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Oligomerization properties of GCN4 leucine zipper *e* and *g* position mutants

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Abstract

Putative intersubunit electrostatic interactions between charged amino acids on the surfaces of the dimer interfaces of leucine zippers (***g-e′*** ion pairs) have been implicated as determinants of dimerization specificity. To evaluate the importance of these ionic interactions in determining the specificity of dimer formation, we constructed a pool of >65,000 GCN4 leucine zipper mutants in which all the ***e*** and ***g*** positions are occupied by different combinations of alanine, glutamic acid, lysine, or threonine. The oligomerization properties of these mutants were evaluated based on the phenotypes of cells expressing λ repressor–leucine zipper fusion proteins. About 90% of the mutants do not form stable homooligomers. Surprisingly, approximately 8% of the mutant sequences have phenotypes consistent with the formation of higher-order (>dimer) oligomers, which can be classified into three types based on sequence features. The oligomerization states of mutants from two of these types were determined by characterizing purified fusion proteins. The Type I mutant behaved as a tetramer under all tested conditions, whereas the Type III mutant formed a variety of higher-order oligomers, depending on the solution conditions. Stable homodimers comprise less than 3% of the pool; several ***g-e′*** positions in these mutants could form attractive ion pairs. Putative repulsive ion pairs are not found among the homodimeric mutants. However, patterns of charged residues at the ***e*** and ***g*** positions do not seem to be sufficient to predict either homodimer or heterodimer formation among the mutants.

Keywords: dimerization specificity; leucine zippers; protein structure; recombinant fusion proteins; site-directed mutagenesis

α-Helical coiled coils are involved in the assembly of a wide variety of proteins. A subclass of the coiled-coils known as leucine zippers are found as short dimerization motifs in many eukaryotic transcription factors. Leucine zipper sequences are characterized by leucine appearing in every seventh position (***d***) over four to five heptad repeats $(\boldsymbol{abcdefg})_n$ (for a review, see Hurst, 1994). Leucine zippers fold into dimeric, parallel coiled-coils, where each heptad forms two α-helical turns. Because of their small size and simple structure, leucine zippers and other short α-helical coiled coils have been used extensively as a model system to study how amino acid sequences specify structure (e.g., see Hodges et al., 1988; DeGrado et al., 1989; Hu et al., 1990; O'Shea et al., 1992, 1993; Zhou et al., 1992; Pu & Struhl, 1993; Monera et al., 1996; Zeng et al., 1997). Leucine zipper-containing transcription factors function as a variety of different pairs of homodimeric and heterodimeric subunits. The specificity of dimer formation is thus of special interest not only as an instance of the general problem of how protein–protein interactions are determined, but also for understanding the construction and operation of the regulatory circuitry of the cell.

The core of the dimer interface is formed by the predominantly hydrophobic residues at the ***d*** and ***a*** positions. The solvent-accessible ***e*** and ***g*** positions (Fig. 1), which flank the dimer interface, are occupied frequently by charged amino acids in naturally occurring leucine zippers (Hu & Sauer, 1992; Hurst, 1994). In the crystal structures of GCN4 homodimers and Jun-Fos heterodimers, ***g-e′*** intersubunit salt bridges are visible between oppositely charged amino acids in the $\boldsymbol{g}_i$ and $\boldsymbol{e}'_{i+5}$ positions (O'Shea et al., 1991; Ellenberger et al., 1992; Konig & Richmond, 1993; Glover & Harrison, 1995).

Changing the amino acids at the ***e*** and ***g*** positions can be sufficient to change the dimerization specificity of a leucine zipper (Schuermann et al., 1991; O'Shea et al., 1992, 1993; Vinson et al., 1993; John et al., 1994; Zhou et al., 1994a, 1994b). Studies on the role of these residues in conferring dimer stability and specificity have focused on their ability to form attractive and repulsive in-

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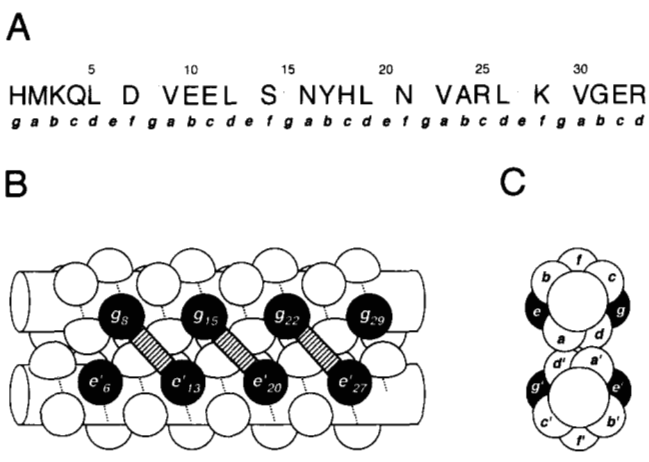

Fig. 1. The leucine zipper of GCN4. The leucine zipper is arranged as a parallel coiled-coil. **A:** Sequence of the leucine zipper as it occurs in the λ repressor fusion system. The ***e*** and ***g*** positions are indicated by outline font. Lower-case letters indicate positions in the heptad repeat. **B:** Side view of the dimer. Amino acid backbones in a helical conformation are represented by cylinders, with the path of the polypeptide chain indicated by the stippled lines. Side chains are represented as knobs. The ***e′*** and ***g*** positions are highlighted; hatched bars connecting ***g*** positions with following ***e′*** positions indicate possible positions of interchain ion pairs. **C:** End view showing how different heptad positions are arranged in the dimer.

terchain ionic interactions. Thermodynamic cycle analysis of mutant coiled coils showed that charged residues at a ***g*** position at residue i can interact favorably or unfavorably with a charged residue at the ***e′*** position at $i + 5$ (Krylov et al., 1994; Zhou et al., 1994a, 1994b; Kohn et al., 1995), consistent with the orientation of ion pairs observed in the crystal structures.

Initially, the dimerization specificities of some natural and designed leucine zippers were rationalized in terms of ***g-e′*** ionic interactions (O'Shea et al., 1992, 1993). Vinson et al. (1993) proposed that whether a heterodimer can form between two leucine zipper monomers can be predicted by comparing the ***g-e′*** ion pairs in the putative heterodimer to those of the two putative homodimers. Dimers with more attractive interactions between oppositely charged residues and fewer repulsive interactions between like charges would be favored over dimers with fewer attractive and more repulsive interactions. The properties of many leucine zipper mutants appear to support this idea (Schuermann et al., 1991; John et al., 1994; Zhou et al., 1994a, 1994b). Indeed, the stability of a set of two-stranded coiled-coil peptides was related linearly to the number of attractive and repulsive interchain and intrachain interactions (Monera et al., 1994). However, the contributions of ionic interactions between charged end groups remains controversial (Lavigne et al., 1996). NMR measurements of pK_a shifts of glutamates involved in ion pairs in the GCN4 structure suggest that the net contributions of interchain Glu-Lys electrostatic interactions to coiled-coil stability are either insignificant or slightly destabilizing (Lumb & Kim, 1995). The relative and absolute contributions of attractive and repulsive ***g-e′*** ionic interactions are also clearly dependent on solution conditions (Monera et al., 1994; Yu et al., 1996).

Models for how different amino acid side chains at the ***e*** and ***g*** positions determine dimerization specificity should be able to predict the formation of homodimers and heterodimers among a set of related sequences that vary only at these positions. In the experiments described here, we studied GCN4 leucine zipper mutants with different combinations of charged and neutral residues at the ***e*** and ***g*** positions. These mutants include many combinations of potentially attractive and repulsive interchain g_i-e'_{i+5} interactions and should thus provide an experimental test of whether or not the predicted contributions of ionic interactions are sufficient to predict dimerization specificities of leucine zippers.

Results

Identification of homooligomeric mutants

To test the importance of ***g-e′*** ion pairs in determining dimerization specificity, we studied a previously described pool of mutations in the leucine zipper of GCN4 (Hu et al., 1993). In this pool, each of eight ***e*** and ***g*** positions (Fig. 1) are occupied by all possible combinations of alanine (A), glutamic acid (E), lysine (K), or threonine (T). The 65,536 sequences in this pool should include many different combinations of attractive (EK and KE), repulsive (EE and KK), or neutral (EA, AE, ET, TE, KA, AK, KT, TK, AT, TA, AA, and TT) residue pairs at each ***g-e′*** pair (***g***8-***e***13′, ***g***15-***e***20′, or ***g***22-***e***27′; see Fig. 1) in homodimers or heterodimers.

The properties of these mutant leucine zippers can be characterized rapidly in gene fusions to the DNA binding domain of λ *c*I repressor (*c*I$^+$ fusions). *Escherichia coli* cells expressing plasmid-encoded oligomeric *c*I$^+$ fusions are immune to λ phage infection, whereas cells expressing monomeric *c*I$^+$ fusions are sensitive. In a previous study (Hu et al., 1993) of this mutant pool, the fusion proteins were expressed from the uninduced *lacUV5* promoter. Under these conditions, about 73% of the mutants were immune. To provide a more stringent screen for homooligomers, we recloned the mutant library so that fusion proteins were expressed from a weaker constitutive promoter, P7107 (unpubl.).

DNA encoding a mixture of mutant sequences was subcloned from an amplified pool of previously described transformants (Hu et al., 1993). Among 111 independent subclones screened for phage immunity, only about 10% of the mutants formed enough functional homooligomers at this lower expression level to confer immunity to λ (data not shown). Note that, at this expression level, some of the proteins that dimerize weakly may not score as immune; cells expressing control fusions with leucine zippers from GCN4 and Jun are immune to λ, whereas cells expressing a C/EBP fusion are sensitive (Zeng et al., 1997).

To increase the number of functional mutants in our data set, 50 additional mutants were identified by selection for growth on plates seeded with λ. The DNA sequences encoding the mutant leucine zippers were determined and clones with mutations only at the ***e*** and ***g*** positions were chosen for further study. Each mutant is identified by an Isolate Designation (ID) and its sequence in the ***e*** and ***g*** positions, i.e., ***e***6-***e***13-***e***20-***e***27/***g***8-***g***15-***g***22-***g***29.

e and g position mutants can form higher-order oligomers

Preliminary experiments to characterize the ***e*** and ***g*** position mutants that conferred λ immunity led us to suspect that some might form higher-order homooligomers instead of homodimers (data not shown). Dimers can be distinguished from higher-order oligomers in vivo by an assay based on binding of repressor fusions to tandem λ operator sites (Zeng & Hu, 1997). In $\lambda 112O_sP_s$ (Beckett et al., 1993), two DNA-binding sites for λ repressor lie upstream of a synthetic promoter controlling expression of chloramphenicol

Table 1. *Oligomerization stoichiometry assays for representative mutants*[a]

Leucine zipper	*e/g* sequence	%*lacZ* in λ112O$_s$P$_s$	%*lacZ* in λXZ970	XZ970/λ112O$_s$P$_s$	Note
wt GCN4	ELEK/KKEL	42 ± 1	58 ± 1	1.4	Dimer
GCN4-aIdL	ELEK/KKEL	19 ± 3	56 ± 3	2.9	Dimer
GCN4-aLdI	ELEK/KKEL	7 ± 0	41 ± 5	5.8	Tetramer
n34	KKTK/AAEA	30 ± 1	52 ± 1	1.7	Type D
s39	TEKT/AEAK	3 ± 1	40 ± 1	13.3	Type I
s36	AKKA/ATTK	5 ± 1	63 ± 6	12.6	Type II
n31	TAAA/AAAT	7 ± 0	82 ± 2	11.7	Type III

[a]Expression of *lacZ* in reporter strains JH607 (for λ112O$_s$P$_s$) and XZ980 (for λXZ970) was normalized in each case to isogenic strains without any repressor plasmids. Data were averaged over at least two experiments.

acetyl transferase (*cat*) and β-galactosidase (*lacZ*). The distal site, which does not overlap the promoter, is a high-affinity binding site, whereas the proximal site, which does, is a weak binding site.

If a cI^+ fusion protein forms tetramers, binding of repressor domains to the upstream site in λ112O$_s$P$_s$ increases the occupancy of the promoter-proximal site by increasing the local concentration of DNA binding domains. For comparison, we measured the ability of each mutant to repress expression from λXZ970, an isogenic control reporter where the distal binding site in λ112O$_s$P$_s$ has been removed. Thus, significantly increased repression of λ112O$_s$P$_s$ compared to λXZ970 indicates that a fusion protein forms higher-order oligomers either in solution or after DNA binding. Data for representative mutants and controls are shown in Table 1. The fusion to wild-type (wt) GCN4 leucine zipper, which forms dimers (Hope & Struhl, 1987; O'Shea et al., 1989, 1991), represses moderately in both reporters; the fusion to GCN4-aIdL, a dimeric mutant of GCN4 (Harbury et al., 1993), represses a little better in λ112O$_s$P$_s$ than in λXZ970. By contrast, the fusion to GCN4-aLdI, a tetrameric mutant of GCN4 (Harbury et al., 1993), represses *lacZ* expression from λ112O$_s$P$_s$ to one-sixth the level expressed from λXZ970. Of the four mutants shown in Table 1, three repress *lacZ* expression from λ112O$_s$P$_s$ much more (11–13-fold) efficiently than from λXZ970. This property of these mutants is inconsistent with dimers being their major oligomeric form.

Among 25 mutants tested in both reporters, every mutant that expressed *lacZ* from λ112O$_s$P$_s$ at 10% or less of the unrepressed control and was unable to grow on 150 μg/mL Cm in the λ112O$_s$P$_s$ strain showed a pattern of repression consistent with formation of higher-order oligomers. Therefore, for the other 30 mutants, we only used assays for *lacZ* expression and Cm sensitivity in λ112O$_s$P$_s$ to screen for formation of higher-order oligomers.

Using these tests, 42 of 55 functional mutants were classified as forming higher-order oligomers. All but one of these, the s12 (AAAT/AAEK) mutant, fall into one of three types based on sequence features (Table 2A,B,C).

The remaining 13 mutants repressed *cat* and *lacZ* from λ112O$_s$P$_s$ less than 10-fold. Eight of them were further tested for *lacZ* expression in λXZ970 (data not shown). The pattern of repressor activities for these mutants (Type D) was similar to the dimeric controls (Table 1). The sequences of the Type D mutants are shown in Table 3.

Higher-order oligomers form in vitro

To determine whether the assays performed in vivo identified novel oligomeric forms of the leucine zipper, two mutant fusion proteins were purified and characterized in vitro by gel filtration, crosslinking, and sedimentation velocity and equilibrium experiments.

Figure 2 shows sedimentation equilibrium data for mutant and control repressor fusion proteins in 200 mM KCl at 20–25 °C. Sedimentation of fusion proteins containing the wild-type GCN4 leucine zipper is consistent with a mixture of monomer–dimer and monomer–tetramer equilibria with dissociation constants of about 3.6×10^{-6} M and 2×10^{-15} M3, respectively (Fig. 2A). Assuming these values, 60–70% of the total protein is in dimers under the conditions used in these experiments, whereas tetramers account for only 3–7% of the total protein. Sedimentation of the fusion protein containing the control ***a***L***d***I tetrameric leucine zipper is well described by a monomer–tetramer equilibrium, with a small amount of aggregation to higher-order complexes (Fig. 2B). Sedimentation of s09, a Type I mutant, was also consistent with a monomer–tetramer equilibrium, but more higher-order oligomers were observed than with the ***a***L***d***I tetrameric control protein (Fig. 2C). Crosslinking and size-exclusion chromatography experiments were consistent with the s09 fusion proteins being predominantly tetrameric, whereas analysis of sedimentation velocity data also suggested that the protein is predominantly tetrameric with small but significant amounts of monomers and higher-order oligomers (data not shown).

By contrast, the class III mutant n01 behaved as if it formed higher-order aggregates. Figure 3 shows the behavior of this mutant in size-exclusion chromatography. At 200 mM KCl, the n01 fusion protein elutes as a broad peak with an apparent molecular weight of about 400 kDa, corresponding to 20 or more subunits in the complex (Fig. 3A). Note, however, that the broad tail of the peak extends beyond the elution position expected for tetramers and dimers, suggesting dissociation of the aggregates as the protein is diluted through the column. At 500 mM KCl, the n01 fusion protein eluted as a more symmetrical peak, but the elution position varied with the concentration of protein loaded (Fig. 3B,C). The peaks elute at apparent molecular weights on the order of 140–200 kDa. Sedimentation velocity experiments indicate that the n01 fusion protein consists of a complex mixture of different oligomeric species (data not shown).

Dimerization specificity assay

We next examined the specificity of interactions among eight of the dimer-forming Type D mutants, using an assay based on challenging the activity of a repressor fusion with a dominant negative fusion protein (Bunker & Kingston, 1995; Hu, 1995; Joung et al., 1995; Marchetti et al., 1995; Zeng et al., 1997). Mutations in λ

Table 2. *Mutants that form higher-order oligomers*[a]

A. Type I									B. Type II									C. Type III								
Mut	*e* positions				*g* positions				Mut	*e* positions				*g* positions				Mut	*e* positions				*g* positions			
n32	**A**	**E**	**K**	T	**A**	T	E	T	s05	**A**	**K**	K	T	**A**	T	**A**	**K**	n01	A	A	A	T	**A**	**A**	**A**	**T**
s01	**T**	**E**	**K**	A	**A**	**A**	A	K	s10	**A**	**K**	T	T	**A**	A	**A**	**K**	n31	T	A	A	A	**A**	**A**	**A**	**T**
s06	**A**	**E**	**K**	K	**A**	**A**	A	K	s17	**A**	**K**	A	T	**A**	A	**A**	**K**	n33	A	K	T	K	**A**	**A**	**A**	**A**
s09	**T**	**E**	**K**	K	**A**	**E**	K	A	s34	**A**	**K**	K	K	**A**	T	T	**K**	s02	T	A	T	K	**A**	**A**	**A**	**A**
s21	**A**	**E**	**K**	T	E	**E**	T	K	s36	**A**	**K**	K	A	**A**	T	T	**K**	s08	A	A	T	A	**A**	**A**	**A**	**A**
s22	**T**	**E**	**K**	E	**A**	**E**	E	E	s62	**A**	**K**	T	K	**A**	A	**A**	E	s14	T	E	T	K	**A**	**A**	**A**	**A**
s25	**A**	**E**	**K**	T	**A**	**E**	K	E										s18	A	K	K	A	**A**	**A**	**A**	**T**
s30	**A**	**E**	**K**	K	**A**	**E**	K	T										s31	E	A	A	A	**A**	**A**	**A**	**T**
s32	**T**	**E**	**K**	K	E	**E**	E	T										s35	K	E	T	A	**A**	**A**	**A**	**T**
s37	**T**	**E**	T	K	**A**	**A**	A	E										s40	K	E	A	K	**A**	**A**	**A**	**A**
s39	**T**	**E**	**K**	T	**A**	**E**	A	K										s42	T	K	E	K	**A**	**A**	**A**	**A**
s48	**A**	**E**	T	K	**A**	**E**	E	A										s44	E	E	A	T	**A**	**A**	**A**	**T**
s52	**A**	**E**	**K**	A	**A**	**A**	A	K										s51	T	A	K	K	**A**	**A**	**A**	**A**
s58	**A**	**E**	**K**	K	T	**A**	E	A										s54	T	K	K	A	**T**	**A**	**A**	**A**
s59	**A**	**E**	**K**	K	**A**	K	E	A										s57	T	T	T	K	**A**	**A**	**A**	**A**
s70	**A**	**E**	**K**	T	**A**	**E**	A	K										s75	A	A	A	K	**A**	**A**	**A**	**A**
s72	**A**	**E**	**K**	A	E	**E**	T	E																		
s73	**A**	**E**	**K**	A	**A**	**A**	E	A																		
A	2	0	0	4	14	6	6	5	A	6	0	1	1	6	3	4	0	A	5	7	6	6	15	16	16	10
T	6	0	2	5	1	1	2	3	T	0	0	2	3	0	3	2	0	T	7	1	6	2	1	0	0	6
E	0	18	0	1	3	10	7	4	E	0	0	0	0	0	0	0	1	E	2	4	1	0	0	0	0	0
K	0	0	16	8	0	1	3	6	K	0	6	3	2	0	0	0	5	K	2	4	3	8	0	0	0	0
cons	At	E	K	x	A	Ea	x	x	cons	A	K	x	x	A	x	A	K	cons	x	x	x	x	A	A	A	At

[a]Each mutant is listed by its ID and *e* and *g* sequence. The consensus sequence is assigned when the occupancy of an amino acid at a position is >50%, and it is capitalized in the consensus. Consensus residues in the *e* and *g* positions of each mutant are indicated in boldface. In A and C, if the second most frequent amino acid is more than 30%, it is also included into the consensus, but not capitalized.

repressor DNA-binding domain that destroy specific DNA binding have a dominant negative phenotype (Nelson et al., 1983). When a cI^+ fusion and a dominant negative fusion are expressed in the same cell, the immunity of the cell is determined by the interaction or the lack thereof between the two leucine zippers. If the leucine zippers in the two fusion proteins do not interact with each other, the activity of the cI^+ fusion will be insensitive to the presence of the other fusion protein and the cell will be immune to phage infection. However, if stable heterodimers can form, homooligomers of the cI^+ fusion will be titrated into inactive heterodimers, and the cell will lose immunity. Note that the sensitivity of a cI^+ repressor fusion to interference by a dominant negative fusion is dependent on the absolute and relative expression levels of the two proteins. In our implementation of this scheme, titration is observed whenever the same homodimeric leucine zipper is used in both fusion proteins (Zeng et al., 1997).

Table 3. *Type D mutants*

Mutant ID	*e/g* sequence	net *g-e'*[a]
n34	KKTK/AAEA	2
s03	KEKT/KEAE	4
s11	TTKK/AEEK	4
s20	EEAE/KKAA	2
s24	AKKK/TETA	2
s26	AEAE/KKKE	4
s33	KAKK/EEEE	4
s38	KEKK/EEET	2
s45	AKKK/EAEK	4
s56	EKTA/EKTK	2
s63	EKKA/AEAT	2
s64	ATKK/AEET	4
s67	KKEA/EKEA	4

[a]Calculated from *g*1-*e*2', *g*2-*e*3', and *g*3-*e*4' ion pairs per homodimer, with EK or KE equals 1, EE or KK equals −1 (Vinson et al., 1993).

The eight Type D mutants and wt GCN4 were cloned into a dominant negative fusion vector and were coexpressed with the cI^+ fusions. The results of phage immunity tests on strains expressing 81 pairwise combinations of wt and mutant leucine zippers are shown in Figure 4. As had been observed previously for fusions to other homodimeric leucine zippers (Zeng et al., 1997), each of the Type D mutants was inactivated by an excess of the dominant negative fusion protein containing the same leucine zipper.

For any pair of leucine zipper mutants, the results of the dimerization specificity assays fall into one of three patterns. In the first pattern, neither dominant negative fusion protein detectably affects the activity of the cI^+ fusion to the other leucine zipper. Specifically, none of the eight Type D mutants can titrate or be titrated by GCN4 and mutants s56 and s64 do not titrate each other. In the second pattern, each dominant negative fusion titrates out the activity of the cI^+ fusion to the other leucine zipper. All combinations of the mutants n34, s03, s20, s24, and s56 titrate each other. Other mutually titrating pairs are n34 + s67, s20 + s67, s56 + s67, and s26 + s67.

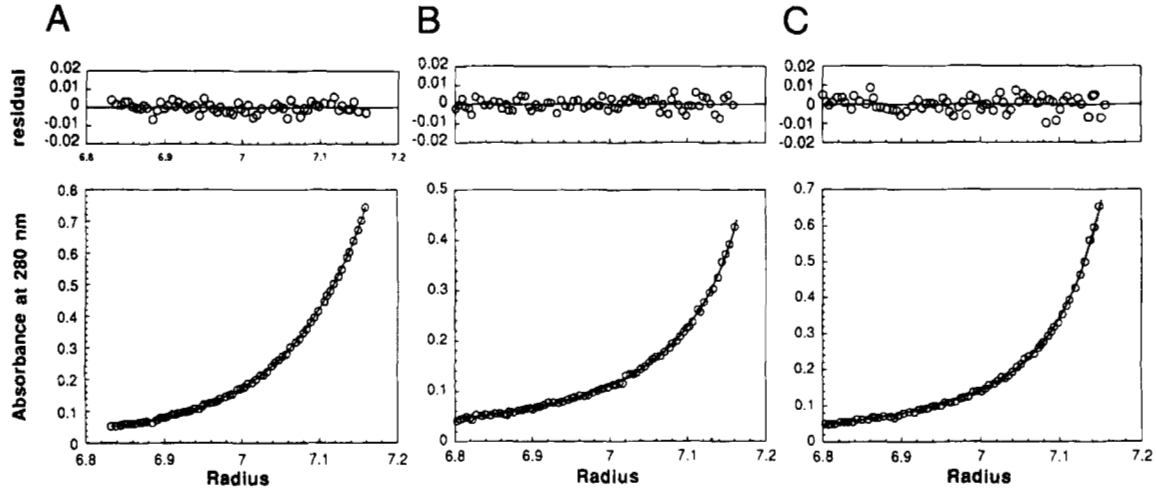

Fig. 2. Sedimentation analysis of fusion proteins. Samples were prepared and analyzed as described in Materials and methods. **A:** cI-GCN4 fusion protein. **B:** cI-aLdI. **C:** cI-s09 mutant. For each panel, the lower plot shows the raw data and the best fit to a specific model. The upper plot shows residuals from fitting panel A to a mixture of monomer–dimer and monomer–tetramer equilibria and fitting panels B and C to a mixture of monomer–tetramer and monomer–octamer equilibria.

Finally, the relationship between two leucine zippers can be asymmetric. "One-way titration" occurs when the dominant negative fusion to leucine zipper A can titrate the cI^+ fusion to leucine zipper B, but the dominant negative fusion to B cannot titrate cI^+-A. This is observed in several of the pairs: n34 + s26, s24 + s26, s24 + s67, s56 + s26, s64 + n34, s64 + s03, s64 + s20, s64 + s24, s64 + s26, s64 + s67, and s67 + s03.

Under the conditions used to study the Type D mutants, cI^+ repressor fusions to mutants that form higher-order oligomers were not titrated by dominant negative fusions to the same leucine zipper mutant. This can be understood if only two cI^+ domains in a mixed tetramer are sufficient to retain repressor activity. In this case, a higher molar ratio of dominant negative to cI^+ protein would be needed to titrate enough of the latter into heterotetramers with only one cI^+ DNA binding domain.

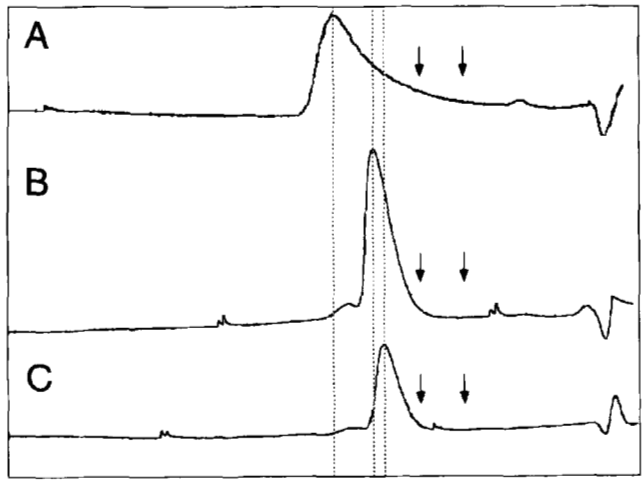

Fig. 3. Size-exclusion chromatography of mutant n01 fusion protein. The n01 fusion protein was resolved on a Superdex 200 HR FPLC column as described in Materials and methods. **A:** 2 mg/mL loaded and eluted in 200 mM KCl. **B:** 2 mg/mL loaded and eluted in 500 mM KCl. **C:** 1 mg/mL loaded and eluted in 500 mM KCl. Dashed lines indicate the positions of the peaks in the three traces; arrows indicate the positions where tetrameric and dimeric fusions elute in parallel experiments.

Discussion

Although this study was initiated to examine the role of ionic interactions in dimerization specificity, the DNA binding properties of repressor fusions in vivo suggest that in our mutant pool only the Type D mutants form homodimers, whereas most of the other mutants that form functional fusion proteins at all form higher-order homooligomers. For one class I mutant and one class III mutant, we have been able to show formation of higher-order oligomers in vitro.

Although our data indicate that one Type I mutant forms predominantly tetramers and one Type III mutant forms oligomers ranging from tetramers to large aggregates, the structural nature of these oligomers is certainly not yet clear. The significance of the consensus residues is demonstrated by those mutants where one or two amino acid changes at the ***e*** and ***g*** positions can be correlated with a difference in the oligomerization properties of a pair of mutants. For example, a single substitution at the ***e*1** position away from the Type I consensus converts s32 (Type I) to s38 (Type D). In several other examples, mutants with different oligomerization properties differ by only one or two amino acid changes. None of the 45 nonfunctional mutants (not shown; Hu et al., 1993), or the 13 Type D mutants (Table 2), or the 67 naturally occurring leucine zippers (Hurst, 1994) match any of the three consensus sequences.

Type D mutants comprise only about one-fourth of the functional oligomers, or less than 3% of the original mutant pool. Although this set of sequences is small, common features among these mutants may reflect important determinants of the parallel dimeric structure of the wild-type leucine zipper. Homodimers of the wt GCN4 leucine zipper and the 13 Type D mutants all have at least one pair of attractive ***g*-*e'*** and ***g'*-*e*** ionic interactions (KE or EK). Among the Type D mutants (Table 3), only one repulsive ***g*-*e'*** ionic interaction, between ***g*8E** and ***e*13E** of mutant s38, is found. In contrast, most of the 45 mutants that are sensitive to phage

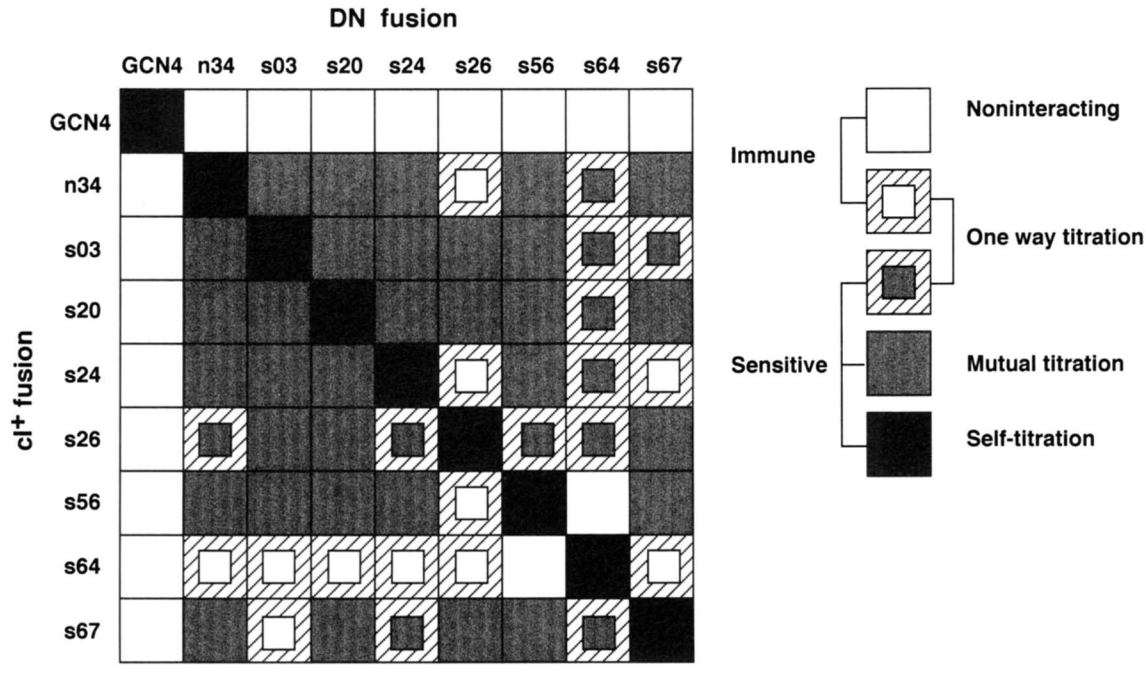

Fig. 4. Dimerization specificity of Type D mutants. Results of the dimerization specificity assay are shown as a matrix. Wild-type GCN4 and mutants are listed by their ID designations (see Table 3). Each row shows results for one cI^+ fusion protein expressed with each of the dominant negative fusions (DN fusions). Shading indicates sensitivity or immunity to phage λ. Black boxes along the diagonal show combinations where the leucine zipper is the same in both fusion proteins. Solid white boxes indicate a combination that is immune to phage infection when either combination of cI^+ and dominant negative fusions are coexpressed. Solid gray boxes indicate a combination that is sensitive to phage infection with either combination of cI^+ and dominant negative fusions. Hatched boxes with insets indicate combinations where different results are obtained depending on which leucine zipper is fused to the cI^+ and dominant negative DNA binding domains. White insets indicate that immunity is retained for this specific configuration and shaded insets indicate that the pair is sensitive to λ.

infection, indicating weak or no oligomer formation, have repulsive (EE or KK) ***g-e*** ionic interactions; none has more than one set of EK or KE ***g-e*** ion pairs (not shown; Hu et al., 1993).

The relative stabilities of the Type D mutants have not been measured directly. However, the direction of "one-way titration" often reflects the relative stabilities of homodimers and heterodimers in a pair of leucine zippers (Zeng et al., 1997). One-way titration has been observed in cases where heterodimers are allowed, but one of the homodimers is more stable than the other. If one-way titration is taken as evidence for the relative stabilities of pairs of leucine zippers, then the data in Figure 4 allows a partial ranking of some of the mutants. Using A > B to indicate that AA homodimers are more stable than BB homodimers, s64 > n34 > s26, s64 > s20, s64 > s24 > s26, s64 > s24 > s67 > s03, and s56 > s26.

Note that, although s64, which can form two pairs of favorable ***g-e′*** and ***g′-e*** ionic interactions, behaves as if it is more stable than any other mutant it interacts with, n34 and s56, which have only one favorable pair of ionic interactions, behave as if they are more stable than s26, which should be able to form two pairs of ionic interactions. Other factors beyond the number of favorable interactions between ionic groups in a pair of ***g*** and ***e*** positions must contribute comparable favorable or unfavorable energies to stabilizing the dimers. Mutant s26 seems to be relatively unstable despite having the potential to form two pairs of K-E ionic interactions. However, one of the favorable ionic interactions involves positions ***g***22K and ***e***27E; both of these sequence features were shown previously to be underrepresented among oligomeric mutants, consistent with unfavorable local interactions (Hu et al., 1993). ***g***22K can form repulsive intrahelical ionic interactions with ***c***18H and ***c***25R, whereas ***e***27E could interact unfavorably with the C-terminal end of the helix macrodipole.

The charge complementarity hypothesis (Vinson et al., 1993) predicts which pairs of leucine zippers should or should not form heterodimers based on the net increase or decrease in ***g-e′*** ion pairs formed in homodimers and heterodimers. Indeed, a linear relationship between stability and the number of attractive and repulsive ionic interactions has been shown in vitro for a set of model coiled coils (Monera et al., 1994). The patterns of which mutants can titrate each other's activities should provide an additional test of the idea that charge complementarity is sufficient to predict dimerization specificity. If the hypothesis is correct, we would expect that, in the combinations of mutants that retain immunity, the heterodimers should be predicted to be more unstable relative to the homodimers than in pairs where immunity is lost.

In fact, this effect is not observed. If we calculate the net change in ion pair formation based on 1 mol of each homodimer giving rise to 2 mol of heterodimers, the net change in ionic interactions upon heterodimer formation for both interacting and noninteracting pairs ranges from 0 to −16 (Fig. 5). Even if we exclude those pairs where one-way titration occurs, the distributions for interacting and noninteracting pairs of mutants are overlapping. Similar tests were performed examining the change in individual types of ion pairs (EE, KK, EK, KE) or the change in different combinations of possible pairs upon formation of heterodimers. In no case did we observe a clear correlation between the number of repul-

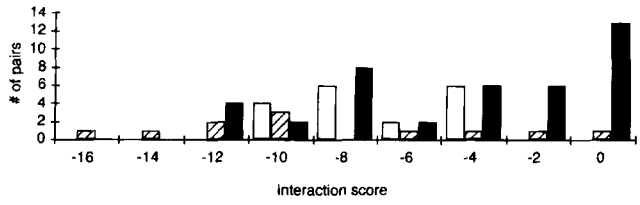

Fig. 5. Distribution of putative ion pairs for different classes of interactions. For each pair of leucine zipper sequences tested in Figure 4, an interaction score was calculated based on the number of attractive and repulsive ionic interchain interactions that might occur between ***g*** and ***e*** residues. Each favorable ionic interaction that could form in heterodimers was given a score of +2 (because 2 mol of heterodimers would be formed by stoichiometric conversion of 1 mol of each homodimer), and each unfavorable interaction was given a score of −2. Similarly, an interaction that would favor either homodimer was given a score of −1, and an interaction that should destabilize either homodimer was given a score of +1. The number of combinations with a particular interaction score is shown for each class of phenotypic effect shown in Figure 4. Open bars show pairs where immunity is retained in either combination of cI^+ and dominant negative fusions. Closed bars show pairs that are sensitive to λ in either combination. Hatched bars indicate pairs where "one-way" titration is observed.

sive, attractive, or the net ion pairs and the ability to form heterodimers (not shown). The failure of putative repulsive interactions to prevent heterodimer formation presumably reflects differences in the sensitivities of the homodimerization and heterodimerization assays in vivo.

It should be noted that, because our genetic test currently requires that the mutants we tested be able to form fusion proteins with strong enough dimerization to be detected as functional repressors, our data set includes no examples of pairs where the formation of heterodimers leads to a net increase in the number of attractive ion pairs, as would be observed with Fos-Jun heterodimers and in the designed heterodimers with acidic and basic monomers (O'Shea et al., 1992, 1993; Graddis et al., 1993; Zhou et al., 1994a). In these cases, the pH dependence of homodimer and heterodimer formation suggests that charge–charge interactions play a role in determining specificity. The salt dependence of leucine zipper stability also indicates that ionic effects are involved in stabilizing and/or destabilizing leucine zippers (Monera et al., 1994; Zhou et al., 1994b; Kohn et al., 1995; Yu et al., 1996). However, our results indicate that other factors involving the ***e*** and ***g*** positions can also play a significant role in determining dimerization specificity. Whatever these other factors are, they are significant enough to prevent us from predicting the dimerization specificities of the mutants.

The 81 pairwise interactions in Figure 4 show that all eight of the Type D mutants tested have dimerization specificities that are different from that of wt GCN4. Although all combinations of mutants s34, s03, s20, s24, and s56 are sensitive to phage, suggesting that these four sequences can form any combination of heterodimers, each mutant has a unique pattern of interactions with the other four mutant leucine zippers. Thus, each mutant can be thought of as having an overlapping but distinct dimerization specificity.

Although interactions among the mutant leucine zippers are specific, they are also relatively promiscuous. Among 64 pairwise combinations involving only mutants, only 13 retain immunity to λ. Only one pair of mutants, s56 and s64 are completely noninteracting, and every mutant has some significant heterodimer formation with at least six of the other seven mutants. This suggests that only a few combinations of sequences in our mutant set destabilize heterodimers enough to prevent their detection by this assay. Stabilizing interactions between the ***a*** and ***a*** positions, which are the same in homodimers and heterodimers, presumably provide the driving force to allow the many different combinations of heterodimers to form. In contrast, the wt GCN4 leucine zipper fails to titrate or be titrated by any of the mutants tested. Although it is possible that the pattern of charged residues in GCN4 has been selected for this property, the most striking difference between GCN4 and the mutants is the presence of leucines at ***e***2 and ***g***4. The role of these residues in the selectivity of dimerization warrants further investigation.

The enrichment of complementary-charged residues at the ***e*** and ***g*** positions of naturally occurring leucine zippers has been taken as evidence that the stabilizing energy of complementary ion pairs makes a critical contribution to leucine zipper stability and dimerization specificity (Cohen & Parry, 1990; Vinson et al., 1993). Although opposite charges must contribute some attractive binding energy, the observed enrichment for complementary charges could be due mainly to evolutionary constraints required to avoid sequences that will have either too many repulsive interactions or the tendency to form higher-order oligomers.

Caution should be exercised in using patterns of surface charge interactions to predict the oligomerization properties of sequences derived from either natural sources or de novo protein design. Further study is clearly needed to understand how sequence features determine molecular recognition in even this simple model protein–protein interaction.

Materials and methods

Bacterial strains and plasmids

Experiments described in this paper were performed on LB agar plates (Miller, 1972) supplemented with ampicillin (200 μg/mL), tetracycline (20 μg/mL), kanamycin (30 μg/mL), and/or chloramphenicol as appropriate. Plasmids were introduced by electroporation or M13-mediated transduction (Vershon et al., 1986) into *E. coli* strain AG1688 [F′128 *lacI*^{Q} *lacZ*::Tn*5*/*araD*139, Δ(*ara-leu*)7697, Δ(*lac*)*X74*, *galE15*, *galK16*, *rpsL*(Str^R), *hsdR2*, *mcrA*, *mcrB1*] (Hu et al., 1993) or derivatives bearing reporter genes as lysogens of specialized transducing phages. JH607 and XZ980 are lysogens of λ112O_sP_s and λXZ970, respectively, in AG1688 (Zeng & Hu, 1997).

Plasmid constructions were done by standard molecular biology methods. Mutant leucine zipper sequences were subcloned from a library of mutants described in Hu et al. (1993). Ampicillin-resistant plasmids expressing low constitutive levels of *c*I-leucine zipper fusion proteins were constructed by subcloning an *Nsi* I–*Bam*H I fragment from a mixed population of plasmids derived from amplifying a frozen aliquot of the library of mutants into the *Nsi* I–*Bam*H I-cut plasmid backbone of pXZ240, a plasmid designed for construction of cI^+ fusions expressed from the P7107 promoter, a constitutive mutant of the *lac*UV5 promoter (unpubl.). pXZ240 has the origin of replication and ampicillin resistance from pBR322 and, with the exception of the promoter region, is identical to pJH391 (Hu et al., 1993). As in pJH391, a stuffer fragment between the *Sal* I and *Bam*H I sites facilitates purification of the doubly cut plasmid backbone away from singly cut partial digestion products. Sequences of individual mutants were deter-

mined by dideoxy sequencing using M13 phagemid DNA as a template as described (Hu et al., 1993).

Dominant negative fusion proteins were constructed by subcloning individual leucine zipper segments from the ampicillin-resistant clones into a *Sal* I–*Bam*H I-cut backbone of pXZ610, a stuffer plasmid that expresses the fusion proteins under the control of the *tac* promoter. The DNA binding domain of λ *c*I repressor in pXZ610 contains the QL44 and IS84 mutations and a Flag-M2 epitope tag (IBI) in the linker region. The epitope tag does not affect repressor activity (data not shown). The origin of replication and tetracycline resistance gene in pXZ610 are from pJH550 (Kim & Hu, 1995), a modified version of pACYC184.

In vivo activity assays

Repressor activity of fusion proteins was determined by their ability to confer immunity to phage λ. Plasmid-containing cells were tested for immunity by cross-streaking against λKH54 at 37 °C (Hu et al., 1990, 1993). Cells that are immune to killing by λKH54 are assumed to carry plasmids that encode oligomeric fusion proteins.

To determine whether the mutants behaved as dimers or higher-order oligomers in vivo, M13 phagemids carrying the cI^+ fusions were made from mutant plasmids derived from pXZ240, and were used to transduce JH607 or XZ980 (Zeng & Hu, 1997). The resulting strains were assayed for *lacZ* activity and Cm resistance. β-Galactosidase was measured by the $CHCl_3$-SDS lysis method of Miller (1972) from log phase cultures grown in M9 glucose minimal medium supplemented with casamino acids. Expression of *lacZ* was normalized to β-galactosidase activity in an isogenic control strain without any plasmids, and is given as the average of at least two independent experiments.

Specificity of dimer formation for dimeric mutants was determined in a negative dominance assay. QL44 and IS84 are mutations in the DNA-binding domain of λ repressor that act as dominant negative alleles in intact λ repressor (Hecht et al., 1983; Nelson et al., 1983). QL44 has also been shown to act as a dominant negative inhibitor in leucine zipper fusions (Zeng et al., 1997). The QL44, IS84 double mutant is a better dominant negative inhibitor of repressor activity than QL44 alone and was therefore used in these studies (unpubl.). Cells carrying two plasmids, one encoding a cI^- fusion protein and the other with a dominant negative mutant fusion protein were tested for phage immunity. Sensitivity to phage infection indicates formation of enough heterodimers to deplete the concentration of active homodimers below a threshold required for immunity.

Purification of fusion proteins

Fusion proteins were purified from the plasmids expressing the dominant negative QL44 mutation. Expression of the fusion protein is controlled by the *tac* promoter in these plasmids. Plasmid-containing cells were grown with shaking at 37 °C in LB broth to an OD_{600} of 1.0. Isopropyl-thio-D-galactoside was added to a final concentration of 100 μg/mL and growth was continued for 2.5 h. Cells were harvested by centrifugation and resuspended in 30 mL lysis buffer (100 mM Tris HCl, pH 8, 200 mM KCl, 1 mM EDTA, 2 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM DTT). PMSF was added to 1 mM and the cells were lysed by sonication. The lysates were adjusted to 50 mL with the same buffer and insoluble material was removed by centrifugation. Polyethyleneimine (PEI) was added to 0.2% to the supernatant and the PEI precipitate was removed by centrifugation. Proteins were precipitated by addition of two volumes of saturated ammonium sulfate, resuspended in SB buffer (50 mM Tris HCl, pH 8.0, 0.1 mM EDTA, 5% glycerol) containing 50 mM KCl (SB50), dialyzed extensively against the same buffer, and loaded on a 2.5 × 5 cm Bio-Rex 70 column. The column was washed with SB50 and bound proteins were eluted with a linear gradient from 50 mM to 1 M KCl. Fractions containing the fusion protein were identified by running aliquots on SDS gels, pooled, dialyzed against SB buffer containing 200 mM KCl (SB200), and loaded onto a heparin agarose column. Proteins were eluted with a linear gradient from 50 mM to 1 M KCl and fusion protein-containing fractions were pooled and concentrated using an Amicon pressure cell and a YM3 membrane.

Proteins purified in this way were homogeneously pure by inspection of stained gels. Purified proteins were stored in SB200 at 4 °C or at −20 °C.

Characterization of purified fusion proteins

For size-exclusion chromatography, 50 μL samples of purified proteins in SB200 were injected onto a Superdex 200 HR 10/30 FPLC gel filtration column and eluted at 1 mL/min with 10 mM Tris HCl, pH 8.0, and 0.1 mM EDTA containing either 200 mM or 500 mM KCl. The apparent molecular weights of complexes were determined by comparison to standard proteins (Sigma).

Oligomerization states and association constants were obtained by sedimentation equilibrium analysis of fusion proteins with either wt GCN4, the ***a*L*d***I tetrameric control, or the s09 mutant. Data were collected on a temperature-controlled Beckman XL-A analytical ultracentrifuge equipped with a An60Ti rotor and photoelectric scanner. Samples were prepared by dialysis in Slide-a-lyzer 10K cassettes (Pierce) against 10 mM Tris HCl, pH 8.0, 0.1 mM EDTA, 200 mM KCl. A double sector cell, equipped with a 12-mm epon centerpiece and quartz windows was loaded with 130–150 μL of sample using a blunt-end micro syringe. Dialysis buffer from outside the cassette was used in the reference cell. Data were collected at rotor speeds of 3,000–18,000 rpm in step mode at 20 °C or 25 °C, and all scans were performed at 280 nm with a step size of 0.001 cm; 30 scans were averaged. Samples were allowed to equilibrate for 34 h, and duplicate scans 3 h apart were overlaid to determine that equilibrium had been reached. The partial specific volume of the mutants was calculated based on their amino acid composition by the method of Cohn and Edsall as implemented in the XL-A software (Laue et al., 1992). The data were analyzed by a nonlinear least-squares analysis using the Origin software provided by Beckman. The data were then fitted to two classes of models. First, the data were fitted to a single ideal species model using Equation 1:

$$C_r = \exp[\ln(C_0) + (M(1 - \bar{\nu}\rho)\omega^2/2RT)\cdot(x^2 - x_0^2)] + E, \quad (1)$$

where C_r is the absorbance at radius x, C_0 is the absorbance at a reference radius x_0 (usually the meniscus), M is the molecular weight of the single species, $\bar{\nu}$ is the partial specific volume of the protein (mg/mL), ρ is the density of the solvent (g/mL), ω is the angular velocity of the rotor (radian/sec), R is the gas constant (8.314×10^7 erg/mol), T is the temperature in K, and E is a baseline error correction factor.

In the experiments described here, the apparent molecular weights from fitting to single species were not integer multiples of the

monomer molecular weight, indicating that mixed species were present. The data were then fitted to a self-associating system of monomer to n-mer equilibria using Equation 2:

$$C_r = \exp[\ln(C_0) + (M(1 - \bar{\nu}\rho)\omega^2/2RT)\cdot(x^2 - x_0^2)] + \sum(\exp[n\ln(C_0) + \ln(K_{an}) + nM((1 - \bar{\nu}\rho)\omega^2/2RT) \times (x^2 - x_0^2)]) + E, \quad (2)$$

where K_{an} is the association constant for the formation of n-mer from monomers, and M is the monomeric molecular weight. A series of models was tested to examine various possibilities, involving dimers, trimers, tetramers, and octamers. In Figure 2, the fits are shown to the model with the best fit, based on the randomness and the magnitude of the residuals.

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