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Thermodynamically Stable Aggregation-Resistant Antibody Domains through Directed Evolution

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Protein aggregates are usually formed by interactions between unfolded or partially unfolded species, and often occur when a protein is denatured by, for example, heat or low pH. In earlier work, we used a Darwinian selection strategy to create human antibody variable domains that resisted heat aggregation. The repertoires of domains were displayed on filamentous phage and denatured (at 80 °C in pH 7.4), and folded domains were selected by binding to a generic ligand after cooling. This process appeared to select for domains with denatured states that resisted aggregation, but the domains only had low free energies of folding (ΔG^{o}_{N-D} = 15–20 kJ/mol at 25 °C in pH 7.4). Here, using the same phage repertoire, we have extended the method to the selection of domains resistant to acid aggregation. In this case, however, the thermodynamic stabilities of selected domains were higher than those selected by thermal denaturation (under both neutral and acidic conditions; ΔG^{o}_{N-D} = 26–47 kJ/mol at 25 °C in pH 7.4, or ΔG^{o}_{N-D} = 27–34 kJ/mol in pH 3.2). Furthermore, we identified a key determinant (Arg28) that increased the aggregation resistance of the denatured states of the domains at low pH without compromising their thermodynamic stabilities. Thus, the selection process yielded domains that combined thermodynamic stability and aggregation-resistant unfolded states. We suggest that changes to these properties are controlled by the extent to which the folding equilibrium is displaced during the process of selection.

Keywords: aggregation; directed evolution; phage display; antibody domains; dAbs

Proteins have an inherent tendency to aggregate[1] and, in doing so, they lose function and can become toxic. Folded proteins avoid aggregation by burying hydrophobic residues in the core and exposing a largely polar and hydrophilic protein surface to solvent[2] and by capping off or breaking up aggregation-mediating structural elements such as β-sheet edges.[3,4] However, with unfolded proteins present in thermodynamic equilibrium with the folded state and during protein synthesis, the hydrophobic core is more exposed. *In vivo*, aggregation of these unfolded species is often prevented by extrinsic factors such as molecular chaperones.[5,6] When proteins are expressed in heterologous organisms or purified *in vitro*, as in biotechnological processes, they are typically without such protective extrinsic factors and often at high concentrations, making the unfolded states particularly vulnerable to aggregation.[7] In principle, this leaves two distinct ways to engineer aggregation-resistant proteins: to make mutations that (a) increase the thermodynamic stability of the native state (thus reducing the concentration of the unfolded species), and/or (b) reduce the tendency of the unfolded species to aggregate.

Human antibody variable domains (dAbs) derived from the V_H3 family are typical of many proteins *in vitro*: natively folded dAbs resist aggregation, whereas denatured dAbs generally aggregate.[8]

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Abbreviations used: dAb, human antibody variable domain; T_m, melting temperature; PBS, phosphate-buffered saline.

Furthermore, these domains display apparent two-state unfolding when heated, providing a simple system to study aggregation.[8,9] In earlier work, we presented a phage-display method for selecting dAbs resistant to heat aggregation from a library based on DP47d, an aggregation-prone heavy-chain variable domain wherein diversity was introduced in the loops comprising the complementarity-determining regions.[10] The domain library was displayed on phage by fusion to the phage minor coat protein (pIII) present in about five copies on the infective tips of filamentous fd phage, thereby yielding high local concentrations of domains.[10,11] Aggregation was then promoted by heating the phage to 80 °C for 10 min in phosphate-buffered saline (PBS: 25 mM phosphate buffer at pH 7.4 with 150 mM NaCl), a temperature 19 °C above the apparent melting temperature T_m of DP47d,[8] and phage was selected after subsequent cooling by binding to protein A,[10] which was expected to bind only to domains that had escaped aggregation and refolded.

All the selected domains, when isolated as soluble fragments, resisted aggregation upon heating to 80 °C, but had melting temperatures similar to or lower than that of DP47d. Furthermore, the average ΔG^{o}_{N-D} for the selected clones (20 kJ/mol) was much lower than that of DP47d (35 kJ/mol) and several other aggregation-prone domains in the V_H3 family (40–53 kJ/mol).[12] The aggregation resistance of these domains must therefore be due to the properties of the denatured state. These results contrasted with those of others, who, using a similar process close to the melting temperature (50–60 °C) of the library members, had isolated a single-chain Fv fragment with improved thermodynamic stability but that aggregated irreversibly on heating.[13]

Antibody domains are also known to aggregate when exposed to acidic pH at physiological temperatures.[14] Here we first explored the effect of these conditions on the aggregation of model heavy-chain variable domains that were expressed as soluble fragments in the bacterial periplasm. Purified DP47d aggregated within a few hours upon exposure to 37 °C at pH 3.2 (Fig. 1a). These aggregates were amorphous with no amyloid component, as determined by the absence of an increase in thioflavin T fluorescence and the absence of fibrils when negatively stained and visualised by transmission electron microscopy (data not shown). At the same concentration, HEL4, a hen–egg lysozyme-binding domain previously shown to be aggregation-resistant upon heating in PBS,[8,10] showed greater aggregation resistance than DP47d at acidic pH, but did aggregate after an initial lag phase (Fig. 1a).

The thermal unfolding of the two domains at pH 3.2 was then followed by circular dichroism (CD) measurements. The melting curves were consistent with two-state transitions for both DP47d and HEL4, with the unfolding transition predominantly above 37 °C (Fig. 1b). The far-UV CD spectrum for HEL4 at 37 °C in pH 3.2 overlapped well with the

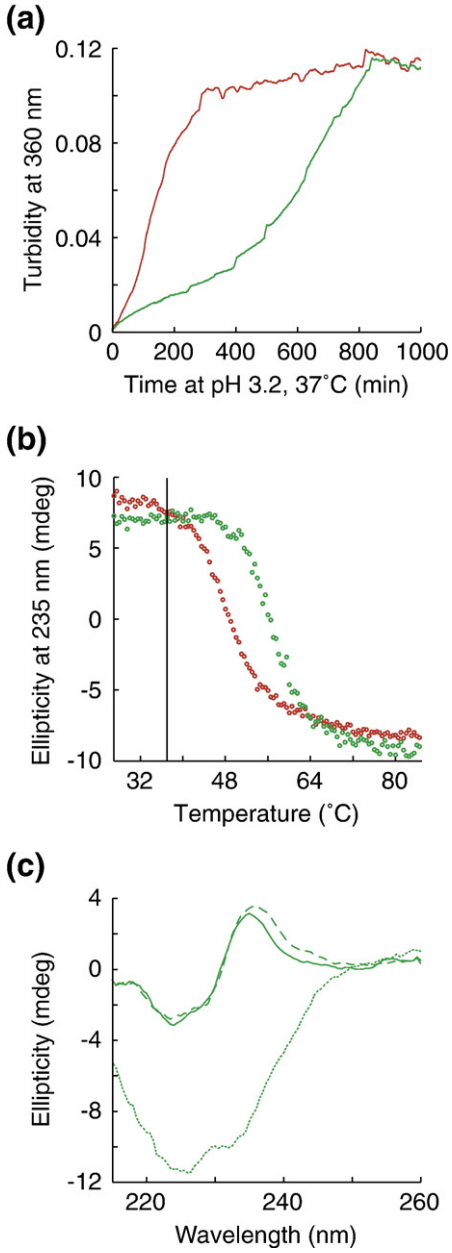

Fig. 1. Aggregation and biophysical properties of soluble DP47d (red) and HEL4 (green) at pH 3.2. (a) Turbidity measurements at 360 nm on 5 μM stirred protein solutions at 37 °C in pH 3.2 (HNC: 5 mM Hepes, 5 mM NaCl, and 100 mM citrate at pH 3.2). (b) Thermal unfolding at pH 3.2 (HNG: 5 mM Hepes, 5 mM NaCl, and 100 mM glycine at pH 3.2), followed by CD measurements at 235 nm. The vertical line marks the temperature (37 °C) used during the turbidity measurements in (a). The melting curves are consistent with two-state equilibrium unfolding transitions.[15] HEL4 reversibly unfolds. DP47d partially aggregates upon the unfolding with a downshift and 20% amplitude reduction upon repeated thermal melting. (c) Far-UV CD spectra for 5 μM HEL4 at 37 °C in pH 7.0 (HN: 10 mM Hepes and 10 mM NaCl at pH 7.0) (solid line), at 37 °C in pH 3.2 (HNG) (dashed line), and at 80 °C in pH 7.4 (PBS) (dotted line). Proteins were expressed and purified as described by Jespers *et al.*[10] No visible aggregates were formed during collection of the spectrum at 80 °C, and a native spectrum without amplitude reduction was restored upon subsequent cooling.

spectrum at 37 °C in pH 7.0, but the two spectra were distinctly different from the one for the thermally denatured HEL4 at 80 °C in PBS (Fig. 1c). These spectra suggest that the aggregation-promoting condition at 37 °C in pH 3.2 does not significantly perturb side-chain environments or secondary structures compared to neutral pH. Furthermore, 8-anilino-1-naphthalenesulfonic acid, a fluorescent hydrophobic dye known to bind to mobile hydrophobic regions typical of partially folded intermediates,[16] did not show increased binding to the domains at 37 °C in pH 3.2 compared to lower temperatures or neutral pH.

Taken together, the findings above suggest a two-state folding equilibrium in pH 3.2, with the domains largely natively folded at 37 °C. The latter is in contrast to heating at[13] or above the melting temperature,[10] as used in earlier work. Nevertheless, the folding equilibrium is displaced compared to physiological pH, as the natively folded state is destabilised at pH 3.2 (with decreases in apparent T_m: 16 °C for DP47d and 10 °C for HEL4), and we suggest that, at this pH, the concentration of the denatured state increases to a level that nucleates aggregation. The aggregation profiles with initial lag phases (Fig. 1a) are characteristic of such nucleation-dependent aggregation.[1]

We then examined the behaviour of phage displaying high local concentrations of DP47d and HEL4 at 37 °C in acidic pH. For DP47d, these conditions led to the formation of tip-to-tip clusters of phage visualised by transmission electron microscopy (Fig. 2a), where aggregate nuclei are formed at each phage tip, as previously noted for the heat-induced aggregation.[10] This was entirely consistent with the large loss of binding of DP47d phage to protein A (Fig. 2b). By contrast, for the HEL4 phage, there was little evidence of phage aggregation or failure to bind protein A after exposure to the same conditions (Fig. 2b).

To see whether the exposure of phage to acidic conditions could be used as the basis for a selection process for domains that resist acid aggregation, we mixed HEL4 phage with DP47d phage in a ratio of 1:1000 and operated several rounds of selection. The HEL4 phage could be enriched sevenfold per selection round (as in Jespers *et al.* [10], but here with the aggregation-promoting condition above). Operating the same selection process without the incubation step at 37 °C in pH 3.2, by contrast, led to no enrichment of HEL4 phage. This confirmed that the enrichment was due to the higher aggregation resistance of HEL4 at 37 °C in pH 3.2.

We now turned to the DP47d-based domain repertoire[10] used previously for the isolation of

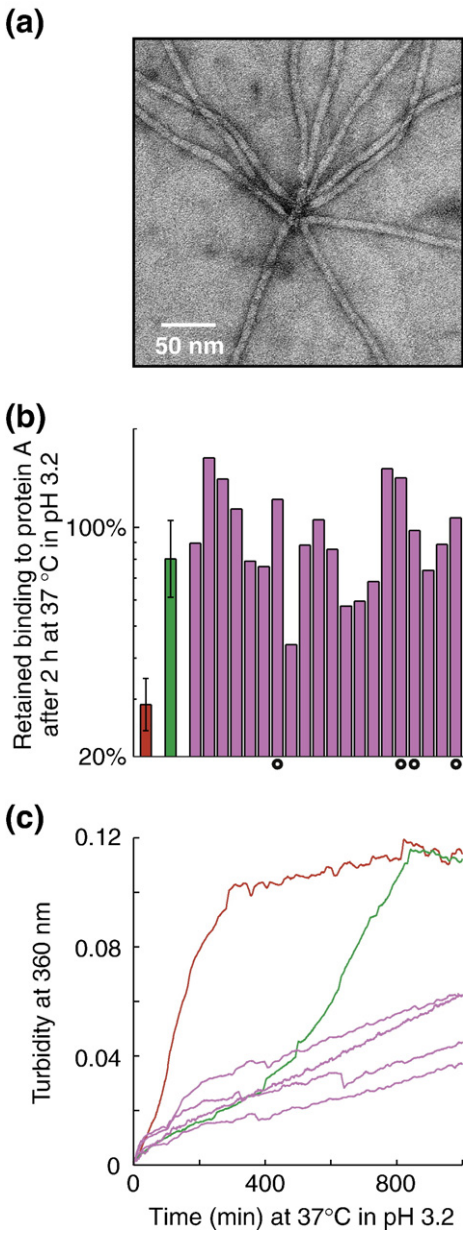

Fig. 2. Aggregation properties on phage and of selected domains in solution. The phage constructs were based on the fdDOG1 phage vector,[17] with the dAbs fused to all copies of the pIII protein separated by a c-*myc* tag linker.[10] Phage were prepared, purified, and stored as described by McCafferty *et al.*[18] (a) Transmission electron micrograph showing negatively stained tips of phage displaying DP47d after incubation at 37 °C in pH 3.0 (20 mM acetate) for 2 h. Phage were stained and visualised as described by Jespers *et al.*[10] (b) Aggregation propensities when displayed on phage for DP47d, HEL4, and 20 selected domains at 37 °C in pH 3.2. Purified phage at 1.0×10^{12} TU/ml was incubated for 2 h at 37 °C in pH 3.2 (HNC). After neutralisation, the level of aggregation, as determined by retained binding to protein A, was assayed by ELISA.[10] For DP47d (red) and HEL4 (green), the figure displays the means of logarithmic values ($N=6$) and 95% confidence intervals. A retained binding above 100% corresponds to a stronger phage-binding signal after incubation than before. Domains pH3–10, pH3–23, pH3–24, and pH3–28 [further characterised in (c)] are labeled with circles below the corresponding bars. (c) Turbidity measurements at 360 nm of 5 μM stirred samples upon incubation at 37 °C in pH 3.2 (HNC) (DP47d, green; HEL4, red; pH3–10, pH3–23, pH3–24, and pH3–28, purple).

domains that resisted aggregation on heating. We operated three rounds of selection at 37 °C in pH 3.2 for 2 h as the aggregation-promoting condition. The aggregation properties of the displayed domains on 20 selected phage clones were characterised by assessing the binding to protein A upon exposure to acidic pH[10] (Fig. 2b). All showed increased aggregation resistance at 37 °C in pH 3.2 compared to DP47d. Four domains were then expressed as soluble fragments, and their aggregation properties were evaluated (Fig. 2c). The selected domains all showed superior aggregation resistance to DP47d and HEL4 at 37 °C in pH 3.2 without any detectable exponential phase in the measured turbidity.

The apparent melting temperatures of these domains and of four of the heat-aggregation-resistant domains that were isolated previously[10] were determined by thermal unfolding at pH 3.2 using CD. Under these conditions, all the acid-selected domains had higher apparent melting temperatures and thermodynamic stabilities, and the differences between the two sets of domains largely persisted at neutral pH (Table 1). The results suggest that, in contrast to the heat-selected domains, the domains selected here have gained, at least in part, their aggregation resistance by increased thermodynamic stability.

We wondered whether we could identify the amino acid residues contributing to the resistance to acid aggregation. We therefore compared the sequences of 22 acid-selected domains to those of 20 domains from the unselected library. We noted significant changes (with numbering according to Kabat *et al.*[20]) at residue 27 (phenylalanine or valine, $P<0.01$, chi-square test), residue 28 (arginine or lysine, $P<0.01$), and residue 31 (aspartic acid or asparagine, $P<0.01$). Of these, arginine or lysine rarely occurs at residue 28 in natural antibody sequences and is not present in any of the human germ-line V_H sequences[21,22] (and only in about 1% of the rearranged sequences available in the Kabat antibody sequence database[23]). We therefore wondered if this change might contribute to resistance to acid aggregation.

We also noted that residue 28 represents threonine and arginine, respectively, in our two model domains DP47d (aggregates at pH 3.2) and HEL4 (resists aggregation at the same pH). We therefore mutated residue 28 to arginine in DP47d, and to threonine in HEL4 (and also in pH3–10, one of the acid-selected domains). The wild-type and mutant proteins were then compared for aggregation resistance. The domains with arginine at residue 28 were more resistant to aggregation than the corresponding domains with threonine (Fig. 3a), but were not more thermodynamically stable (Fig. 3b, Table 1). This confirms that arginine 28 contributes to resistance to acid aggregation, but does so by increasing the resistance of the denatured state to aggregation.

In conclusion, we have selected antibody domains that resist aggregation at acidic pH. In general, the resistance can be accounted for by the thermo-

Table 1. Thermodynamic stabilities of selected domains in solution

	PBS		pH 3.2	
Domain	Apparent T_m (°C)	Apparent ΔG^o_{N-D} (kJ/mol)	Apparent T_m (°C)	Apparent ΔG^o_{N-D} (kJ/mol)
DP47d	64	35	47	16
HEL4	65	29	55	21
pH3–10	71	26	60	27
pH3–23	77	–	65	30
pH3–24	72	–	58	24
pH3–28	81	47	68	34
HEAT13	56	18	43	7
HEAT36	63	21	42	7
HEAT47	63	19	52	18
HEAT59	55	19	51	7
DP47d T28R			48	16
HEL4 R28T			55	24
pH3–10 R28T			58	30

Apparent T_m and ΔG^o_{N-D} values derived from CD measurements at 235 nm upon thermal unfolding in PBS and pH 3.2 (HNG). Apparent T_m values refer to temperature at the midpoint transition for domains with reversible unfolding and to temperature at the start of aggregation for DP47d, pH3–23, and pH3–24 in PBS. The unfolding was considered reversible in cases where a second melting curve (1 °C/min) fully overlapping with the first could be collected after cooling of the protein sample. For domains with reversible unfolding, the melting curves were fitted to a two-state model,[10,19] and the best fit was used to calculate the apparent ΔG^o_{N-D} value at 25 °C using a ΔC_p contribution of 12 cal per amino acid residue.[10] For DP47d, pH3–23, and pH3–24 in PBS, the apparent ΔG^o_{N-D} value could not be derived as the domains aggregated during melting. For DP47d in PBS, the ΔG^o_{N-D} value was instead derived from urea-induced denaturation curves.[10] In all measurements, 5 μM unstirred protein was used (except for DP47d wt and DP47d T28R in pH 3.2, for which 1 μM was used to avoid aggregation during the measurements). The calculated apparent ΔG^o_{N-D} value for HEL4 (29 kJ/mol) was in good agreement with the value previously calculated based on urea-induced denaturation curves[8] (28.4 kJ/mol). For the remaining domains, the calculated apparent ΔG^o_{N-D} values should be treated with adequate care until confirmed with complementary methods and should primarily be used to assess differences between the analysed domains. Furthermore, as the purpose was to detect differences between the groups of selected domains, single data sets were collected for multiple domains, but error margins for individual data points were not assessed.

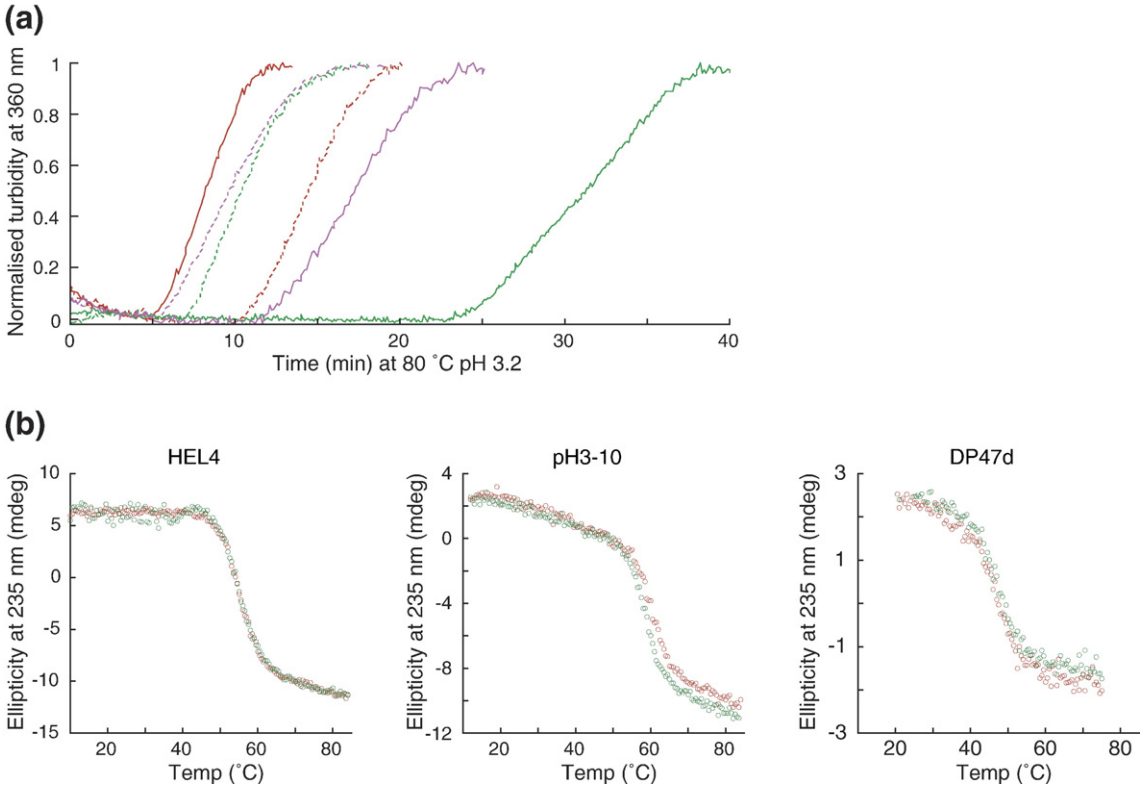

Fig. 3. The role of Arg28 in the stability and aggregation resistance of the denatured state. (a) Turbidity measurements at 360 nm of 5 μM stirred samples upon incubation at 80 °C in pH 3.2 (HNC). The turbidity measurements where stopped when the readings reached plateau levels (HEL4 wt, green solid; HEL4 R28T, green dashed; DP47d wt, red solid; DP37d T28R, red dashed; pH3–10 wt, purple solid; pH3–10 R28T, purple dashed). (b) Thermal unfolding at pH 3.2 (HNG), followed by CD at 235 nm (wt domains, red circles; point mutants, green circles). The measurements were performed on 5 μM unstirred protein, except for DP47d wt and DP47d T28R, which were performed at 1 μM to avoid aggregation of the former.

dynamic stability of the native state and also, at least for the domains comprising Arg28, by the improved properties of the denatured state. Taken together with previous findings using thermal unfolding,[10,13] we suggest that the outcome of the selections is determined by the displacement of the folding equilibrium induced by the selection condition. If conditions are such as to fully denature all members of the repertoire (as with extreme heat), then there is little selective advantage for those of greater thermodynamic stability, but a great advantage for those with aggregation-resistant denatured states. However, if conditions only lead to a partial unfolding of most members of the repertoire (as with acidic conditions, or heating below the melting temperatures), then the selection process will favour those domains that are thermodynamically stable (as well as those with aggregation-resistant denatured states). Indeed, as shown here, it is possible to combine both properties within the same protein.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.10.075

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