A Designed Buried Salt Bridge in a Heterodimeric Coiled Coil

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Received February 17, 1997

Protein structures are stabilized by a balance of energetic components including hydrophobic interactions, van der Waals packing, electrostatics, and H-bonding.[1] Hydrophobic interactions provide a powerful driving force for compacting the peptide chain and stabilizing the final three-dimensional structure.[2−5] By contrast, hydrogen bonds and salt bridges are believed to be less important for overall stability.[6,7] However, because these latter interactions are more sensitive to the distance and geometry of the groups in contact, they help provide specificity for a given conformation over other folds. For instance, a buried hydrogen bond between two Asn residues in the coiled coil of GCN4 specifies a dimeric conformation: substituting these residues with nonpolar residues leads to the formation of higher order aggregates.[8,9]

Exposed salt bridges have been engineered into monomeric helices[10,11] and coiled coils to provide thermodynamic stability, or to specify the formation of heterooligomers relative to homooligomers.[12−15] The free energy associated with each exposed salt bridge in these systems is very small (0 to 0.5 kcal/mol).[16−18] Thus, a very large number of exposed salt bridges is required to produce a significant effect. Such interactions are also very sensitive to ionic strength, switching from favorable to unfavorable as the electrolyte concentration is increased from 0.01 to 0.5 M.[19] By contrast, the interaction energy between charged groups within the interior of a protein is often an order of magnitude larger[20,21] and remains favorable at high ionic strength. Thus, buried salt bridges represent a potentially important element for engineering conformational specificity into designed proteins. However, the introduction of a buried salt bridge has been described only once, and this design was not successful.[6]

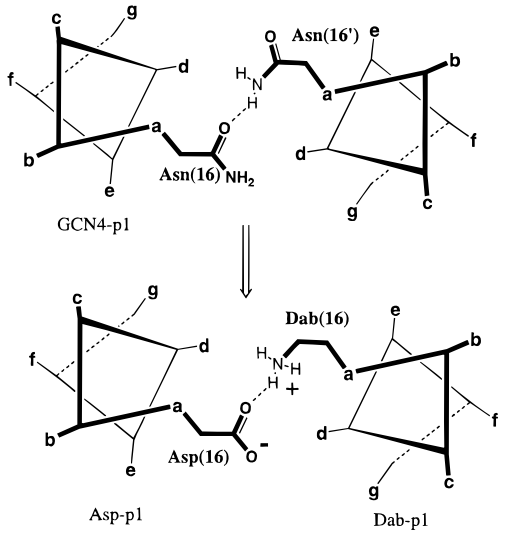

Figure 1. Heptad arrangement of GCN4-based peptides showing the formation of a buried salt bridge at position 16.

We introduced an interior salt bridge at the helix/helix interface of a peptide spanning the homodimeric coiled coil of GCN4 (GCN4-p1), an extensively studied experimental system.[8,9,17,19] A buried interhelical hydrogen bond between the carboxamide groups of Asn16 and Asn16′ can be converted to a salt bridge by conceptual hydrolysis of one Asn to an Asp and reduction of the other Asn to 2,4-diaminobutyric acid (Dab) (Figure 1). Also, computer modeling suggested that a peptide containing a 2,3-diaminopropionic acid residue (Dap) might interact favorably with Asp16 of a neighboring helix. Thus, three variants of GCN4-p1, designated Asp-p1, Dab-p1, and Dap-p1, were synthesized[22] and evaluated.

Circular dichroism spectroscopy (CD) indicated that the individual peptides form helices in a pH-dependent manner (Figure 2A), with the helical content decreasing at pH's beyond the intrinsic pK_a of the Dab, Dap, and Asp side chains. By contrast, GCN4-p1 is fully helical between pH 2 and 10 (Figure 2A). These data indicate that the side chain introduced at position 16 must be in a neutral, non-ionized form to allow formation of homooligomers. Further, sedimentation equilibrium ultracentrifugation shows that the folded forms of Asp-p1 and Dab-p1 are dimeric. Dap-p1 exists as a mixture of dimers and trimers at pH 9.0.[23] However, the trimeric state is not significantly populated at pH 6.3 where heterodimer formation was investigated.

The thermodynamic stabilities of the Dab-p1/Asp-p1 heterodimer and the corresponding Dap-p1/Asp-p1 pair (at pH 6.3, 0.15 M NaCl) were determined from the temperature and concentration dependence of their CD spectra (Supporting Information). At this pH, the side chains of the Asp, Dab, or Dap residues should be ionized in the unstructured monomers,

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(22) Peptides related to the GCN4-p1 peptide[27] of sequence Ac-RMKQLEDKVEELLSK**X**YHLENEVARLKKLVGER-CONH$_2$ (X = Asn, GCN4-p1; X = Asp, Asp-p1; X = L-2,3-diaminopropionic acid, Dap-p1; X = L-2,4-diaminobutyric acid, Dab-p1) were synthesized by using standard Fmoc-protocol, purified to homogeneity by RP-HPLC, and characterized by ESI mass spectroscopy.

(23) Sedimentation equilibrium (Supporting Information) indicates that Asp-p1 sediments as a monomer at pH 9 (50 mM BTP, 150 mM NaCl), where Asp-16 is deprotonated. Similarly, Dap-p1 and Dab-p1 are monomeric at pH 3 (50 mM Gly, 150 mM NaCl) where Dab-16 or Dap-16 is in the charged, protonated state. Asp-p1 and Dab-p1 exist in a monomer−dimer equilibrium at pH 3 and 9, respectively, while Dap-p1 exists in a monomer−dimer−trimer equilibrium at pH 9. Data from equimolar mixtures of Dap-p1/Asp-p1 and Dab-p1/Asp-p1 (pH 6.3, 50 mM MES, 150 mM NaCl) are well described by a monomer−dimer equilibrium, without any detectable higher order aggregates.

S0002-7863(97)00493-9 CCC: $14.00

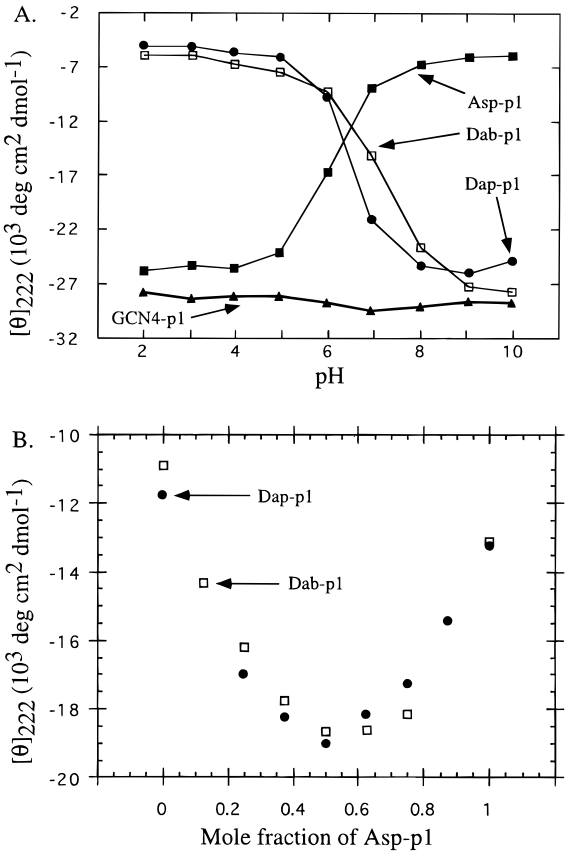

Figure 2. (A) Mean residue ellipticity at 222 nm monitored as a function of pH for 50 μM solutions of Asp-p1, Dab-p1, and Dap-p1 [150 mM NaCl, 50 mM buffer (buffer = glycine at pH 2 and 3; acetate at pH 4 and 5; MES[28] at pH 6; BTP at pH 7, 8, and 9; CAPS at pH 10 and 11)]. The pK_a of methyl 2-(*N*-benzyloxycarbonyl)amino-3-aminoproprionate was independently determined to be 7.8, suggesting that in the case of Dap-p1, helical structure should decrease as the pH is lowered below this value. (B) Mean residue ellipticity at 222 nm versus the mole fraction of Asp-p1 for Dap-p1 and Dab-p1. Minima are observed at 1:1 molar ratios of Dap-p1:Asp-p1 and Dab-p1:Asp-p1 suggesting, in each case, the formation of heterodimers (total peptide concentration held constant at 50 μM, pH 6.3, 150 mM NaCl, 50 mM MES).

Table 1

peptide(s)	[NaCl] (M)	ΔG^o- (37 °C)[a]	$\Delta\Delta G^o$- (spec.)	T_m[b] (1 M)	T_m[c] (100 μM)	ΔH_m[d] (1 M)
Asp-p1	0.15	5.0		79.0	30.4	53.6
Dab-p1	0.15	3.7		66.6	16.5	49.7
Dap-p1	0.15	5.0		78.7	27.6	57.6
(1:1) Dap-p1:Asp-p1	0.15	6.6	1.6	96.3	46.8	57.3
(1:1) Dab-p1:Asp-p1	0.15	6.2	1.9	91.8	42.7	56.9
(1:1) Dab-p1:Asp-p1	0.025	6.2	1.9	95.9	42.8	54.0
(1:1) Dab-p1:Asp-p1	0.5	6.2	1.9	91.4	42.8	57.8

[a] Thermal denaturation data of 100 μM peptide samples (pH 6.3, 50 mM MES[28]) were obtained by monitoring circular dichroism at 222 nm (standard state = 1 M); $K_d = \exp(-\Delta G^\circ/RT)$; units for ΔG^o and $\Delta\Delta G^o$(spec.) are kcal mol^{-1}; the uncertainties in ΔG^o, ΔH_m, and T_m are $\pm$0.2, 0.4 kcal mol^{-1}, and 0.2 °C, respectively, as estimated from sensitivity analysis of the theoretical curves. [b] Midpoint of the thermal denaturation curve extrapolated to the conventional 1 M standard state; units for T_m are °C. [c] Midpoint of the thermal denaturation curve obtained with 100 μM total peptide concentration. [d] The extrapolated enthalpy of the transition at T_m (1 M); units for ΔH_m are kcal mol^{-1}.

thereby inhibiting homodimeric and favoring heterodimeric folding. Indeed, the thermal unfolding curves for these individual peptides indicate that they formed marginally stable coiled coils (Table 1). By contrast, equimolar mixtures consisting of the Asp-p1/Dab-p1 or the Asp-p1/Dap-p1 peptides formed heterodimers with midpoint temperatures (T_m) well above those observed for the individual peptides. The dimeric nature of the heterodimers was confirmed by ultracentrifugation,[23] as well as a titration experiment in which the CD spectrum was monitored as the mole fraction of Asp-p1 was varied from 0 to 1.0. The resulting chevron-shaped plots (Figure 2B) show clear minima at a 1:1 molar ratio indicative of a 1:1 stoichiometry. Thus, oppositely charged groups at position 16 of neighboring helices interact, directing heterodimer formation.

The molar free energies of formation of the homodimers and heterodimers were determined by fitting thermal denaturation data to the Gibbs−Helmholtz equation. As described in the Supporting Information, the dissociation constants for the homodimers were first determined from thermal unfolding curves of individual peptides. Once these constants were known, analysis of the unfolding curves for mixtures of the appropriate peptides provided the dissociation constants for the heterodimers (Table 1). The degree of specificity (free energy advantage) for heterodimerization of an a/b heterodimer relative to the corresponding a/a and b/b homodimers is given by eq 1:

$$\Delta\Delta G^o(\text{spec.}) = \Delta G^o_{ab} - {}^1/_2[\Delta G^o_{aa} + \Delta G^o_{bb}] \qquad (1)$$

The degree of specificity incurred by an Asp/Dab pair was calculated to be 1.9 kcal mol^{-1}, a 22-fold preference for forming heterodimers versus homodimers; the corresponding $\Delta\Delta G^o$-(spec.) for the Asp/Dap pair was 1.6 kcal mol^{-1}. In contrast to earlier studies with exposed salt bridges,[19] the values of $\Delta\Delta G^o$-(spec.) were independent of the salt concentration (Table 1).

These studies clearly show that a single salt bridge provides an impressive degree of specificity for forming heterodimers versus homodimers. However, this specificity occurs at a thermodynamic price; the Dab-p1/Asp-p1 and Dap-p1/Asp-p1 heterodimers are less stable than GCN4-p1 by about 2 kcal mol^{-1}.[24] This result parallels recent findings from the study of a salt bridge in a globular protein.[21] A buried hydrogen bond or hydrophobic interaction often provides a more favorable driving force for molecular recognition relative to a buried salt bridge, even though the interaction energy associated with the salt bridge may be greater.[21,25] This is because the free energy for dimer formation in aqueous solution must include the free energy cost of dehydration associated with transferring the charged residues from water to the helix/helix interface upon folding. Because the neutral groups are uncharged, the free energy cost of dehydration is much less unfavorable than the corresponding dehydration of the charged partners in the salt bridge. Nevertheless, the ability to achieve a high degree of specificity from a *single* interaction is well worth the thermodynamic cost if a unique heterodimer can be specified. Previous attempts to design heterodimeric coiled coils relied on the introduction of charged groups at *each* "e" and "g" position (16 residues),[26] Figure 1. By contrast, the use of a single buried salt bridge provides an impressive degree of specificity under a much greater range of ionic strength. The present strategy also leaves the "e" and "g" positions free to be varied for other structural or functional purposes.

Acknowledgment. This work was supported by the National Institutes of Health (GM54616) (W.F.D.). We thank Mark Melden for his assistance in peptide synthesis.

Supporting Information Available: Experimental details, *r* vs absorbance plots, thermal denaturation data, and plots of temperature vs θ_{222} (19 pages). See any current masthead page for ordering and Internet access instructions.

JA970493G

(24) ΔG^o(37 °C) determined for GCN4-p1 (100 μM, pH 6.3, 150 mM NaCl, 50 mM MES) is 8.4 kcal mol^{-1} compared to 6.6 and 6.2 kcal mol^{-1} for Dap-p1/Asp-p1 and Dab-p1/Asp-p1 mixtures, respectively.

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(28) MES = 2-[*N*-morpholino]ethanesulfonic acid, BTP = 1,3-bis(tris-[hydroxymethyl]methylamino)propane, CAPS = 3-[cyclohexylamino]-1-propanesulfonic acid.