Peptide 'Velcro*': design of a heterodimeric coiled coil

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Background: The leucine zipper is a protein structural motif involved in the dimerization of a number of transcription factors. We have previously shown that peptides corresponding to the leucine-zipper region of the Fos and Jun oncoproteins preferentially form heterodimeric coiled coils, and that simple principles involving electrostatic interactions are likely to determine the pairing specificity of coiled coils. A critical test of these principles is to use them as guidelines to design peptides with desired properties.

Results: Based on studies of the Fos, Jun and GCN4 leucine zippers, we have designed two peptides that are predominantly unfolded in isolation but which, when mixed, associate preferentially to form a stable, parallel, coiled-coil heterodimer. To favor heterodimer formation, we chose peptide sequences that would be predicted to give destabilizing electrostatic interactions in the homodimers that would be relieved in

the heterodimer. The peptides have at least a 10^5-fold preference for heterodimer formation, and the dissociation constant of the heterodimer in phosphate-buffered saline is approximately 30 nM at pH 7 and 20 °C. Studies of the pH and ionic strength dependence of stability confirm that heterodimer formation is favored largely as a result of electrostatic destabilization of the homodimers.

Conclusions: Our successful design strategy supports previous conclusions about the mechanism of interaction between the Fos and Jun oncoproteins. These results have implications for protein design, as they show that it is possible to design peptides with simple sequences that have a very high preference to pair with one another. Finally, these sequences with 'Velcro'-like properties may have practical applications, including use as an affinity reagent, in lieu of an epitope tag, or as a way of bringing together two molecules in a cell.

Background

Structural and energetic studies of leucine-zipper peptides [1–4] and analyses of coiled-coil sequences [5–8] suggest that simple principles may determine the specificity of pairing for coiled coils. A critical test of these principles is to use them as guidelines in the design of peptides with desired properties.

One strategy to promote preferential pairing between two peptides is to design their sequences so that the heterodimer has specific stabilizing interactions. For example, sequences could be designed to pack preferentially at the hydrophobic interface in a heterodimer. However, attempts to design heterodimeric coiled coils with leucine–alanine interactions at the dimer interface did not result in heterodimers that are more stable than both corresponding homodimers [9].

Sequences that result in favorable electrostatic interactions between the helices in a heterodimer could also be designed. Circular dichroism (CD) studies of the leucine zipper of the yeast transcription factor GCN4,

however, suggest that it may be difficult to stabilize a leucine zipper with interhelical ionic interactions. Studies of the GCN4 leucine zipper have shown its thermal stability does not depend strongly on pH or ionic strength (the melting temerature, T_m, in solution in PBS is 51 °C, 57 °C and 49 °C at pH 2,7 and 11, respectively, and the T_m is 57 °C ± 2 °C from 15 mM to 1.5 M NaCl at pH 7). This suggests that ion pairs observed in the crystal structure of this peptide [3] may not be significantly stabilizing.

A less obvious strategy to promote heterodimer formation is illustrated by the Fos/Jun leucine-zipper heterodimer, in which preferential pairing of the Fos and Jun peptides is driven primarily by the destabilization of the Fos homodimer [1,4]. Unfavorable interhelical electrostatic interactions substantially destabilize the Fos homodimer, thereby favoring heterodimer formation. We chose to design a heterodimeric coiled coil consisting of two peptides that would have many unfavorable interhelical electrostatic interactions in the homodimeric states.


Results and discussion

Peptide design

The coiled-coil structure consists of two right-handed α-helices wrapped around one another with a slight left-handed superhelical twist [10–12]. This structural motif is found in many proteins, including tropomyosin, keratin, bacterial surface proteins, leucine-zipper proteins and tumor suppressor gene products [12–16]. The sequences of coiled-coil proteins consist of heptad repeats, where positions of the heptad are labeled with the letters **a**–**g** [5,17]. Analysis of these sequences has shown that coiled-coil proteins have a characteristic 4–3 hydrophobic repeat, with hydrophobic amino acids spaced every four and then every three residues [5,17]. The hydrophobic residues occur at positions **a** and **d** of the heptad repeat, whereas residues at positions **e** and **g** are predominantly charged (Fig. 1a).

These sequence preferences were rationalized by McLachlan and Stewart [5] with a three-dimensional model for the coiled coil. Experimental support for this model is provided by the X-ray structure of the GCN4 leucine-zipper homodimer [3], a two-stranded, parallel coiled coil. The structure of the coiled coil can be represented as a ladder, in which the helix backbones are the sides of the ladder and the rungs are comprised of residues at the dimer interface (Fig. 1a). There are two types of rung, each containing four residues, which alternate along the superhelical axis. One type of rung consists of residues from positions **a**, **a′**, **g**, **g′**, and the second type of rung has residues from positions **d**, **d′**, **e**, **e′**. Residues at positions **e** and **g** can also participate in interhelical ionic interactions (Fig. 1a).

The stability of homodimers containing residues of like charge at positions **e** and **g** is strongly pH-dependent [1,4], which can be explained by reference to the crystal structure of the GCN4 leucine zipper [3]. Side-chain methylene groups of residues at positions **e** and **g** pack against the predominantly hydrophobic residues at positions **a** and **d**, completing the dimer interface (Fig. 1b) and bringing together charged groups of side chains from opposing subunits (Fig. 1a). Thus, if positions **e** and **g** are occupied by residues of like charge, destabilization of the dimer is expected to result from electrostatic repulsion as well as loss of hydrophobic contacts.

The two peptides that we have designed differ only at positions **e** and **g** (Fig. 2). Glutamic acid was chosen for these positions in one peptide, designated ACID-p1, whereas lysine was chosen in a second peptide, designated BASE-p1. The ACID-p1 and BASE-p1 homodimers are expected to be destabilized at neutral pH by unfavorable electrostatic interactions. Leucine was chosen for positions **a** and **d** because it is the most common amino acid at these positions in coiled-coil proteins [6–8] and because Hodges [18,19] and DeGrado [20] have placed leucine at these positions
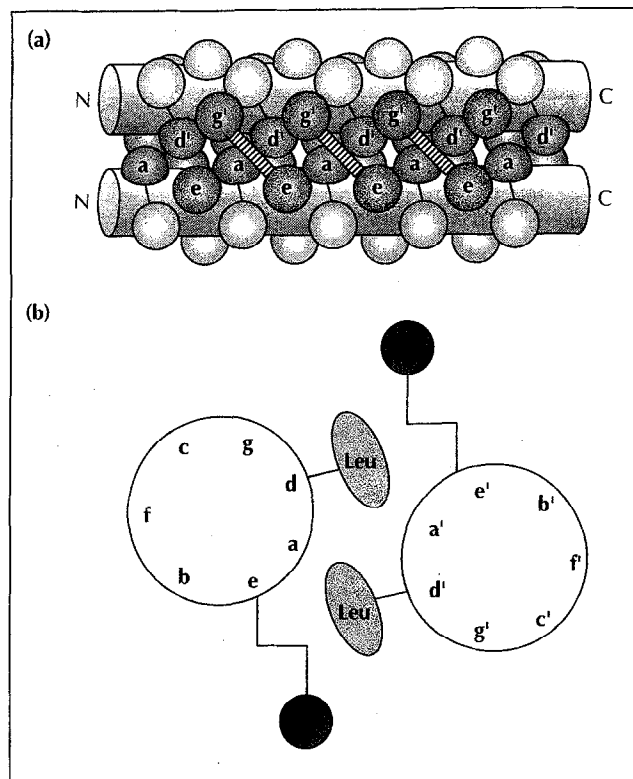
Fig. 1. Schematic representations of the coiled-coil structure illustrating the interactions between residues at the dimer interface. (a) The coiled-coil structure can be thought of as a ladder with sides formed by the helix backbones and alternating rungs composed of four residues. A side view of a coiled coil is shown: the positions of the heptad of one subunit are labeled with the letters **a**–**g**; residues **a′**–**g′** are on the other subunit. One set of rungs (red) consists of side chains from positions **a**, **a′**, **g**, **g′**, whereas the second set (blue) consists of side chains from positions **d**, **d′**, **e**, **e′** (only **a**, **g′**, **d** and **e′** are marked). Residues at positions **e** and **g** pack against positions **a** and **d**, as well as participating in interhelical electrostatic interactions, which are indicated with bridges. For simplicity, the supercoiling of the helices is not depicted. (b) Schematic cross section through the dimer, illustrating the interactions between four residues in a rung. The large circles represent the helical backbone and line segments represent bonds between carbon atoms. As an example, a rung consisting of leucine at position **d** and glutamate at position **e** is shown. Residues at positions **d** and **d′** make side-to-side interactions, as in a handshake. Additionally, methylene groups from residues at the charged positions **e** and **e′** make contacts with the hydrophobic residues at positions **d** and **d′**. These types of interaction are also seen in the other type of rung, in which methylene groups from residues at positions **g** and **g′** make contacts with residues at positions **a** and **a′**.

in simple designed homomeric coiled coils. A single asparagine residue was placed at position **a** in the second heptad to favor the parallel orientation of helices. Replacing a hydrophobic residue (at position **a**) with an asparagine in a leucine zipper that has little intrinsic preference for helix orientation results in a marked preference for the parallel orientation (R Rutkowski, EKO and PSK, unpublished observations).

A polar residue is often found at an **a** position in the sequences of leucine-zipper proteins, including GCN4 [21]. In the crystal structure of the GCN4 leucine
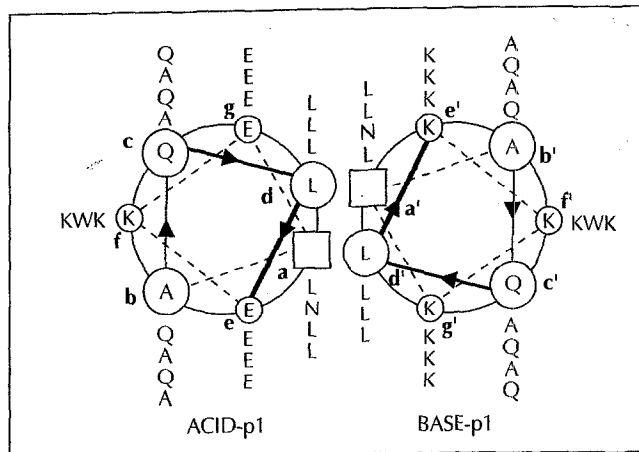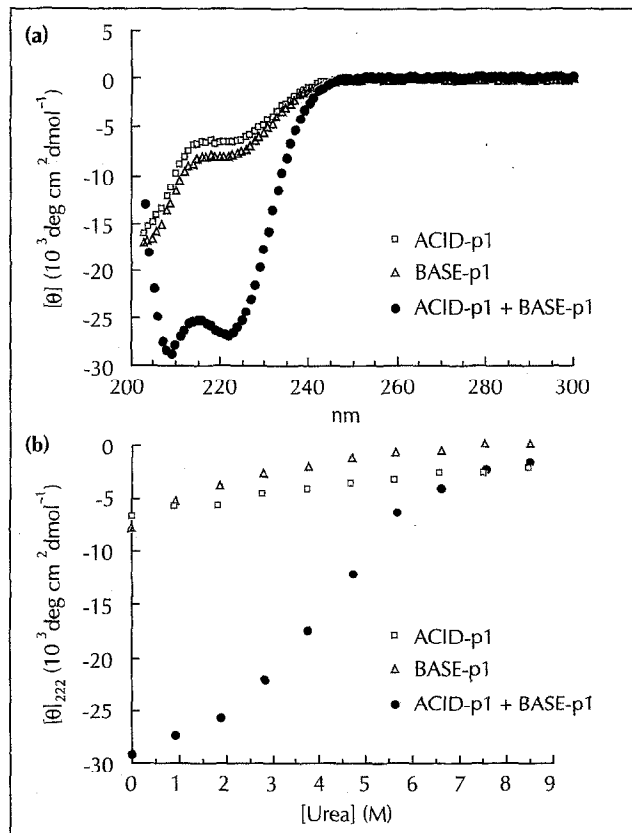
Fig. 2. Helical wheel representation [5,17] of the ACID-p1/BASE-p1 heterodimer. ACID-p1 differs from BASE-p1 only at positions **e** and **g**, where ACID-p1 contains glutamate and BASE-p1 contains lysine. The homodimers of these peptides are expected to be destabilized by unfavorable intrahelical and interhelical electrostatic interactions arising from residues at positions **e** and **g**. This destabilization is expected to be relieved in the heterodimer. The view shown is from the amino termini, looking down the superhelical axis. The peptide sequences begin at position **b** and end at position **c**. The sequence of peptide ACID-p1 is: Ac–AQLEKELQALEKENAQLEWELQALEKELAQ–NH₂; the sequence of BASE-p1 is Ac–AQLKKKLQALKKKNAQLKWK–LQALKKKLAQ–NH₂.

Fig. 3. The ACID-p1 and BASE-p1 peptides show little evidence of structure in isolation, but when mixed form a stable, helical structure. **(a)** The ACID-p1 and BASE-p1 peptides are predominantly unfolded in PBS (pH 7.0, 37 °C), but a mixture of the peptides forms a highly helical structure characterized by CD minima at 222 nm and 208 nm. **(b)** The structure of the ACID-p1 plus BASE-p1 mixture is stable and undergoes a cooperative unfolding transition with a midpoint at approximately 4.5 M urea (PBS, pH 7.0, 37 °C). In contrast, isolated ACID-p1 and BASE-p1 peptides show no evidence for significant folding.

zipper, in which the helices have a parallel orientation, an asparagine is part of the dimer interface and forms a buried hydrogen bond with the corresponding asparagine from the opposing subunit [3]. An asparagine at position **a** is expected to disfavor an antiparallel orientation of helices, because each asparagine (at position **a**) would be paired with a leucine (position **d**) from the opposing subunit. Based on similar reasoning, an asparagine at position **a** is expected also to favor an unstaggered arrangement of the helices [3].

The peptides contain alternating alanine and glutamine residues at positions **b** and **c**. Uncharged residues were desired at these positions in order to avoid substantial electrostatic interactions with adjacent residues at positions **e** and **g** (Fig. 2). In addition, alanine and glutamine residues promote helix formation [20,22,23] and the polar glutamine side chain is expected to increase solubility [24]. Lysine was chosen for the residues on the outside of the dimer at position **f** to increase the solubility of the peptides and to discourage aggregation. One residue at position **f** was chosen to be a tryptophan in order to facilitate concentration-determination by absorbance [25].

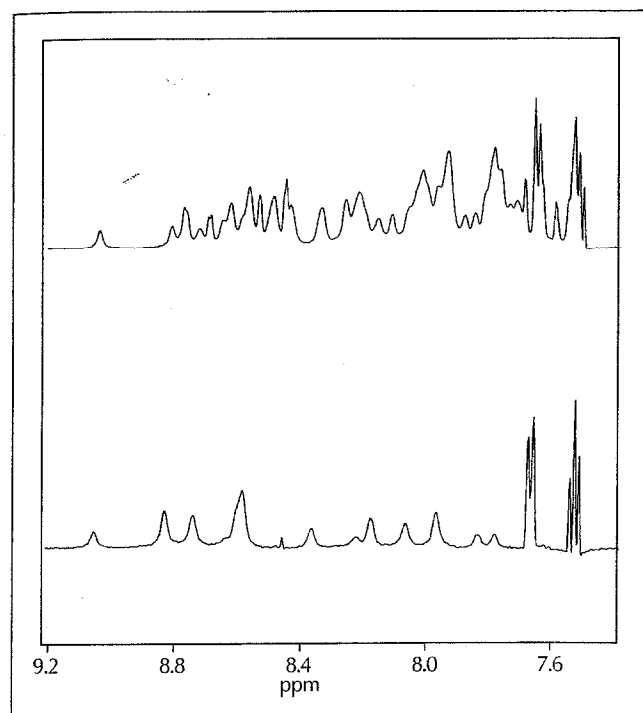**The ACID-p1/BASE-p1 heterodimer is helical and stable**

CD spectra indicate that the individual ACID-p1 and BASE-p1 peptides are predominantly unfolded at 37 °C and pH 7 in phosphate-buffered saline (PBS) (Fig. 3a). In contrast, the CD spectrum of an equimolar mixture of ACID-p1 and BASE-p1 indicates that the mixture is highly helical (Fig. 3a). This helical structure is stable: at a heterodimer concentration of 5 μM, the T_m is 65 °C

in PBS at pH 7. In addition, whereas a urea-induced unfolding transition is observed for the heterodimer, the isolated peptides show no evidence for cooperative unfolding (Fig. 3b). Sedimentation equilibrium studies of an ACID-p1 plus BASE-p1 mixture at 20 °C in PBS, pH 7 indicate definitively that it has the molecular weight expected for the heterodimer: the observed molecular weight is 7200 D (the expected molecular weight is 7123 D). Thus, the ACID-p1 and BASE-p1 peptides associate preferentially and fold as a stable, helical heterodimer.

The amide proton exchange behavior of the ACID-p1/BASE-p1 heterodimer was characterized by nuclear magnetic resonance (NMR) spectroscopy. A coiled-coil dimer with a well-packed hydrophobic core is expected to have some protons protected from exchange by factors approaching the equilibrium constant for folding of the molecule. For example, several amide protons in a GCN4 leucine-zipper peptide have protection factors of 10^5–10^6, comparable to the equilibrium constant for folding [26]. In contrast, slowly exchanging protons in the designed four-helix bundle

Fig. 4. Several amide protons are protected from exchange by a factor of at least 10⁴, suggesting that the interface between helices in the ACID-p1/BASE-p1 heterodimer has a well-defined structure. A region of the one-dimensional ¹H-NMR spectrum containing resonances arising from the amide and aromatic protons is shown. The top spectrum is taken in H_2O (0 hours) and the bottom spectrum is taken 70 hours after the sample was dissolved in D_2O (pH 4.7, 20 °C). The dissociation constant measured by urea denaturation (see later, Fig. 8a) indicates that a 1 M solution of peptide would have a free energy of unfolding at 20 °C, pH 7.0 of $-10.1\,\text{kcal mol}^{-1}$. The concentration dependence of stability is given by $\Delta G^0 = \Delta G + RT\ln(c)$, where ΔG° is the standard state free energy and c is the concentration of peptide. Thus, a 3 mM peptide solution at 20 °C, pH 7.0 is expected to have a free energy of unfolding of $-6.7\,\text{kcal mol}^{-1}$. Because the stability of the ACID-p1/BASE-p1 heterodimer is independent of pH from pH 7 to pH 4.5 (Fig. 5a), the free energy of unfolding under the exchange conditions (pH 4.7, PBS) is estimated to be $-6.7\,\text{kcal mol}^{-1}$, corresponding to an equilibrium constant of 10⁵. At pH 4.7, 20 °C the half-time for exchange of an unprotected amide proton is approximately 0.4 minutes [43]; thus, protons that are half-exchanged after 70 hours have protection factors of 10⁴.

α4 are protected from exchange by factors of only 10³–10⁵, compared to the maximum of 10¹⁶ expected from the global stability of the molecule [27,28]. These NMR data and the results of other experiments suggest that α4 contains fluctuating tertiary structure [28,29].

The global stability of the ACID-p1/BASE-p1 heterodimer, under the conditions in which hydrogen exchange was studied, is $-6.7\,\text{kcal mol}^{-1}$. Thus, the maximum degree of protection from exchange is expected to be about 10⁵. At a time when protons that are protected by a factor of approximately 10⁴ are expected to be half-exchanged, twelve amide proton resonances are present in the spectrum (Fig. 4). This suggests strongly that the interface between the ACID-p1 and BASE-p1 helices is well-packed [28,29].

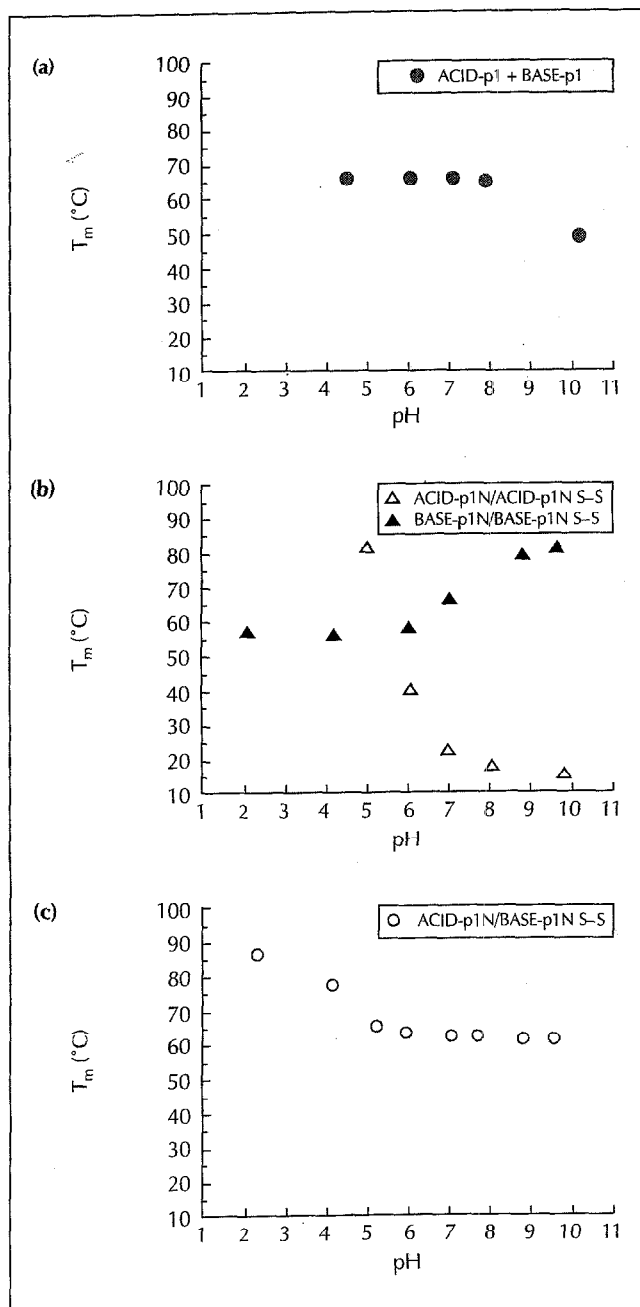## Characterization of the disulfide-bonded heterodimer and homodimers

Our design predicts that the ACID and BASE peptides will fold as parallel, helical dimers but, at practical peptide concentrations, the ACID-p1 and BASE-p1 homodimers are too unstable to study. One way to stabilize dimeric coiled-coil peptides is to join the peptides with a disulfide-bond linker [1,2,19,30,31]. Therefore, versions of the ACID and BASE peptides containing an amino-terminal cysteine followed by two glycines, designated ACID-p1N and BASE-p1N, were made and studied. The glycines allow disulfide-bond formation without distortion of the coiled-coil structure [2]. CD studies demonstrate that the disulfide-bonded heterodimer and homodimers are greater than 80 % helical at 0 °C and that a cooperative thermal unfolding transition is observed for each dimer, indicating that the ACID and BASE homodimers can fold when stabilized with a disulfide-bond linker.

To determine if the orientation of the helices is parallel, the concentration dependence of stability was studied for each of the disulfide-bonded peptide dimers [1,2]. A dimer in which the helices are joined in the preferred orientation is expected to have stability and structure that is independent of peptide concentration. Alternatively, a dimer in which the helices are covalently linked in the unfavorable orientation is expected to have concentration-dependent stability and structure, arising from the intermolecular association of dimers.

When the ACID-p1N and BASE-p1N peptides are joined with a disulfide bond (that is, with a parallel orientation), the T_m of the heterodimer and each homodimer was found to be independent of peptide concentration from approximately 2.5 to 170 μM (see Fig. 5 legend). In contrast, when the heterodimer is disulfide-bonded in the antiparallel orientation (by joining the peptide ACID-p1N to a BASE peptide that has a carboxy-terminal sequence Gly–Gly–Cys), the CD signal is dependent on peptide concentration, indicating that higher-order oligomers are formed. In PBS, pH 7.0 containing 2.25 M GuHCl at 0 °C, the $[\theta]_{222}$ values of the antiparallel heterodimer change from $-11\,100\,\text{deg cm}^2\,\text{dmol}^{-1}$ at 9 μM to $-25\,300\,\text{deg cm}^2\,\text{dmol}^{-1}$ at 25 μM. Collectively, these results indicate that the folded conformations of the ACID and BASE heterodimer and homodimers are parallel.

Mechanism of specificity

Our design strategy sought to drive heterodimer formation by electrostatic repulsion in the homodimers. Nonetheless, because the ACID and BASE peptides contain oppositely charged residues at the **e** and **g** positions of the coiled-coil repeat, it is very plausible instead that electrostatic attraction between these peptides provides a major driving force for heterodimer formation.

(a)

T_m (°C)

100
90
80
70
60
50
40
30
20
10

1 2 3 4 5 6 7 8 9 10 11
pH

● ACID-p1 + BASE-p1

(b)

T_m (°C)

100
90
80
70
60
50
40
30
20
10

1 2 3 4 5 6 7 8 9 10 11
pH

△ ACID-p1N/ACID-p1N S–S
▲ BASE-p1N/BASE-p1N S–S

(c)

T_m (°C)

100
90
80
70
60
50
40
30
20
10

1 2 3 4 5 6 7 8 9 10 11
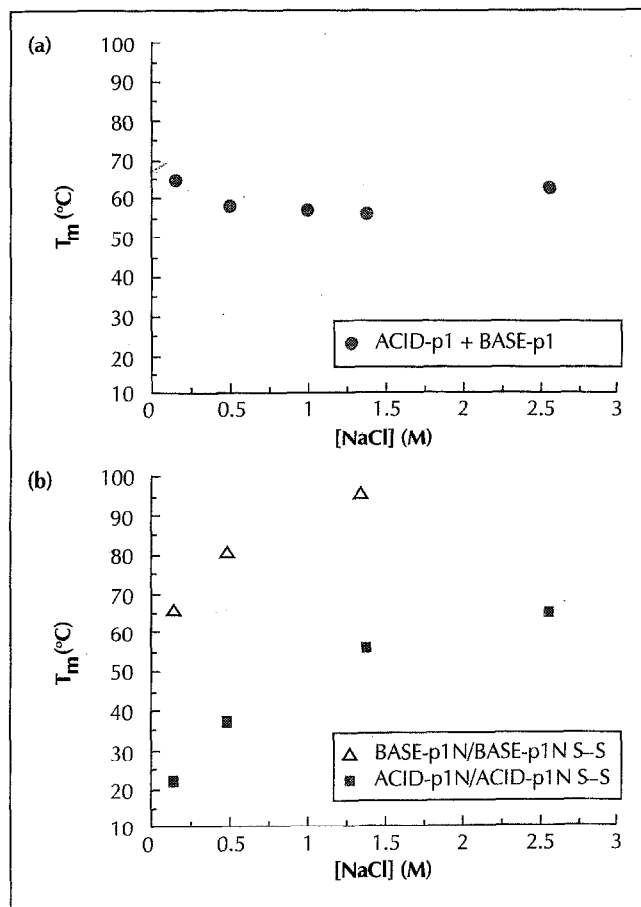pH

○ ACID-p1N/BASE-p1N S–S

Fig. 5. The pH-dependence of stability indicates that the ACID and BASE homodimers are destabilized by unfavorable electrostatic interactions, whereas the net electrostatic contribution to the stability of the heterodimer is minimal. (a) The stability of the heterodimer, measured in the absence of a disulfide bond, is relatively independent of pH. (b) In contrast, the T_m of the disulfide-bonded ACID-p1N homodimer increases at low pH and the T_m of the disulfide-bonded BASE-p1N homodimer increases at high pH. (c) The stability of the disulfide-bonded ACID-p1N/BASE-p1N heterodimer in 3 M GuHCl increases at low pH, suggesting that the heterodimer is somewhat destabilized by acidic residues at neutral pH. In PBS and at pH 7, the T_m values of the disulfide-bonded ACID-p1N/ACID-p1N, BASE-p1N/BASE-p1N, and ACID-p1/BASE-p1 dimers are 21 ± 1 °C, 66 ± 1 °C and > 100 °C, respectively, over the concentration range 2.5–170 μM. Because the disulfide-bonded ACID-p1N/BASE-p1N heterodimer is too stable to denature thermally, the T_m of a 2.5 μM solution of heterodimer was measured in PBS containing 3 M GuHCl and determined to be 62 ± 1 °C.

Assuming that one of these mechanisms predominates, some simple predictions can be made about the pH- and ionic strength-dependence of stability. If electrostatic destabilization of homodimers is dominant, then the homodimers will be more stable when the charges are removed by titration at extremes of pH or when the ionic strength is increased. In contrast, if electrostatic attraction between the ACID and BASE peptides in the heterodimer provides the dominant driving force for preferential heterodimer formation, then the heterodimer will be less stable as charges are removed by titration at pH extremes (that is, a bell-shaped curve for the pH dependence of stability would result) and the stability of the heterodimer should decrease at higher ionic strength.

The stability of both homodimers in the more stable disulfide-bonded species is highly dependent on both pH and ionic strength (Figs 5 and 6). The T_m of the ACID-p1N/ACID-p1N homodimer is more than 80 °C higher at acidic pH than at pH 7, indicating clearly that it is destabilized by negatively charged residues at neutral pH (Fig. 5b). Similarly, the BASE-p1N/BASE-p1N homodimer is approximately 20 °C more stable at basic pH than at pH 7, indicating that it is destabilized at neutral pH by positively charged basic residues. Both homodimers are stabilized substantially by increasing ionic strength at neutral pH (Fig. 6b), indicating that they are destabilized by repulsive electrostatic interactions.

At neutral pH, the BASE-p1N/BASE-p1N homodimer is substantially more stable than the ACID-p1N/ACID-p1N homodimer (Fig. 5b). It is likely that part of the reason for this difference in stability is the difference in the length of the side chains of residues at positions **e** and **g**. Whereas ACID-p1N has glutamate, containing two methylene groups, at position **e** and **g**, BASE-p1N has lysine, which contains four methylene groups. The longer lysine side chain allows for more flexibility and better solvation of the terminal charged group. This is supported by studies of BASE peptides containing the non-natural amino acid ornithine (ORN-p1; three methylene groups) or diaminobutyric acid (DAB-p1; two methylene groups) at positions **e** and **g**. Unlike BASE-p1N, disulfide-bonded homodimers containing these non-natural amino acid do not show evidence for structure at 0 °C, nor for a cooperative thermal unfolding transition. Additionally, the stability of the corresponding ACID/BASE heterodimer decreases with decreasing side chain length: in PBS at pH 7, the disulfide-bonded ACID-p1N/DAB-p1N and ACID-p1N/ORN-p1N heterodimers have T_m values of approximately 63 °C and 80 °C, respectively (the ACID-p1N/BASE-p1N heterodimer has a T_m over 100 °C).

Because the disulfide-bonded ACID-p1N/BASE-p1N heterodimer is exceedingly stable, the pH and ionic

Fig. 6. The ionic strength-dependence of stability indicates that the ACID and BASE homodimers are destabilized by unfavorable electrostatic interactions, whereas the net electrostatic contribution to the stability of the heterodimer is minimal. (a) The stability of the heterodimer, measured in the absence of a disulfide bond, is relatively independent of ionic strength. (b) In contrast, the stability of the disulfide-bonded homodimers increases at high ionic strength.

strength-dependence of stability for the heterodimer was studied in the absence of a disulfide bond. At pH 7, the stability of the ACID-p1/BASE-p1 heterodimer is relatively independent of ionic strength (Fig. 6a). In addition, there is little change in the stability of the heterodimer between pH 4.5 and pH 9 (Fig. 5a). It is, however, difficult to assess the stability of the heterodimer at extremes of pH (< 3.5 and > 10.5) because at these pH extremes the thermal denaturation curves contain evidence for more than one transition, suggesting that other species, such as homodimers, are formed or that folding is not two-state.

To evaluate the stability of the heterodimer at extremes of pH, the pH-dependence was also studied in the presence of the disulfide bond but in 3 M GuHCl to destabilize the heterodimer (Fig. 5c). A comparable concentration of NaCl has little effect on the stability of the heterodimer (Fig. 6a). The striking result is that the stability of the disulfide-bonded heterodimer increases by approximately 25 °C at low pH (Fig. 5c). Sedimentation equilibrium studies show definitively that the

amino-terminal disulfide-bonded ACID-p1N/BASE-p1N heterodimer remains dimeric at pH 2: the observed molecular weight was 7200 D at pH 2 and 7700 D at pH 7 (the expected value is 7556 D). Thus, the negatively charged glutamate residues have a net destabilizing effect in the heterodimer.
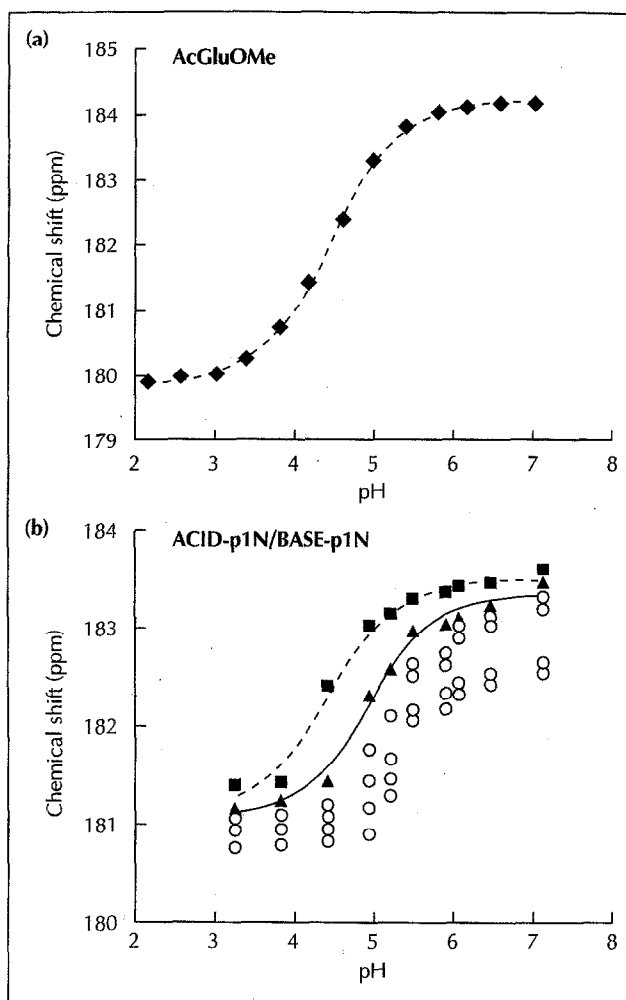
Fig. 7. ^{13}C-NMR spectroscopy indicates that the pK_a values for the glutamic acid side chains in the heterodimer are close to normal, or slightly increased, in PBS at 20 °C. (a) The changes in chemical shift with pH of the carboxylate ^{13}C resonances of AcGluOMe indicate that the pK_a of the carboxylic acid group of AcGluOMe is 4.5. (b) The changes in chemical shift with pH of the carboxylate ^{13}C resonance of disulfide-bonded ACID-p1N/BASE-p1N indicates that the pK_a values of the glutamic acid residues are either close to normal ($pK_a = 4.4$, ■, and $pK_a = 4.9$, ▲) or slightly increased ($pK_a = 5.2$–5.3, ○).

The measurements described above do not, however, rule out extremely large pK_a shifts for some ionizable groups in the heterodimer, which can result from stable salt bridge formation (for example, see [32]). The pK_a values for the glutamic acid residues in the heterodimer were therefore measured directly by natural-abundance ^{13}C NMR. The disulfide-bonded form of the heterodimer was studied to prevent formation of alternate species such as the ACID homodimer at low pH. In contrast to the decrease in pK_a expected if the glutamic

acid residues were involved in stabilizing salt bridges, the observed pK_a values for all glutamic acid residues in the heterodimer are close to or higher than the pK_a for blocked glutamic acid measured under the same conditions (Fig. 7).

The finding that some of the glutamic acid residues have increased pK_a values is consistent with the observation that the heterodimer is more stable at low pH (Fig. 5c). The most likely explanation for the increased stability at low pH is that electrostatic repulsion between the glutamic acid residues in the individual ACID helix is destabilizing, even in the heterodimer. Similarly, the Fos/Jun leucine-zipper heterodimer [1,4] and previously designed coiled coils that contain acidic residues at positions **b** and **e** [18,19] are more stable at low pH than at neutral pH, suggesting that there is substantial electrostatic repulsion within the helices of these coiled coils at neutral pH [1,4]. In general, however, intrahelical destabilization can be minimized in designed coiled coils by utilizing a 14-residue repeat in which residues at a particular position in the heptad repeat are different.

It is puzzling that these studies do not indicate that interhelical salt bridges substantially stabilize the heterodimer, given that the heterodimer has charged side chains that might be expected to form salt bridges of the same type seen in the crystal structure of the GCN4 leucine zipper [3]. It is possible that salt bridges do not form in the heterodimer. Alternatively, the salt bridges may form but not be significantly stabilizing, as appears to be the case for the GCN4 leucine-zipper peptide. Finally, the stabilizing effect of salt bridges could be offset by destabilizing factors. For example, the protonated form of a glutamic acid residue has a higher helix propensity than the ionized form [33, 34] and, as discussed above, electrostatic repulsion between residues of like charge within the ACID peptide helix is destabilizing even within the heterodimer.

Even if stabilizing salt bridges are present in the heterodimer, these results indicate that the net electrostatic contribution to specificity from the heterodimer *per se* is either minimal or unfavorable. In contrast, both homodimers have stabilities that are dependent strongly on pH and ionic strength, in a manner that would be expected if the homodimers have substantial net destabilizing electrostatic interactions at neutral pH. We conclude that our design strategy was successful: preferential heterodimer formation is driven primarily by destabilization of the homodimers.

Quantitation of specificity

Because the preference for heterodimer formation in a mixture of the ACID and BASE peptides is so large, the ratio of heterodimer to homodimer cannot be measured readily from an equilibrium mixture of the two peptides. However, the degree of specificity can be estimated because K_{spec}, the equilibrium constant
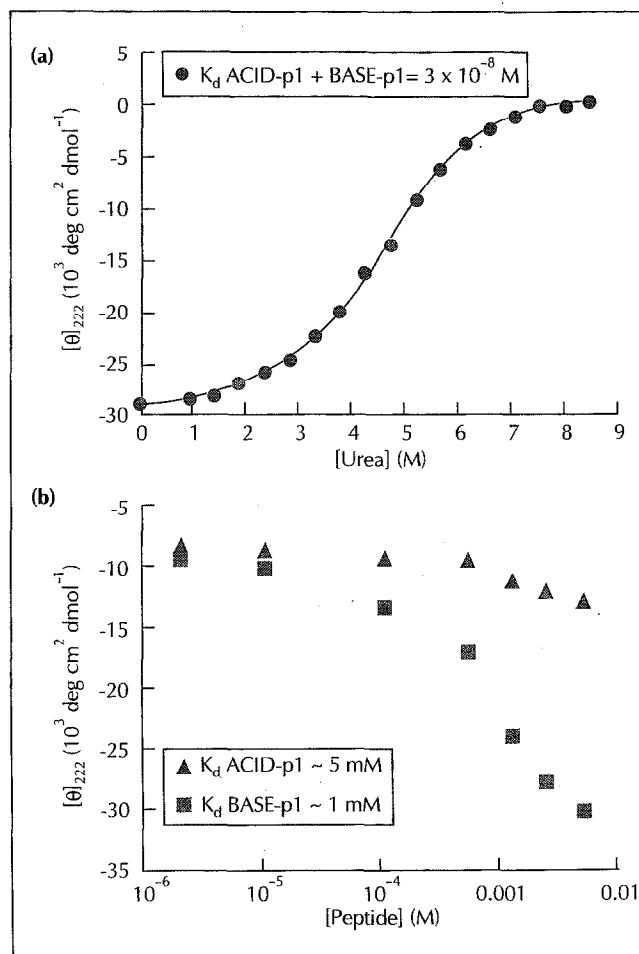
Fig. 8. Estimation of the dissociation constant for the ACID-p1/BASE-p1 heterodimer and homodimers. **(a)** The dissociation constant for the ACID-p1/BASE-p1 heterodimer is estimated to be 3×10^{-8} M from a curve fit of the urea dependence of the CD signal (the line from this fit is shown). **(b)** The dissociation constants for the homodimers of ACID-p1 and BASE-p1, estimated from plots of the CD signal at 222 nm as a function of peptide concentration, are greater than 5×10^{-3} M and approximately 1×10^{-3} M respectively.

describing the ratio of heterodimer to homodimers, can be expressed in terms of the dissociation constants for each dimer [4]. Assuming a two-state model for monomer-dimer equilibrium,

$$K_{spec} = K_d (AB)/[K_d (AA)^{1/2} \times K_d (BB)^{1/2}]$$

where K_d (AB), K_d (AA) and K_d (BB) are the dissociation constants of the ACID-p1/BASE-p1 heterodimer, the ACID-p1 homodimer and the BASE-p1 homodimer, respectively.

The dissociation constant for each dimer was determined so that the degree of specificity, ΔG_{spec}, given by $-RTlnK_{spec}$, could be estimated. A dissociation constant of 3×10^{-8} M for the ACID-p1/BASE-p1 heterodimer was determined by fitting the CD signal as a function of urea concentration to a monomer–dimer equilibrium (Fig. 8a). An estimate of the dissociation constant for each homodimer was obtained from measurements of the helical CD signal as a function of

peptide concentration. The dissociation constant for the BASE-p1 homodimer is approximately 1×10^{-3} M and that for the ACID-p1 homodimer is greater than 5×10^{-3} M (Fig. 8b). These dissociation constants place a lower limit on the degree of preference for heterodimer, ΔG_{spec}, of -6.5 kcal mol^{-1} ($> 10^5$-fold preference for heterodimer).

A different, albeit less reliable, estimate of specificity can be made by measuring the difference between the T_m of the heterodimer and the average of the T_m values for the homodimers [4]. This difference (ΔT_m) has been measured for other disulfide-bonded leucine-zipper peptides and has been shown to be related to ΔG_{spec} by a proportionality constant of $7.4\,^{\circ}$C/kcal mol^{-1} [4]. ΔT_m for the amino-terminally disulfide-bonded ACID-p1N and BASE-p1N peptides is greater than $56\,^{\circ}$C (see Fig. 5 legend). If the same proportional relationship between ΔT_m and ΔG_{spec} exists with the ACID and BASE peptides, this lower limit for ΔT_m implies that ΔG_{spec} is at least -7.5 kcal mol^{-1} (greater than $10^{5.6}$-fold preference for heterodimer).

Thus, the heterodimer is preferred over the ACID-p1 and BASE-p1 homodimers by at least 10^5-fold. This degree of specificity is much greater than that observed for the Fos and Jun peptides, which show only a 10^2-fold preference for heterodimer formation [4]. The increased specificity of the ACID/BASE heterodimer is likely to result from the increased number of repulsive interactions in the designed homodimers.

Conclusions

Our results indicate that the designed ACID and BASE peptides fold as parallel, helical dimers that have a very high preference for the heterodimeric state. As the ACID and BASE peptides were designed using principles learned from the study of preferential heterodimer formation in the Fos and Jun peptide system, our results support previous conclusions about the basis and mechanism of Fos/Jun specificity [1,4]. In both cases, unfavorable electrostatic interactions in the homodimers contribute more to dimerization specificity than favorable electrostatic interactions in the heterodimer.

Our results have implications for protein design, because they demonstrate that it is possible to design simple helical sequences that pair with substantial specificity. The design of simple molecules consisting of two secondary structural elements may lead to stepwise strategies for the design of larger proteins. In contrast to many other designed peptides, the ACID/BASE heterodimer does not contain fluctuating tertiary structure, as judged by amide proton exchange studies, possibly because a polar group was introduced into the otherwise hydrophobic interface [3,29,35,36].

Finally, the ACID-p1 and BASE-p1 peptides can be thought of as peptide 'Velcro': the individual peptides have little self-affinity under physiological conditions of temperature, pH and ionic strength, but the two peptides have high affinity for each other. It may be possible to use these properties for practical applications, including use in affinity reagents, in place of an epitope tag or as a way to bring together two molecules in a cell.

Materials and methods

Peptide synthesis

Peptides were synthesized using small-scale FMOC HBTU reaction cycles and acetic anhydride capping on an Applied Biosystems Model 431A peptide synthesizer [37]. Peptides were cleaved from the resin with trifluoroacetic acid (TFA) using standard cleavage methods and were desalted on a Sephadex G-10 column in 5% acetic acid. Final purification was by reverse-phase high performance liquid chromatography (HPLC) with a Vydac preparative C18 column (2.2×25 cm) at $25\,^{\circ}$C using linear acetonitrile/H$_2$O gradients in the presence of 0.1% TFA. Peptide purity was greater than 90%, as judged by analytical HPLC. The identity of each peptide was confirmed by laser desorption mass spectrometry on a Finnegan Lasermat and each was found to be within 2 D of the expected mass. All peptides are acetylated at the amino terminus and amidated at the carboxyl terminus.

CD spectroscopy

CD studies were performed on an Aviv Model 60DS or 62DS CD spectrophotometer equipped with a thermoelectric controller. Peptide concentrations were determined by absorbance at 280 nm in 6 M GuHCl [25] and samples were prepared in PBS (150 mM NaCl, 10 mM sodium phosphate). CD spectra (Fig. 3a) were measured at $37\,^{\circ}$C, pH 7.0 using a peptide concentration of 10 µM ACID-p1, 10 µM BASE-p1, or 5 µM ACID-p1 plus 5 µM BASE-p1. Urea denaturation curves shown in Figure 3b were determined at $37\,^{\circ}$C, pH 7.0 using a peptide concentration of 20 µM ACID-p1, 20 µM BASE-p1, or 10 µM ACID-p1 plus 10 µM BASE-p1. Thermal melting curves were determined by monitoring the CD signal at 222 nm as a function of temperature. For studies of the pH-dependence of stability (Fig. 5), melting curves were recorded with 5 µM homodimer or 5 µM heterodimer in PBS with the pH adjusted to the desired value. For studies of the ionic strength-dependence (Fig. 6), melting curves were recorded at pH 7.0 with 5 µM heterodimer or with 2.5 µM homodimer in 10 mM sodium phosphate in the presence of the indicated concentration of NaCl. The T_m values were estimated by taking the first derivative of the CD signal with respect to temperature^{-1} and finding the minimum of this function [38]. All thermal melts performed at pH values below pH 8 are reversible; more than 90% of the CD signal at $0\,^{\circ}$C was recovered after the melt. The urea denaturation curve shown in Figure 8a was determined at $20\,^{\circ}$C, pH 7.0 with 10 µM ACID-p1 plus 10 µM BASE-p1. A two-state model for unfolding was assumed and the urea denaturation curve for the heterodimer was fit with a non-linear least-squares fitting program (KaleidaGraph, Synergy Software) to the following equation that describes the urea dependence of the CD signal for a monomer-dimer equilibrium:

$$\theta = \theta_d + (\theta_m - _d)[\exp((\Delta G^\circ + m[urea])/RT)/4C_T] +$$
$$[\exp((\Delta G^\circ + m[urea])/RT)^2/16C_T^2] +$$
$$[\exp((\Delta G^\circ + m[urea])/RT)/2C_T^{1/2}]$$

where $\theta =$ CD signal at 222 nm; θ_d and θ_m are linear equations describing the urea dependence of the CD signal of the dimer and unfolded monomer respectively; $\Delta G^\circ =$ free

energy of folding under standard conditions of 20 °C and 1 M concentration of peptide chains; m = the dependence of the free energy of folding on denaturant concentration; R = gas constant; T = temperature in K; C_T = total concentration of peptide chains in M. Measurements of the CD signal at 222 nm as a function of peptide concentration for the ACID-p1 and BASE-p1 homodimers were performed at 20 °C, pH 7.0 (Fig. 8b).

Analytical centrifugation

Sedimentation equilibrium was performed at 20 °C using a Beckman XL-A ultracentrifuge at rotor speeds between 30 and 40 krpm. Three samples at a heterodimer concentration of 22 µM, 44 µM and 66 µM in PBS were analyzed. Samples were dialysed (> 12 hours) against the reference buffer (PBS). Data were fit to an ideal model plot of radial distance squared versus log(concentration). The residuals were analyzed to reveal the presence of species other than the dimer; in no case were systematic deviations of the residuals observed. Partial molar volumes and solvent densities were calculated as described by Laue et al. [39].

NMR spectroscopy

One-dimensional ^{1}H-NMR spectra were recorded on a Bruker AMX 500 spectrometer operating at 500.1 MHz for ^{1}H at 20 °C with a sweep width of 6024.1 Hz and a delay of 1.2 seconds. The sample was approximately 3 mM ACID-p1/BASE-p1 in PBS, pH 4.7 and internally referenced with trimethylsilylpropionic acid [40]. 8192 points were collected and water was suppressed by continuous irradiation. The pH values were uncorrected for the isotope effect [41]. Data were processed with the FTNMR software package (Hare Research Inc.) and spectra were apodized with a Gaussian window.

One-dimensional ^{13}C-NMR spectra were recorded at natural abundance on a Bruker AMX 500 spectrometer operating at 125.8 MHz for ^{13}C at 20 °C with a spectral width of 26315.79 Hz and a recycle delay of 3 seconds. Samples were prepared in deuterated PBS and were either 38 mM in AcGluOMe (Bachem Feichemikalien AG, Bubendorf, Switzerland) or approximately 2 mM in disulfide-bonded ACID-p1N/BASE-p1N. ^{1}H were decoupled using WALTZ-16 broadband decoupling [42]. Data sets consisting of 4096 to 25000 transients defined by 4096 points were collected and zero-filled twice before Fourier transformation. The pH values were uncorrected for the isotope effect [41].

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