The Energetic Contribution of Backbone−Backbone Hydrogen Bonds to the Thermodynamic Stability of a Hyperstable P22 Arc Repressor Mutant

Thomas E. Wales and Michael C. Fitzgerald*

Department of Chemistry, Duke University
Durham, North Carolina, 27708

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Hydrogen bonds involving backbone C═O and backbone NH groups constitute a large number of the native contacts in folded proteins, and it has been suggested that such backbone−backbone hydrogen-bonding interactions play an important role in protein folding and stability.[1] However, the exact nature and magnitude of their contributions to protein folding reactions have been difficult to determine experimentally. This is in large part due to the inherent difficulties associated with introducing backbone modifications into proteins using conventional site-directed mutagenesis experiments. Recently, two new technologies (one relying on chemical synthesis strategies and one relying on a specialized *in vitro* translation technique) have been developed that permit modification of the peptide backbone in proteins.[2,3] Here we employ a total chemical synthesis strategy to study the role of backbone−backbone hydrogen-bonding interactions in P22 Arc repressor, a well characterized model protein system for protein folding studies. In this work we report the total chemical synthesis and initial biophysical characterization of wild-type Arc repressor and a backbone-modified, ester bond-containing analogue of Arc repressor, (POL8)Arc. The (POL8)Arc analogue is designed to effectively delete two backbone−backbone hydrogen-bonding interactions that are formed in the intersubunit, β-sheet region of a hyperstable Arc repressor analogue, (PL8)Arc (Figure 1). Our results indicate that these two backbone−backbone hydrogen-bonding interactions contribute a total of 2.5 kcal/mol to the folding free energy of the (PL8)Arc dimer.

In earlier mutational studies on the P22 Arc repressor system it was found that a proline-to-leucine mutation at position 8 in Arc repressor's 53 amino acid polypeptide chain increased the protein's thermodynamic stability by 2.5 kcal/mol.[4] It is also important to note that Arc repressor is a symmetric homo-dimer and that a single mutation in the protein's 53-amino acid peptide chain occurs twice in the protein's final, folded structure. X-ray crystallographic data obtained on (PL8)Arc revealed that its three-dimensional structure was nearly identical to that of the wild-type protein with the exception that there were eight backbone−backbone hydrogen bonds formed in the intersubunit β-sheet region of the (PL8)Arc structure compared to only six in this region of the wild-type structure (Figure 1 A and B).[5] The increased thermodynamic stability of the (PL8)Arc analogue has been largely attributed to the presence of these two additional hydrogen bonds (one per monomer unit) in the protein's three-dimensional structure.[5] However, in these earlier mutational studies it was not possible to define the exact magnitude of the stabilizing effects of these backbone−backbone hydrogen bonds in (PL8)Arc because it was unclear to what degree, if any, the mutant leucine side chain stabilized or destabilized the (PL8)Arc structure relative to the wild-type protein. The (POL8)Arc analogue in this study contains an amide-to-ester bond mutation between residues 7 and 8 which effectively deletes the two additional backbone−backbone hydrogen bonds that are thought to stabilize the (PL8)Arc dimer structure (Figure 1 C). We reasoned that if the ester bond is a useful amide bond isostere for deleting backbone−backbone hydrogen-bonding interactions in proteins and that if the increased stability of the (PL8)Arc analogue was due entirely to the additional backbone−backbone hydrogen-bonding interactions, then the thermodynamic stability of (POL8)Arc should match that of the wild-type protein.

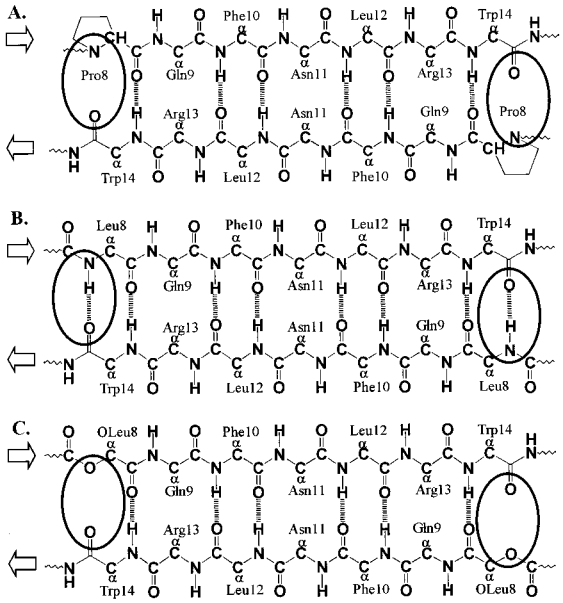

Figure 1. Schematic representation of the putative backbone−backbone hydrogen-bonding interactions involved in the intersubunit β-sheet region of (A) wild-type Arc repressor, (B) (PL8)Arc, and (C) (POL8)Arc.

The 53-amino acid polypeptide chains of wild-type Arc and (POL8)Arc were assembled in stepwise fashion using manual solid-phase peptide synthesis (SPPS) methods and highly optimized *in situ* neutralization protocols for Boc chemistry as described elsewhere.[6] The ester bond was incorporated into the polypeptide chain of (POL8)Arc by coupling leucic acid at position 8 using previously established protocols.[7] The crude synthetic product that we obtained from each synthesis was readily purified by reversed-phase HPLC (Supporting Information); and the purified polypeptide products were readily folded in buffer (50 mM Tris-HCl, 100 mM KCl, and 0.2mM EDTA, pH 7.4) at room temperature over the course of 30 min.

Our preliminary characterization of the synthetic wild-type Arc repressor and (POL8)Arc constructs prepared in this work indicates that the folded structures of these proteins are very similar to each other and nearly identical to wild-type Arc repressor protein obtained by conventional recombinant DNA-based methods. Far ultraviolet circular dichroism (UV-CD) spectra that we recorded for our synthetic wild-type Arc repressor and (POL8)Arc constructs were very similar to each other (Supporting Information) and consistent with far UV-CD spectra previously reported for recombinantly derived Arc repressor protein.[8]

* Corresponding Author: Dr. Michael C. Fitzgerald, Department of Chemistry, Box 90346. Duke University, Durham, NC 27708-0346, Telephone: 919-660-1547. Fax: 919-660-1605. E-mail: mfitz@chem.duke.edu.

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Table 1. Thermodynamic Parameters for the Guanidine-Induced Equilibrium Unfolding of wt Arc and Mutants

	ΔG_u[a] (kcal/mol of dimer)	m[a] [kcal/(mol M)]
synthetic wt Arc	10.6 ± 0.4	3.6 ± 0.1
synthetic (POL8) Arc	10.4 ± 0.2	3.3 ± 0.1
recombinant wt Arc	10.21 ± 0.34[b]	3.01 ± 0.25[b]
recombinant (PL8) Arc	12.75 ± 0.18[b]	3.16 ± 0.13[b]

[a] Results were obtained as in ref 5 using a two-state model involving native dimer and unfolded monomer (Supporting Information). The values reported here represent an average of the results from three trials. Errors are standard deviations. [b] Data taken from ref 5.

Fluorescence emission spectra (with excitation at 280 nm) recorded for our synthetic Arc repressor and (POL8)Arc constructs in the presence and absence of Arc repressor's cognate DNA also showed a 7 nm blue-shift in the center of spectral mass consistent with DNA binding (Supporting Information). This shift in the center of spectral mass was similar to that previously reported in a similar experiment using wild-type protein obtained by recombinant DNA methods.[9]

Significantly, the guanidine-induced equilibrium unfolding curves that we generated for our synthetic wild-type Arc repressor construct and for our (POL8)Arc analogue using far UV-CD spectroscopy were also identical to each other (Supporting Information) and consistent with equilibrium unfolding curves previously reported in similar experiments on the recombinantly derived wild-type protein.[8] The unfolding free energies, ΔG_u, and m-values that we extracted from the unfolding curves are summarized in Table 1. The ΔG_u and m-values that we calculated (Supporting Information) for our synthetic wild-type Arc repressor are essentially identical to those previously reported for the wild-type protein.[5] The ΔG_u value that we calculated for our (POL8)Arc analogue is exactly 2.5 kcal/mol lower than the value previously reported for (PL8)Arc.[5] The amide-to-ester bond mutation in our (POL8)Arc analogue effectively deletes the two backbone−backbone hydrogen bonds in the intersubunit β-sheet of the (PL8)Arc dimer that are thought to be responsible for the increased thermodynamic stability of this hyperstable mutant. The results of our equilibrium studies on (POL8)Arc establish that these backbone−backbone hydrogen bonds in (PL8)Arc are entirely responsible for the increased stability of this analogue and that they contribute a total of 2.5 kcal/mol to the folding free energy of this hyperstable mutant.

Protein analogues containing amide to ester bond mutations in their polypeptide backbone have been used in several other studies to determine the contribution of backbone−backbone hydrogen bonds to protein reactions.[10−15] Several characteristics of the ester bond make it an attractive amide bond isostere for modulating the hydrogen-bonding characteristics of a protein's backbone (e.g., it favors a *trans* configuration, it is planar, and its bond lengths are very similar to those of an amide bond).[16−18] However, several complicating issues regarding the use of backbone amide-to-ester bond mutations to delete hydrogen bonds in proteins have also been noted.[7,10,11,13,14] For example, in addition to effecting backbone−backbone hydrogen-bonding interactions, amide-to-ester bond mutations in the polypeptide backbone of a protein can alter the solvation properties of a chemically-denatured polypeptide chain. They can also create unfavorable electrostatic and van der Waals interactions between the oxygen ester and neighboring carbonyl groups in a protein's final folded structure. Interestingly, our results with (POL8)Arc suggest that these complicating issues did not significantly factor into our thermodynamic analyses. For example, if the amide-to-ester bond mutation in (POL8)Arc was significantly destabilizing due to unfavorable interactions between the oxygen ester and the neighboring carbonyl group C-terminal to Trp14 (Figure 1C), then we would have expected the (POL8)Arc construct to be less stable than the wild-type protein. However, it is possible that the amide-to-ester bond mutation in (POL8)Arc changes multiple properties of the protein which affect its thermodynamic stability and that the compensatory effects of these changes on (POL8)Arc fortuitously result in a 2.5 kcal/mol destabilization of the protein relative to (PL8)Arc.

The energetic contributions of backbone−backbone hydrogen-bonding interactions involved in α-helices, in β-sheets, and in protein−protein interfaces have been reported in several other studies.[10−14] In one study the contribution of individual hydrogen bonds at various positions in an α-helix to the overall stability of T4 lysozyme was determined to be in the range of 0.8 to 1.7 kcal/mol.[10] In another study individual backbone−backbone hydrogen bonds in a β-sheet were found to contribute between 1.5 and 2.5 kcal/mol to the stability of Staphylococcal nuclease.[11] Investigations on the complexation of serine proteinases and their protein inhibitors have evaluated the energetic contribution of backbone−backbone hydrogen bonds in these protein−protein interactions to be between 0 and 4.5 kcal/mol.[7,12,13] A four-ester bond-containing analogue of chymotrypsin inhibitor 2 (CI2) which resulted in the deletion of three hydrogen bonds in an α-helix of this protein was destabilized by close to 1 kcal/mol per hydrogen bond.[14]

The results reported here indicate that the two backbone−backbone hydrogen bonds at the ends of the intersubunit β-sheet in the (PL8)Arc structure each contribute ~1.25 kcal/mol to the thermodynamic stability of this hyperstable Arc mutant. The stabilizing effects of this particular type of backbone−backbone hydrogen-bonding interaction (i.e., one which is involved in an intersubunit β-sheet) have not been previously studied. However, it is noteworthy that the stabilizing effect we measured for this type of backbone−backbone hydrogen bond is in the same range as that reported for other types of backbone−backbone hydrogen-bonding interactions in proteins (0−4.5 kcal/mol).[7,10−14] Interestingly, this stabilizing effect is slightly lower than that previously reported for hydrogen-bonding interactions in an intra-subunit β-sheet (1.5−2.5 kcal/mol).[11] More studies on other backbone−backbone hydrogen-bonding interactions in the Arc system and in other model protein systems are needed to determine if this is a general trend.

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Supporting Information Available: Reversed-phase HPLC and ESI-MS analysis; far UV-CD analysis; qualitative results from fluorescence-based DNA binding assays; and guanidine-induced equilibrium unfolding curves for synthetic wild-type Arc and (POL8)Arc (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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