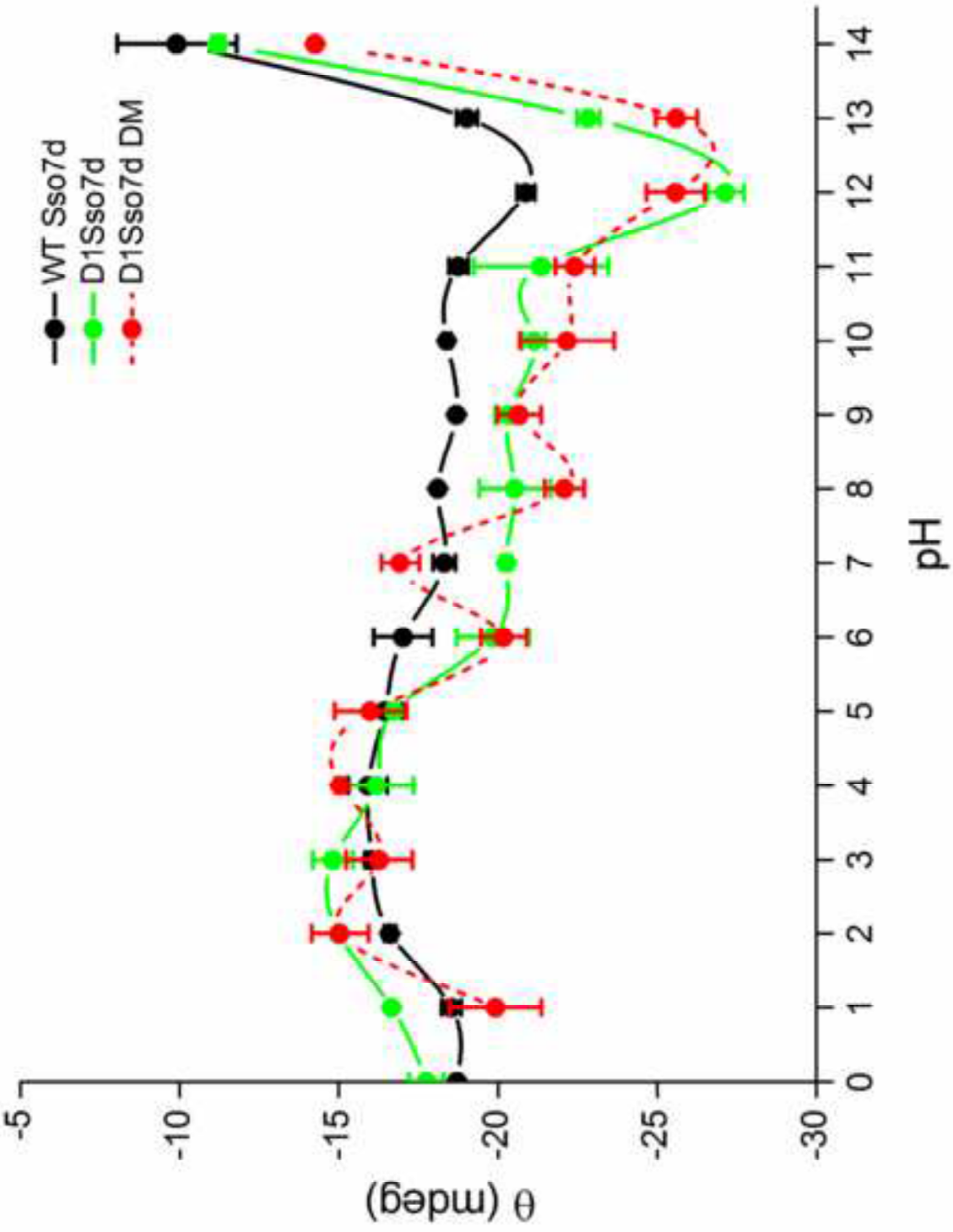

Figure 5
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Figure 6

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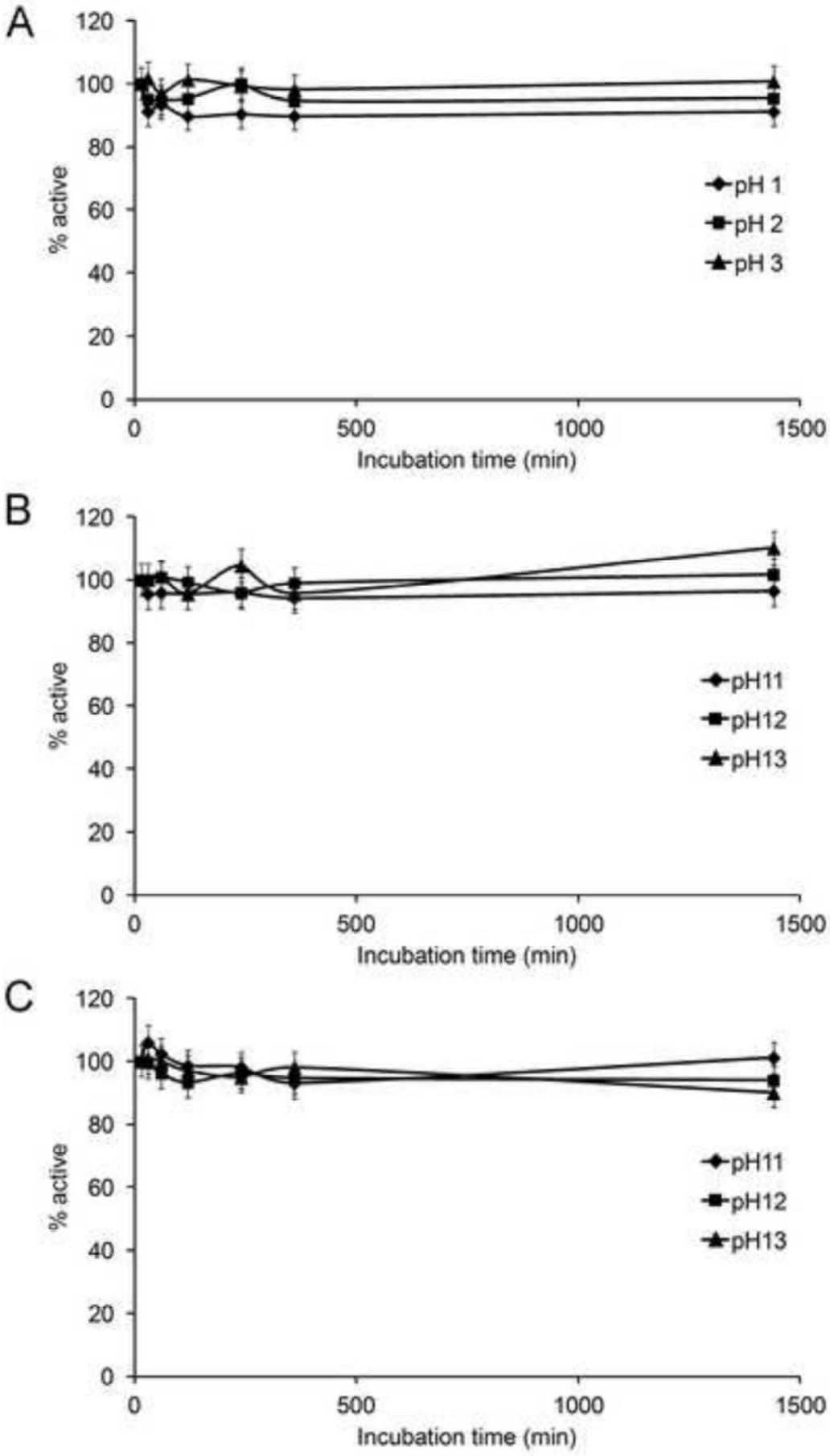

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