Preferential Heterodimer Formation via Undercompensated Electrostatic Interactions

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The design of new protein structures and activities will require a detailed understanding of energetics and the development of a robust set of design principles. Here we demonstrate the successful design of mutants of P22 Arc repressor that form a preferential heterodimer. To achieve the requisite specificity, we make use of *complementary* patterns of hydrophobic and hydrophilic groups at the heterodimer interface juxtaposed with *noncomplementary* patterns in the homodimers. The heterodimer constructed in this way is predicted to be −2.5 kcal/mol more stable than the average of the homodimers; experiments have verified a strong heterodimer preference.

Compensated interactions are a dominant theme in protein structure. Buried hydrophobic groups pack against one another and provide much of the driving force for protein folding. Likewise, polar and charged groups occur in arrangements that permit mutual hydrogen bonding. While the relative strength of these interactions is subject to debate,[1−3] it is generally agreed that both are more favorable than uncompensated polar and charged groups removed from solvent. In fact, the large penalty incurred by buried, uncompensated (or undercompensated) polar and charged groups is a likely source of specificity.[2,4] A key feature of our preferential heterodimer design is the use of negative determinants of specificity to disfavor formation of homodimers through undercompensated electrostatic interactions.

Arc repressor is a 53-residue protein that folds as a homodimer (Figure 1A). The structure of Arc[5] reveals an electrostatic network that crosses the dimer interface. Arg31, Glu36, and Arg40 form a salt bridge triad (RER) in one monomer that links to Ser44′ and Glu48′ (SE) in the other monomer (Figure 1B). Through symmetry, the related Arg31′, Glu36′, Arg40′, Ser44, and Glu48 interactions are also present. These interfacial interactions contribute to the stability of the Arc homodimer; the SA44 and EA48 mutations destabilize Arc by 1.6 and 2.4 kcal/mol per dimer, respectively.[6] Replacing the triad by MYL (Met31, Tyr36, and Leu40) enhances stability by −3.9 kcal/mol per dimer[3] but leaves Ser44′ and Glu48′ undercompensated. In the MYL background,

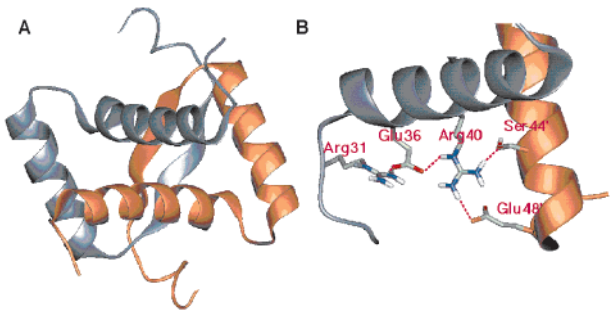

Figure 1. (A) Arc repressor dimer with each monomer colored gray or red. (B) Hydrogen bonds between Arg31, Glu36, and Arg40 in one monomer and Ser44′ and Glu48′ in the other indicated by dashed lines.

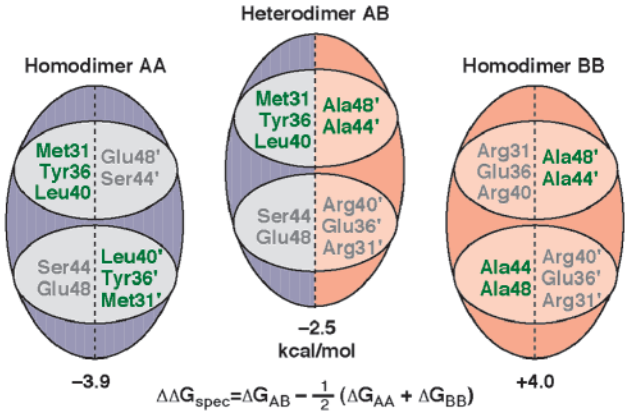

Figure 2. Homodimer and heterodimer interactions involving charged and polar residues (gray) and hydrophobic residues (green).

the SA44 and EA48 mutations are stabilizing by −0.98 and −0.13 kcal/mol per dimer, respectively.[7] Hence, the effects of mutating SA44 and EA48 are context dependent—destabilizing in the RER (wild-type) background and stabilizing in MYL. This behavior forms the crux of our design.

In principle, a preferential heterodimer could be formed with one all-electrostatic network (RER)−(SE)′ and one all-hydrophobic network (MYL)′−(AA). This design generates one homodimer with two undercompensated (RER)−(AA)′ interactions in the interface and another homodimer with two undercompensated (MYL)−(SE)′ interactions. The net effect is electrostatic and hydrophobic complementarity at the heterodimer interface but polar groups across from hydrophobic groups at each homodimer interface, leaving these electrostatic groups undercompensated.

Assuming additive effects for multiple mutations, one can predict the relative stability of the heterodimer and each homodimer from experimental data (Figure 2).[3,7] Relative to wild type, the RER−AA homodimer should be 4.0 kcal/mol per dimer less stable, the MYL−SE homodimer should be −3.9 kcal/mol per dimer more stable, and the heterodimer should be −2.5 kcal/mol per dimer more stable. Because the average homodimer stability is near zero [(4.0−3.9)/2)] relative to wild type, this results in a predicted heterodimer preference over homodimer of −2.5 kcal/mol per dimer. Interestingly, this preference holds even though the heterodimer is predicted to be intermediate in stability between the two homodimers. The equilibrium constant between the homodimers and the heterodimer depends on the difference between the heterodimer stability and the average of the two homodimer stabilities (−2.5 kcal/mol) plus a statistical constant (*RT* ln 2) because there are two ways to form the heterodimer and only one way to form each homodimer. Given a mixture of

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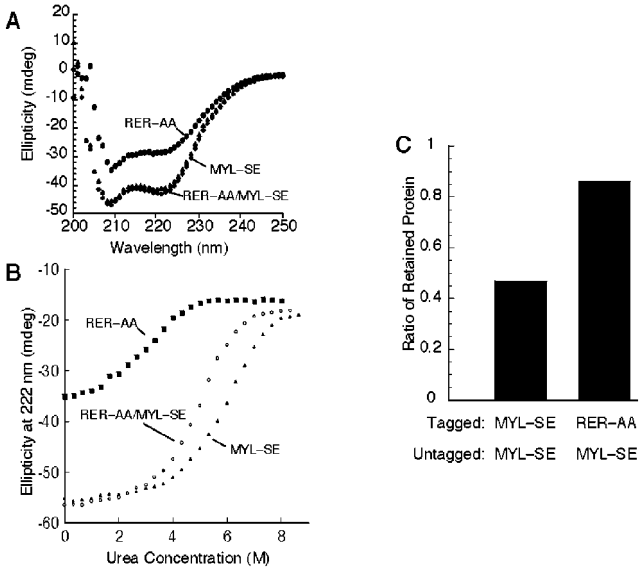

Figure 3. Preferential heterodimer formation. (A) CD spectra of Arc variants RER−AA and MYL−SE alone (5 mM) and mixed (2.5 mM each) at 25 °C in buffer A. (B) Urea denaturation of 50 mM total (25 mM of each monomer in the RER−AA/MYL−SE experiment). The final solution was 250 mM KCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA and the indicated amount of urea. (C) Column binding assay. Retained protein has a ratio of 86% of untagged MYL−SE to tagged RER−AA, indicating a significant heterodimer preference. Control studies with untagged MYL−SE alone showed less than 10% retention.

the designed monomers each at 2.5 mM, these values predict 92% heterodimer formation.

The RER−AA and MYL−SE variants were constructed by cassette mutagenesis and purified as described.[3] Far ultraviolet circular dichroism (far-UV CD) is a measure of secondary structure formation. Figure 3A shows the far-UV CD spectra for each variant alone (5 mM) and mixed (2.5 mM each). The smaller signal from RER−AA is due to partial unfolding at this concentration; the far-UV CD spectra of RER−SE and MYL−SE are virtually identical.[3] If there were no heterodimer preference, the RER−AA/MYL−SE mixture would yield a signal roughly the average of that from RER−AA and MYL−SE alone. However, the RER−AA/MYL−SE mixture generates a signal that is comparable to that of MYL−SE alone, indicating a significant degree of heterodimerization.

Figure 3B shows urea denaturation curves of each variant alone and the mixture. As predicted, the RER−AA/MYL−SE system is less stable than MYL−SE. However, the stability of the heterodimeric system is much greater than the average of the 2 homodimers, again reporting a strong heterodimer preference.

To measure directly heterodimeric preference, a Ni−NTA column retention assay was performed,[8] in which one variant had a His_6 tag and the other did not. Thus, untagged variant could be retained by the resin only through interaction with tagged variant. A random 1:2:1 distribution (AA:AB:BB) would give a ratio of 50% of untagged to tagged variant in the retained fraction. A ratio greater than 50% is evidence for heterodimeric preference. The variants were preincubated for 30 min with 25 mM of each monomer and were applied to the resin in buffer A (10 mM Tris, pH 7.5, 150 mM KCl, and 0.1 mM EDTA.) The resin was collected and washed once with buffer, and the retained protein was eluted with buffer plus 250 mM imidazole. The eluted protein was run on a 15% Tris-Tricine denaturing gel and stained with Sypro orange for quantification. The ratio of untagged-to-tagged protein from the gel is reported (Figure 3C). As a control, His_6-tagged MYL−SE was incubated with untagged MYL−SE, and 47% of the retained protein was untagged MYL−SE, in line with the prediction (50%) for a nonpreferential system. Tagged RER−AA resulted in the retained protein consisting of 86% untagged MYL−SE, similar to the percentage predicted (93%). These results demonstrate that the designed monomers do form heterodimers with approximately the preference predicted.

It should be possible to construct an Arc heterodimer, in which neither homodimer is more stable than the heterodimer, using a similar set of mutations. One could simply add destabilizing mutations distant from the dimer interface to the MYL−SE monomer, which forms the more stable homodimer. These mutations should not affect the heterodimer preference (how much the heterodimer is favored over the average of the homodimers) but will lower the stability of the more favorable homodimer (because the mutations occur twice in the homodimer and once in the heterodimer).

A system with similar heterodimer preference is the Jun/Fos pair of leucine-zipper peptides, where the heterodimer is −2.3 kcal/mol more stable than the average of the homodimers (compared to −2.5 kcal/mol predicted for the designed Arc homodimer).[9] Like the designed Arc heterodimer, one of the homodimers, Jun−Jun, is stable in solution, although unlike the Arc heterodimer, Jun−Jun is slightly less stable than the Jun−Fos heterodimer. In the Jun−Fos system, heterodimer preference is attributed to positively charged side chains in Jun and negatively charged side chains in Fos, which form interactions that are favorable in the heterodimer and unfavorable in each homodimer. A heterodimer that was more stable than the average of the homodimers by −6.5 kcal/mol was created by saturating these coiled coil positions with positive and negative charges.[10a] The use of repulsive negative determinants of specificity (like-signed charges) is different from the present study, in which the negative determinants are undercompensated polar or charged groups.[10]

Thus, undercompensated electrostatic groups can be used as a negative design principle to create preferential heterodimers. The Arc-based heterodimer designed here using this mechanism has a similar heterodimer preference to the Jun−Fos pair of natural leucine zipper peptides. Because the design strategy can be used with a combination of charged, polar, and hydrophobic residues, it can be used in the buried portions of interfaces where packing constraints may limit the placement of oppositely charged side chains.

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