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Postional Dependence of the Effects of Negatively Charged Glu Side Chains on the Stability of Two-stranded α-helical Coiled-coils

WAYNE D. KOHN, CYRIL M. KAY and ROBERT S. HODGES

Department of Biochemistry and the Protein Engineering Network of Centres of Excellence, University of Alberta, Edmonton, Alberta, Canada

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Abstract: The effects on protein stability of negatively charged Glu side chains at different positions along the length of the α-helix were investigated in the two-stranded α-helical coiled-coil. A native coiled-coil has been designed which consists of two identical 35 residue polypeptide chains with a heptad repeat $Q_gV_aG_bA_cL_dQ_eK_f$ and a Cys residue at position 2 to allow the formation of an interchain 2-2′ disulphide bridge. This coiled-coil contains no intra- or interchain electrostatic interactions and served as a control for peptides in which Glu was substituted for Gln in the **e** or **g** heptad positions. The effect of the substitutions on stability was determined by urea denaturation at 20 °C with the degree of unfolding monitored by circular dichroism spectroscopy. A Glu substituted for Gln near the N-terminus in each chain of the coiled-coil stabilizes the coiled-coil at pH 7, consistent with the charge–helix dipole interaction model. This stability increase is modulated by pH change and the addition of salt (KCl or guanidine hydrochloride), confirming the electrostatic nature of the effect. In contrast, Glu substitution in the middle of the helix destabilizes the coiled-coil because of the lower helical propensity and hydrophobicity of Glu compared with Gln at pH 7. Taking the intrinsic differences into account, the apparent charge–helix dipole interaction at the N-terminus is approximately 0.35 kcal/mol per Glu substitution. A Glu substitution at the C-terminus destabilizes the coiled-coil more than in the middle owing to the combined effects of intrinsic destabilization and unfavourable charge–helix dipole interaction with the negative pole of the helix dipole. The estimated destabilizing charge–helix dipole interaction of 0.08 kcal/mol is smaller than the stabilizing interaction at the N-terminus. The presence of a 2-2′disulphide bridge appears to have little influence on the magnitude of the charge–helix dipole interactions at either end of the coiled-coil. ©1997 European Peptide Society and John Wiley & Sons, Ltd.

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INTRODUCTION

The α-helical secondary structural motif occurs in great abundance in the majority of both fibrous and globular proteins. For this reason, and because numerous helical peptide models have been successfully designed, the α-helix is also the most frequently and thoroughly studied class of protein secondary structure.

Statistical studies of α-helices in protein structures have shown that, in addition to differing helical

Abbreviations: DTT, dithiothreitol; GdnHCl, guanidine hydrochloride. HPLC, high performance liquid chromatography

Address for correspondence: R.S. Hodges, Dept. of Biochemistry and the Protein Engineering Network of Centres of Excellence, University of Alberta, Edmonton, Alberta, Canada, T6G 2S2.

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preferences of the naturally occurring amino acids, different amino acids have also been found to prefer different regions of the helix. Charged and neutral polar residues occur more often at the ends of helices while non-polar residues occur more frequently near the centre of helices [1–4]. In particular, potentially negatively charged side chains (Asp, Glu) strongly prefer positions near the N-terminal end of helices while potentially positively charged side chains (His, Arg, Lys) have a less pronounced preference for the C-terminal end. Numerous studies have been carried out to determine plausible reasons for these preferences. Explanations for the preferences of polar side chains for the ends of helices fall into two principal models. Firstly, because the first four backbone NH groups and final four backbone CO groups of a helix are not able to form the i to $i + 4$ hydrogen bond to other backbone groups, polar side chains at the ends of the helix are often able to form hydrogen bonds to these unfulfilled backbone groups [5, 6]. This is termed helix capping and has been demonstrated most clearly with the N-cap residue, defined as the N-terminal residue of the helix with partly helical and partly non-helical character [7–9], although such capping may also occur with side chains at other positions near the ends of helices [3]. Secondly, electrostatic 'charge–helix dipole' interactions between the charged side chains and the net dipole moment of the α-helix formed by the alignment of individual peptide backbone dipoles may also stabilize or destabilize the protein. This net 'helix dipole' was originally suggested to result from parallel alignment of the peptide bond dipoles along the entire helix [10] and was shown to be equivalent to the effect of $+0.5$ unit charge at the N-terminus and -0.5 unit charge at the C-terminus of a helix [11], but recent theoretical studies [12–14] suggest it may arise principally from the four uncompensated NH groups with partial positive charge at the N-terminal end and four CO groups with partial negative charge at the C-terminal end of the helix. We therefore refer to the 'helix dipole' as arising from one of these proposed phenomena, but the resulting charge distribution of $+0.5$ and -0.5 remains the same in both cases. Both explanations for the preference of polar residues at the ends of helices have their merits; however, neither is able to explain all the experimental observations. Nor are these mechanisms mutually exclusive, since charged side chains could participate both as hydrogen bond donors or acceptors to the helix backbone as well as in electrostatic interactions with the helix dipole, as recently suggested by Tidor [14], with the resulting effect on stability being due to a combination of these interactions.

In recent years, the effects of charged residues at the termini of helices have been studied both in isolated helical peptides [15–22] and in helices of intact globular proteins [23–27]. In all of these studies, strong evidence is shown to support the charge–helix dipole interaction model. However, both of these approaches for the study of stabilizing or destabilizing substitutions in α-helices have inherent problems. Studying large globular proteins is difficult because of complex tertiary structural interactions, which can complicate the analysis of intrahelical interactions. Peptide models avoid this complication but can suffer from low inherent helicity, which is generally highly dependent on even single residue substitutions, and significant fraying at the helix ends [28, 29]. We have chosen the two-stranded α-helical coiled-coil, a dimer of amphipathic α-helices, as an ideal model system for studying the principles of protein stability and for developing *de novo* design capabilities [30–32]. This motif has the advantages of being a simple molecule with high symmetry and being composed of only one type of secondary structure, thereby simplifying the interpretation of experimental results. In addition, the helices are significantly stabilized compared with monomeric helical models owing to subunit interactions and have less flexibility at the ends. The major advantage of using a coiled-coil model is that a stable structure with very high helicity in a benign medium can be designed and single residue mutations can be carried out without significant change in helical content, unlike that generally obtained with monomeric α-helical peptides [33, 34]. This is important since quantitative comparison of the thermodynamic stability characteristics is most meaningful when there are not dramatic variations in structure between analogues.

Our previous studies of interchain electrostatic repulsions between Glu residues at the **e** and **g** positions of coiled-coils [35, 36] have suggested the potential importance of charge–helix dipole interactions and other position-dependent effects in contributing to the overall effect of a substitution on coiled-coil stability. In the present study, we have investigated comprehensively the effects of Glu substitutions on coiled-coil stability and have studied the modulation of these effects by changes in pH and salt conditions. We find that the effect of Glu at the N- or C-terminus is different from the effect in the middle of the helix and that the positional

dependence of the effect on stability can be explained by the charge–helix dipole interaction model. Specifically, Glu substitution for Gln increases stability at the N-terminus but destabilizes the coiled-coil in the middle; in addition, destabilization is even more marked at the C-terminus. At pH 3, where the substituted Glu residue is protonated, there is a stabilization of the coiled-coil at all positions but to a greater extent at positions in the middle of the coiled-coil. Both GdnHCl and KCl were found to screen the stabilizing effect of an N-terminal Glu substitution at pH 7, also suggesting that this stabilization is an electrostatic effect.

MATERIALS AND METHODS

Peptide Synthesis and Purification

The peptides were synthesized using solid-phase methodology and purified by reversed-phase HPLC. Their identities were confirmed by amino acid analysis and electrospray mass spectrometry. The disulphide bridged coiled-coils were formed by air oxidation in 0.1 M NH_4HCO_3 solution. Details of these procedures were given previously [35].

Size Exclusion Chromatography

The effective molecular weights of the peptides were determined by size exclusion HPLC as previously described [36].

Circular Dichroism Measurements

Circular dichroism spectroscopy was performed on a Jasco J-500C spectropolarimeter at 20 °C. Urea and GdnHCl denaturation curves were obtained by monitoring the loss of ellipticity at 222 nm with increasing denaturant concentration. Peptide concentration was determined by amino acid analysis. These procedures were described previously [35].

Calculation of Differences in Free Energy of Unfolding, $\Delta\Delta G_u$

Assuming a two-state coiled-coil to random coil denaturation model [37, 38], the molar fraction of folded peptide (f_f) was calculated from the equation $f_f = ([\theta]) - [\theta]_u)/([\theta]_f - [\theta]_u)$, in which $[\theta]$ represents the observed mean residue ellipticity at any particular denaturant concentration and $[\theta]_f$ and $[\theta]_u$ are the mean residue ellipticities of the folded and unfolded states, respectively. The free energy of unfolding (ΔG_u) at each denaturant concentration was calculated from $\Delta G_u = -RT \ln(K_u)$ where K_u is the equilibrium constant of the unfolding process. In the case of the disulphide bridged peptides, this is simply given by $K_u = f_u/f_f = (1 - f_f)/f_f$. The reduced coiled-coils fold via a biomolecular process so that K_u is a function of total peptide monomer concentration (P_t) according to $K_u = 2P_t f_u^2/f_f$ [39]. The free energy of unfolding in the absence of denaturant ($\Delta G_u H_2O$) can be obtained by linear extrapolation according to the equation $\Delta G_u = \Delta G_u H_2O - m$[denaturant] [40]. Plots of ΔG_u versus [denaturant] were made incorporating only data points near the transition midpoint where the greatest linearity was displayed, and a linear least-squares analysis was performed (not shown). However, small errors in the slope term, m, lead to large errors in the extrapolated value of $\Delta G_u H_2O$ [41]. The main interest of this study was to determine the difference in free energy of unfolding between analogues ($\Delta\Delta G_u$), which can be determined accurately by use of the equation given by Serrano and Fersht [8]:

$$\Delta\Delta G_u = ([\text{denaturant}]_{1/2,A} - [\text{denaturant}]_{1/2,B})(m_A + m_B)/2$$

This approach gives the value of $\Delta\Delta G_u$ at the denaturant concentration half-way between the $[\text{denaturant}]_{1/2}$ values of the peptides being compared and is much more accurate for determining small differences in protein stability since errors from extended extrapolations are avoided and linearity between ΔG_u and [denaturant] is assumed over only a small range of [denaturant]. In this study, it was found that for a particular sample under given conditions, the $[\text{urea}]_{1/2}$ is within ± 0.02 M between runs. In addition, the slope m of the ΔG_u versus [urea] plots for the different analogues are all within about 10% of the average for all analogues in both oxidized and reduced data sets. Based on these results, the $\Delta\Delta G_u$ values obtained are reliable to approximately ± 0.05 kcal/mol. This is similar to what was observed by Serrano and Fersht [8].

RESULTS AND DISCUSSION

Peptide Design

The coiled-coils in this study were based on the same native (control) sequence as in our previous papers [35, 36]. The coiled-coil consists of two 35

residue peptide chains, each containing five repeats of the heptad sequence $Q_gV_aG_bA_cL_dQ_eK_f$ where positions **a** and **d**, occupied by Val and Leu respectively, form the hydrophobic face involved in dimerization (Figure 1). Positions **e** and **g** flank the hydrophobic face formed by the **a** and **d** residues and also participate in interhelical interactions, commonly containing charged residues which can form ionic attractions or repulsions across the dimer interface [42–44]. With non-charged residues at the **a,b,c,d,e** and **g** positions of the heptad, this coiled-coil is designed to contain no inter- or intrahelical electrostatic interactions and therefore serves as a good control for studying electrostatic interactions. Lys at position **f** is important for aiding solubility and discouraging aggregation. The peptides were synthesized with an N-terminal acetyl group and a C-terminal amide. A Cys at position 2 in the interface of the coiled-coil allows the formation of an interhelical disulphide bridge. The disulphide bridge ensures the formation of a parallel, in-register coiled-coil and removes the effect of peptide concentration on coiled-coil formation and denaturation behaviour [31]. In addition, disulphide bridges at position 2 of similar synthetic coiled-coil structures have been shown to increase coiled-coil stability dramatically [31, 35, 36, 45].

Substitutions of Glu for Gln residues at various positions along the length of the coiled-coil have been carried out as shown by the sequences in Figure 2 and the effects of these substitutions on coiled-coil stability were determined. The native coiled-coil is represented by N and is shown schematically in Figure 1. The mutants are designated by the position at which Glu substitution is made. For example, E6 refers to a single Glu substitution at position 6. The coiled-coils are further designated with an 'r' if the Cys residues are reduced and an 'x' if the disulphide bridge is formed.

Structural Characterization

We have previously demonstrated that the native sequence, N, used for this study forms a stable two-stranded α-helical coiled-coil both in the presence and absence of the interchain disulphide bridge [35, 36]. The peptide appears highly helical by CD spectroscopy and is two-stranded as determined by size-exclusion HPLC and sedimentation equilibrium ultracentrifugation studies.

The various peptides in this study all have CD spectra similar to that of N, and molar ellipticities at 222 nm in a range from −30,000 to −33,000 deg

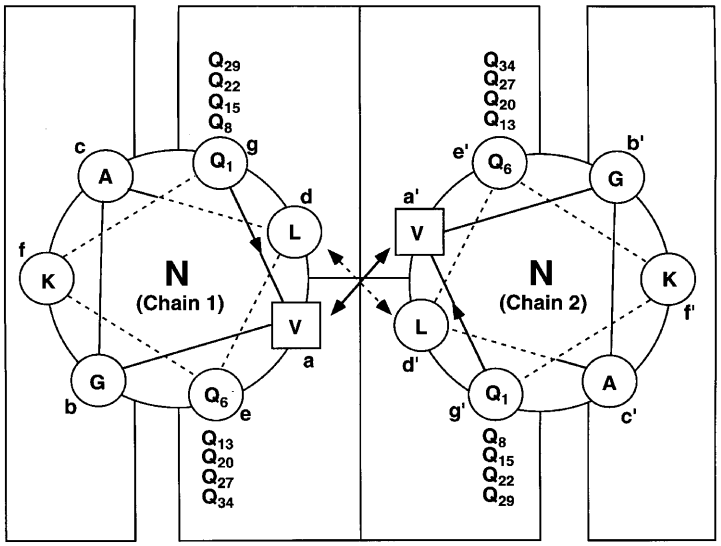

Figure 1 Schematic helical wheel diagram of the 35-residue two-stranded α-helical coiled-coil, in which the coiled-coil is viewed in cross-section from the N-terminus. The interchain **a–a′** and **d–d′** (prime ′ refers to the positions in helix 2) van der Waals interactions between hydrophobic side chains are depicted with arrows. The residues which make up the entire dimerization interface at positions **a**, **d**, **e** and **g** of chain 1 and residues **a′**, **d′**, **e′** and **g′** of chain 2 are shown in the central boxes. The residues in the outer boxes at positions **b**, **c** and **f** of chain 1 and **b′**, **c′** and **f′** of chain 2 do not make any interhelical contacts. In this case, the native (control) coiled-coil, which is referred to as 'N', with Gln residues at all the **e** and **g** positions is shown.

Peptide Name	Amino Acid Sequence
	1 5 10 15 20 25 30 35
	g a b c d e f g a b c d e f g a b c d e f g a b c d e f g a b c d e f
native (N)	Ac-**Q**-C-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-amide
E1	Ac-[**E**]-C-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-amide
E6	Ac-**Q**-C-G-A-L-[**E**]-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-amide
E15	Ac-**Q**-C-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-[**E**]-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-amide
E20	Ac-**Q**-C-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-[**E**]-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-amide
E34	Ac-**Q**-C-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-**Q**-K-**Q**-V-G-A-L-[**E**]-K-amide

Figure 2 Amino acid sequences of the synthetic coiled-coil analogues used in this study. Ac denotes N^{α}-acetyl, and amide denotes C^{α}-amide. 'Native' or 'N' refers to the peptide with glutamine (Q) residues in positions **e** and **g** (highlighted in bold type) of the heptad repeat **abcdefg**. Substitutions in the various analogues are boxed. The peptide nomenclature used in this paper is based on the position of substitutions as detailed under the section on peptide design.

cm^2/dmol in either the reduced or oxidized form, indicative of very high helical content. These results are similar since CD ellipticity measurements and peptide concentration measurements combined are accurate to about ±5%. In addition, all of the analogues, including N, are eluted at very similar times on size exclusion HPLC: between 21.3 and 21.6 min for the reduced form and between 22.3 and 22.7 min for the oxidized form (data not shown). The longer retention times of the oxidized peptides indicate that they are more compact than their reduced counterparts, probably because of the disulphide bridge restricting chain mobility. However, based on these results, it is apparent that no large conformational changes (i.e. changes in oligomerization state) have occurred in the overall structure of the peptides containing the various substitutions.

Stability Studies

Single Glu Substitutions at the N-terminus, C-terminus and Middle of the Helix. The effects on stability of a single Glu for Gln substitution per chain at various positions along the length of the helices making up the coiled-coil are shown in Figure 3. In Figure 3(A), the urea denaturation profiles of the disulphide bridged coiled-coils show that Glu substitutions near the N-terminus (at positions 1 and 6) lead to enhanced stability relative to the control peptide Nx. In contrast, substitutions near the middle of the coiled-coil (positions 15 and 20) and the C-terminus (position 34) lead to reduced stability relative to Nx (see also Table 1). The differing effects of these substitutions on coiled-coil stability is consistent with the charge–helix dipole interaction model. In this model placement of a negatively charged residue near the N-terminus of an α-helix is stabilizing due to favourable interaction with the positive pole of the helix macrodipole, while placement of a negatively charged residue near the C-terminus should have the opposite effect owing to unfavourable interaction with the negative pole of the helix macrodipole [10, 11].

Other factors may also contribute to the change in stability caused by a Gln to Glu substitution. These include intrinsic properties of the amino acid side chains such as helical propensity and hydrophobicity, which have been attributed to the decrease in stability for Gln to Glu substitutions at the **e** and **g** heptad repeat positions of coiled-coils [35, 36, 46]. For example, the coiled-coil E20x, discussed in a previous study [35], has a single Glu substitution for Gln per chain at position 20 (position **e**). This residue is near the middle of the coiled-coil and should have no significant interactions with the helix dipole, but the mutation destabilizes the coiled-coil by 0.2 kcal/mol (0.1 kcal/mol per Glu residue). Similarly, E15x in which Glu is substituted at position **g** of the heptad near the center of the coiled-coil is destabilized to an even greater degree

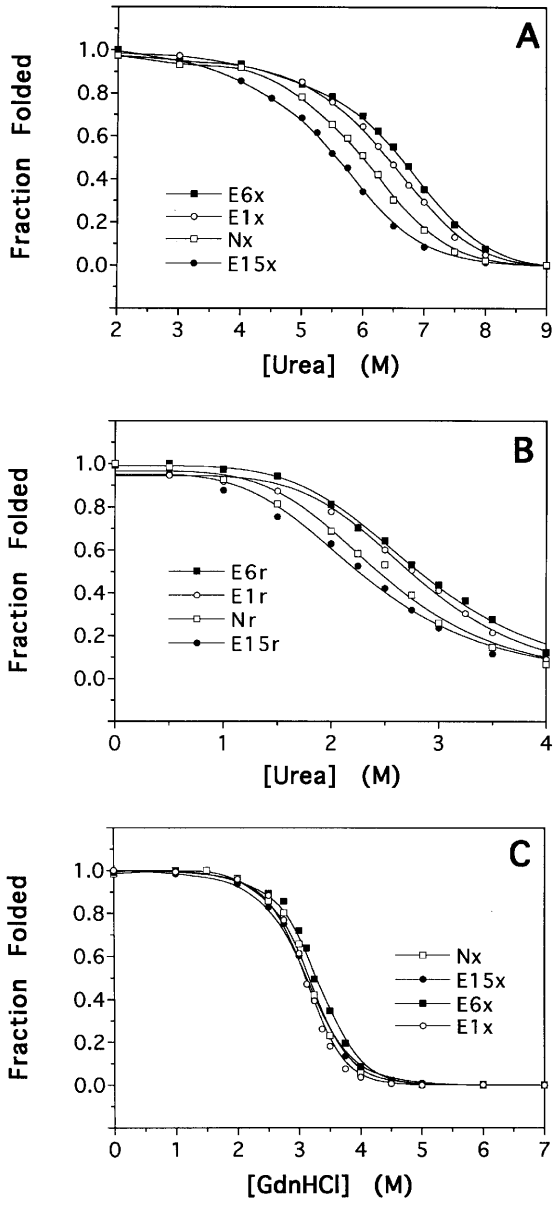

Figure 3 Denaturation profiles at 20 °C in 100 mM KCl, 50 mM PO_4, pH 7 buffer for peptides N, E1, E6 and E15: (**A**) urea denaturation of disulphide bridged peptides; (**B**) urea denaturation of reduced peptides; (**C**) GdnHCl denaturation of disulphide bridged peptides. Buffer contained 5 mM DTT for the reduced samples. The fraction of folded peptide was calculated from the mean residue molar ellipticity at 222 nm, as described under the section on experimental procedures.

(0.38 kcal/mol or 0.19 kcal/mol per Glu substitution, Table 1). In both cases, this can be partly attributed to the lower helical propensity of an ionized Glu compared with Gln [33, 34, 47]. The helical propensity of an amino acid has been shown to affect coiled-coil stability from substitution studies at the outer (**c** and **f**) heptad positions where no interhelical packing contacts occur [48, 49]. In addition, the lower hydrophobicity of an ionized Glu compared to Gln [50–52] will be significant, particularly in the **e** and **g** positions, which can pack against the residues at the hydrophobic core [43] and for which hydrophobicity can profoundly affect coiled-coil stability [53, 54]. However, these effects appear to be much less significant at the ends of the coiled-coil than in the middle [36]. In general, intrinsic properties of the amino acid, such as helical propensity and hydrophobicity, have a lower effect on helicity and stability, as the residue becomes closer to either the N- or C-terminus, presumably because of increased flexibility at the ends [55]. This was shown experimentally in monomeric α-helical peptides with Gly and Ala, which are at opposite ends of the helix propensity scale [28, 56].

Where there is an N-terminal Glu substitution for Gln, as in E1x or E6x, the negative effect on stability of the intrinsic helical propensity and hydrophobicity contributions opposes the positive effect on stability of the proposed charge–helix dipole interaction. Therefore, the observed $\Delta\Delta G_u$ values of + 0.33 and + 0.50 kcal/mol, respectively (Table 1), represent a lower limit for the magnitude of the charge–helix dipole interaction. The actual charge–helix dipole interaction could be estimated by accounting for the intrinsic differences mentioned above through comparisons of peptides E1x and E15x (where the substitution of Glu for Gln at the N-terminus versus the middle of the helix is compared, in both cases, at the equivalent position **g** of the heptad repeat) and peptides E6x and E20x (where the substitution of Glu for Gln at the N-terminus versus the middle of the helix is compared, in both cases, at the equivalent position **e** of the heptad repeat). However, because the intrinsic properties of the amino acids appear, as discussed above, to have less pronounced effects on stability at the ends of an α-helix than in the middle, this estimation probably represents an upper limit for the true value of the charge–helix dipole interaction. This calculated $\Delta\Delta G_u^{\text{helix dipole}}$ (see Table 1, footnote f for details of calculation) is + 0.36 kcal/mol and + 0.35 kcal/mol per Glu residue (per chain) in peptides E1x and E6x, respectively (Table 1). The results indicate that a similar magnitude charge–helix dipole interaction occurs for Glu residues within the first two turns of the helices making up the coiled-coil.

Table 1 pH 7 Stability Data From Urea Denaturation[a]

Peptide[b]	Heptad position	$[Urea]_{1/2}$[c] (M)	m[d]	Observed[e] $\Delta\Delta G_u$ (kcal/mol)	Calculated[f] $\Delta\Delta G_u^{helix\ dipole}$ (kcal/mol)
Nx	–	6.0	0.84	–	–
E1x	**g**	6.4	0.83	+0.33	+0.36
E6x	**e**	6.6	0.81	+0.50	+0.35
E15x	**g**	5.55	0.86	−0.38	–
E20x	**e**	5.8[g]	1.0	−0.19	–
E34x	**e**	5.6	0.89	−0.35	−0.08
Nr	–	2.5	1.47	–	–
E1r	**g**	2.8	1.31	+0.42	+0.35
E6r	**e**	2.8	1.23	+0.41	+0.35
E15r	**g**	2.3	1.38	−0.29	–
E20r	**e**	2.3	1.30	−0.28	–
E34r	**e**	2.2	1.50	−0.45	−0.08

[a] Data were collected at 20 °C in a 50 mM PO_4, 100 mM KCl buffer.
[b] The sequences are given in Figure 1, and nomenclature is described in the section on peptide design.
[c] The $[urea]_{1/2}$ is the concentration of urea at which the peptide is 50% unfolded, as determined by the decrease in molar ellipticity at 222 nm with increasing denaturant concentration.
[d] m is the slope of the assumed linear relationship between ΔG_u and the denaturant concentration.
[e] $\Delta\Delta G_u$ is the observed difference in the free energy of unfolding between the analogue and the native coiled-coil calculated from the $[urea]_{1/2}$ values as described in the section on experimental procedures. A positive $\Delta\Delta G_u$ value indicates that the analogue is more stable than the native coiled-coil.
[f] The free energy change for the charge–helix dipole effect per single Gln to Glu substitution, $\Delta\Delta G_u^{helix\ dipole}$ is calculated as follows:

$$\text{Total } \Delta\Delta G_u^{helix\ dipole} = \Delta\Delta G_u^{observed} - n(\Delta\Delta G_u^{helix\ prop.,\ hydrophobicity})_{\mathbf{e}} - m(\Delta\Delta G_u^{helix\ prop.,\ hydrophobicity})_{\mathbf{g}}$$

where n and m are the number of Gln to Glu substitutions at position **e** and **g**, respectively, of the heptad; $\Delta\Delta G_u^{helix\ prop.,\ hydrophobicity}$ represents the intrinsic effect on stability from a single Gln to Glu substitution and equals −0.1 kcal/mol at position **e** of the heptad and −0.20 kcal/mol at position **g** of the heptad based on the stabilities of E20x and E15x, respectively, versus Nx at pH 7.

Example: total $\Delta\Delta G_u^{helix\ dipole}$ for E1x = + 0.33 − 2(−0.20) = + 0.73 kcal/mol. This gives a charge helix dipole effect of 0.73/2 = + 0.36 kcal/mol per Glu.
[g] Data taken from Kohn *et al.* [35].

The substitution of a Glu residue in each chain of the coiled-coil may lead one to question whether these residues (for example at positions 1 and 1′ in E1x) are in close enough proximity to each other to exert a repulsive destabilizing influence, which would therefore lead to an underestimation of the stabilizing interaction of these Glu residues with the helix dipole. However, as shown in Figure 1, the substituted residues at positions **g** and **g**′ or **e** and **e**′ lie on opposite sides of the dimer interface and therefore interactions between them should be minimal.

Following a C-terminal substitution, as in E34x, the stability decrease due to decreased intrinsic helical propensity and hydrophobicity upon a Glu for Gln substitution should be in addition to the predicted unfavourable charge–helix dipole interaction introduced by a negatively charged residue at the C-terminus; therefore, the overall stability decrease would be predicted to be greater for a C-terminal Glu substitution than one at a position near the middle of the helix. Indeed, the observed $\Delta\Delta G_u$ for E34x is −0.35 kcal/mol compared with −0.19 kcal/mol for E20x in which Glu is substituted at the comparable position **e** of the heptad repeat (Table 1); therefore the value of the calculated $\Delta\Delta G_u^{helix\ dipole}$ is −0.08 kcal/mol per Glu (per chain). In the case of this C-terminal substitution, the observed $\Delta\Delta G_u$ of −0.18 kcal/mol per Glu (−0.35 kcal/mol total) represents the upper limit of the charge–helix dipole interaction since accounting for the intrinsic effects of the substitution on stability at the C-terminus reduces the estimated dipole interaction, while at the N-terminus it has the opposite effect of increasing the predicted helix dipole interaction.

The unfavourable interaction between Glu and the helix dipole estimated for E34x is significantly smaller than the favourable charge–helix dipole interactions calculated for E1x and E6x. This would

suggest that charge–helix dipole interactions at the N-terminus are more important for protein stability and is consistent with the statistical observation that charge distribution in α-helices is more commonly owing to preferential distribution of negative charges at the N-terminus than of positive charges at the C-terminus. This is illustrated by the observation among a large sampling of proteins that the average charge of the side chains near the C-terminus is $+0.34$ but near the N-terminus is -0.64 [3]. One potential reason for less dramatic effects at the C-terminus is that in an α-helix the C^{α}–C^{β} bonds of the side chains are oriented towards the N-terminus, so that the side chains near the N-terminus interact strongly with the positive pole of the helix dipole, while those at the C-terminus point away from the C-terminus and as a result may interact less strongly with the helix dipole [25].

For the reduced coiled-coils (Figure 3(B)), the relative stabilities remain generally the same as for the oxidized coiled-coils. Those containing N-terminal Glu substitutions, E1r and E6r, are more stable than the control Nr, while those with Glu substitutions in positions 15, 20 and 34 are less stable than Nr (Table 1). The values of the $\Delta\Delta G_u^{\text{helix dipole}}$ of $+0.35$, $+0.35$ and -0.08 kcal/mol for E1r, E6r and E34r, respectively, are essentially identical to those obtained for their oxidized counterparts (Table 1). The results for the reduced peptides therefore show that the effect of charge–helix dipole interactions are independent of the presence of the 2-2′ disulphide bridge between the chains of the coiled-coil and merely add to the large effect of the disulphide bridge on coiled-coil stability (indicated by the large increase in $[\text{denaturant}]_{1/2}$ between oxidized and reduced forms and the estimated $\Delta\Delta G_u$ value of 3–4 kcal/mol [35, 36, 57]).

Interestingly, while the stabilities of the oxidized coiled-coils E15x and E20x are substantially different (Table 1), in the reduced form these analogues display equal stability. The difference in these peptides is the substitution of Glu for Gln at heptad repeat position **g** in E15x and at position **e** in E20x. These positions are non-equivalent, with the **g** position packing primarily against the following **a**′ and the **e** position packing against the preceding **d**′ of the other helix [43]. The results for the oxidized peptides suggest that the inferior packing of Glu relative to Gln is more apparent at position **g**, where it packs against Val at position **a**′ rather than at position **e** where it packs against Leu at position **d**′. In the reduced form, the coiled-coil is much more flexible (less compact, as indicated by lower retention times on size-exclusion HPLC) than in its oxidized form and may adjust its structure and interchain packing to accommodate the destabilizing effect of the Glu substitution, such that substitutions at positions **g** and **e** appear similar. The oxidized coiled-coil could be more restricted in structure and less capable of reorganizing its interface packing in response to substitutions, so that the differences in positions **g** and **e** are more apparent. Similar results were observed in the hydrophobic core **a** and **d** positions, where Ala substitutions for Leu were more destabilizing at position **a** than **d** in coiled-coils containing an interchain disulphide bridge (at either the N- or C-terminus), but Ala mutations had the same effect on stability at positions **a** and **d** in reduced coiled-coils [58].

Salt Effects on the Stability of Glu-substituted Coiled-coils. One of the basic tests for determining whether a stabilizing (or destabilizing) substitution is due to electrostatic effects is its ionic strength dependence. Salt is generally believed to screen electrostatic interactions, so raising the ionic strength should reduce stabilizing electrostatic effects. However, the effects of ionic strength on electrostatic interactions in proteins can vary, and indeed electrostatic interactions are not always reduced by increased ionic strength, as indicated by computational [59] and experimental results [60, 61]. The coiled-coils in the present study offer a good model system in which to examine the screening effects of salts on charge–helix dipole interactions at protein surfaces.

The effects of ionic strength on the stability of the coiled-coils have been determined by varying the concentration of KCl from 0 to 3 M. As indicated in Figure 4(A), the $[\text{urea}]_{1/2}$ values of the coiled-coils Nx, E1x and E15x are dramatically increased by an increased ionic strength (increased KCl concentration). As suggested by Mo *et al.* [62] for tropomyosin and by our previous studies on model coiled-coils [36, 60], this stabilization is most probably due to an increase in the hydrophobic effect induced by the added KCl [63]. It may also involve screening of unfavourable electrostatic interactions between helix dipoles caused by parallel packing of the helices, which has been estimated by computational studies [64, 65] and experimental data [66] to account for a 0.5–3 kcal/mol difference in stability of parallel and antiparallel four helix bundles.

In addition, the $[\text{urea}]_{1/2}$ value of E1x, which at low ionic strength is higher than that of N_x pre-

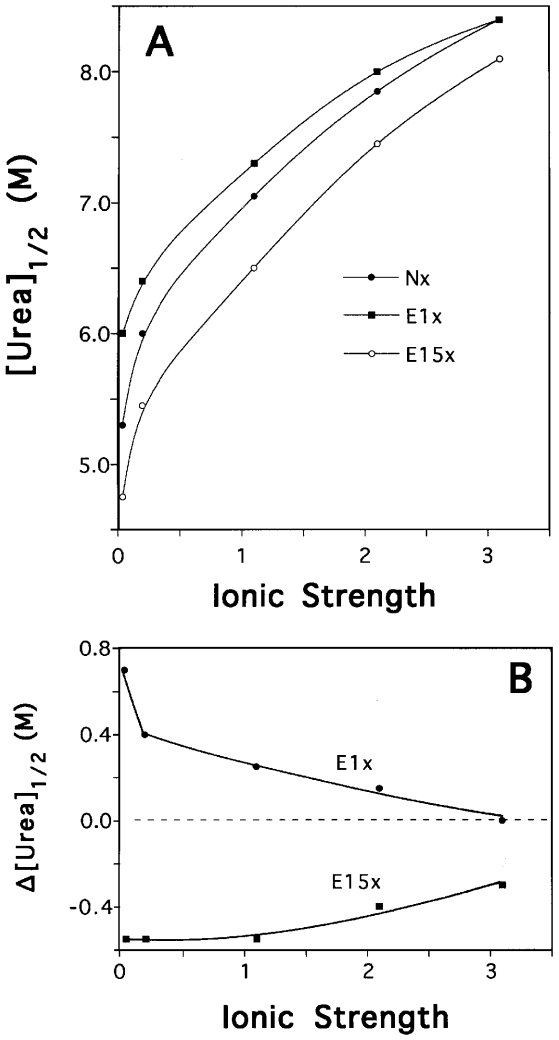

Figure 4 Salt effects on coiled-coil stability. (**A**) Effect of ionic strength on the stabilities (expressed as the $[urea]_{1/2}$ or concentration of urea at which the coiled-coil is 50% unfolded) of the disulphide bridged coiled-coils Nx, E1x and E15x at 20 °C in sodium phosphate, pH 7 buffer. Ionic strength was varied by increasing the KCl concentration from 0 to 3 M. In addition, sodium phosphate concentration was 20 mM for the lowest ionic strength data and 50 mM for the remaining data points. (**B**) $\Delta[urea]_{1/2}$ for E1x and E15x versus Nx as a function of ionic strength.

sumably owing to charge–helix dipole interactions, becomes equal to that of Nx at a high KCl concentration of 3 M (Figure 4(A)). This indicates that the charge–helix dipole interactions are screened at very high salt concentrations and is consistent with the results of previous studies [17–19, 21, 67, 68], which demonstrate large concentrations of NaCl (3–5 M) are required to screen interactions fully between charged side chains and the N-terminal pole of the helix dipole. In contrast, E15x, in which the substituted Glu residue should not interact significantly with the helix dipole as a result of being in the middle of the helix, is much less affected by KCl concentration in terms of its stability relative to Nx. This is clearly demonstrated in Figure 4(B), where the $\Delta[urea]_{1/2}$ values of E15x and E1x relative to Nx are plotted for increasing ionic strength. For E1x, $\Delta[urea]_{1/2}$ decreases dramatically from 0.7 to 0.25 upon changing the ionic strength from 0.04 to 1.1; whereas, for E15x the $\Delta[urea]_{1/2}$ value is unchanged below 1 M KCl (ionic strength 1.1) and is still -0.3 even at 3 M KCl. The inability of KCl to fully remove the $[urea]_{1/2}$ difference between Nx and E15x supports the conclusion that the lower stability of E15x is due to an 'intrinsic' destabilization from the substituted residue rather than a charge–charge or charge–dipole interaction.

The effect of GdnHCl on the charge–helix dipole interactions was also investigated. GdnHCl, as an ionic species, has been shown to behave like a salt in screening interchain electrostatic interactions involving Lys and Glu residues in coiled-coils [35, 36, 38, 60, 69]. As such, GdnHCl would be expected to screen the charge–helix dipole interactions in a similar manner to KCl. The GdnHCl denaturation profiles for the coiled-coils containing Glu substitutions at the N-terminus are essentially identical to Nx (Figure 3(C)), with $[GdnHCl]_{1/2}$ values around 3.1–3.2 M, indicating that GdnHCl has masked the interactions between charged residues and the helix dipole. These results emphasize the importance of choosing the appropriate denaturant to measure the effect of ionic interactions on protein stability. For example, in the recent study of Zhukovsky *et al.* [70], it was concluded that the charge–helix dipole stabilization provided by Glu74 at the N-terminus of helix 2 in human growth hormone is negligible. However, the authors used GdnHCl denaturation to reach this conclusion which, as the present results show, would lead to an incorrect conclusion as to the importance of the charge–helix dipole interaction. In addition, the stability of E15x is equal to that of Nx from GdnHCl denaturation (Figure 3(C)), indicating that GdnHCl 'screens' the intrinsic destabilization of E15x, which 3 M KCl present during urea denaturation was unable to do (Figure 4(A)).

Effects of Glu Substitutions on Coiled-coil Stability at Low pH. Typically, evidence that a particular residue is involved in a stabilizing or destabilizing electrostatic interaction lies in the pH dependence of its effect on stability. In the absence of other

effects, a substitution which introduces a favourable interaction of an acidic side-chain group with the N-terminal end of the helix dipole is expected to have no effect on stability at low pH where the Glu side chain is protonated.

For peptides E1x and E6x, the net effect of the substitution at neutral pH is an increase in stability due to the charge–helix dipole interaction, despite the slightly destabilizing effects of the lower intrinsic helical propensity and hydrophobicity of ionized Glu versus Gln. At pH 3, the Glu side-chain carboxylate should be mostly protonated, and the strength of the charge–helix dipole interaction should be correspondingly reduced. Thus, E1x displays a lower $\Delta\Delta G_u$ (+ 0.09 kcal/mol, Table 2) at pH 3 than at pH 7 (+ 0.33 kcal/mol, Table 1). Protonation of Glu 1 therefore appears to reduce the stabilizing influence of the Gln to Glu substitution, supporting the conclusion that the effect is electrostatic in nature. In contrast, E6x has the same $\Delta\Delta G_u$ at pH 3 and 7 (Tables 1 and 2). In this case, the expected loss of stabilization from the proposed charge–helix dipole interaction due to protonation of Glu 6 appears to be offset by increased intrinsic coiled-coil stabilizing properties of Glu upon protonation. As mentioned above, the substitution of a Glu residue for Gln at pH 7, as in E20x, reduces stability owing to the reduced helical propensity and hydrophobicity of ionized Glu relative to Gln. In contrast, at low pH, Glu 20 is mostly protonated and E20x is more stable than Nx [35]. E20x has a $\Delta\Delta G_u$ of + 0.71 kcal/mol at pH 3, suggesting each Gln to Glu substitutions results in 0.36 kcal/mol stabilization. Both the helical propensity [47, 71] and the hydrophobicity [51, 52] of protonated Glu are higher than for Gln, making protonated Glu a better intrinsically stabilizing residue than Gln. The stability results at pH 3 (Table 2) show the hydrophobic and helical propensity contribution that a protonated Glu makes to stability varies with the position in the peptide with the largest effect in the centre of the coiled-coil (E15x). The results with E15x and E20x show that protonated Glu has significant stabilizing effects at both heptad repeat positions **e** and **g**, but that the stabilization is slightly larger at position **g**. Similarly, the destabilizing effect of ionized Glu was found to be greater at position **g** (in E15x) than at position **e** (in E20x). Overall, the results with Glu substitutions at low pH indicate that the charge–dipole interactions are removed but replaced by other stabilizing effects of the protonated Glu residue.

Table 2 pH 3 Stability Data From Urea Denaturation

Peptide[a]	$[Urea]_{1/2}$ (M)	*m*	Observed $\Delta\Delta G_u$ (kcal/mol)
Nx	6.0	0.91	–
E1x	6.1	0.87	+ 0.09
E6x	6.6	0.79	+ 0.51
E15x	7.25	0.79	+ 1.06
E20x	6.8[b]	0.87	+ 0.71
E34x	6.4	0.81	+ 0.34

[a] The sequences of the peptides are shown in Figure 1.
[b] Data taken from Kohn *et al.* [35].
All symbols were described in Table 1 and under Materials and Methods. Data were collected at 20 °C in a 50 mM PO_4, 100 mM KCl buffer.

Charge–Helix Dipole Interactions Versus Side-chain to Main-chain Hydrogen Bonding. Because both Glu and Gln side chains could act as hydrogen bond acceptors for the main chain NH group of the $i + 3$ residue (the fourth residue), it is possible that a difference in hydrogen bond strength could account for the stability increase observed for E1x. Gln should serve as a good control for Glu because the hydrogen bonding groups of the two amino acids have similar geometry. The charged side chain of Glu could form a stronger hydrogen bond to the main chain because charged hydrogen bond acceptors usually form stronger hydrogen bonds [72]; however, as pointed out by Tidor [14], the ionized Glu must pay a greater desolvation penalty than Gln in order to hydrogen bond to the backbone owing to its charged character, which should roughly offset its stronger hydrogen bonding potential. A recent comprehensive report in which the N- and C-cap propensities of all 20 amino acids were determined [6] shows that Gln is the worst N-capping residue while Asn is the best. Glu was also poorly stabilizing relative to other acidic residues, its pK_a value being raised at the N-terminus, instead of reduced as expected. However, an observed increase in helical content with increased pH was ascribed to stabilization due to interaction of the negatively charged Glu side chain with the positive, N-terminal end of the helix macrodipole. In another study, Lumb and Kim [73] measured the pK_a of two Glu side chains involved in predicted interhelical Lys-Glu ion pairs in the GCN4 coiled-coil. No pK_a change was observed upon coiled-coil formation for one Glu while an increased pK_a was observed for the other. Based on these results, it was concluded that no stabilization due to the ion pair was apparent. The participation

of an acidic side chain in favourable polar/electrostatic interactions need not necessarily be accompanied by a reduced pK_a [74].

Because both Gln and Glu appear to be weak N-capping (hydrogen bonding) residues, the increased stability resulting from a Gln to Glu substitution is probably electrostatic in nature, particularly in light of the results with E6x in which the substituted Glu residue is not at an N-capping position but stabilizes the coiled-coil to a similar extent as it does in E1x. Similarly, the C-terminal helix capping propensities were found to be essentially identical for Glu and Gln [6].

Further, crystallographic studies of mutant lysozymes with Asp substitutions at the N-terminus of α-helices suggested a stabilization due to electrostatic interaction rather than to precise hydrogen bonding between the substituted Asp side chain and the helix backbone [24, 25]. In addition, Chakrabartty *et al.* [75] reported that an N-terminal acetyl group is very effective in forming the helix-capping hydrogen bond between its carbonyl and the $i + 3$ NH group. This interaction is equivalent to the best helix-capping side chain, Asn [6], and cancels the effect on helicity of differences in N-cap propensity of the N-terminal amino acid. This result suggests that in the acetylated peptides employed in the present study, the acetyl group would negate any possible difference in hydrogen bonding effects of the residues substituted at position 1.

The above considerations suggest that precise hydrogen bonding from amino acid side chains to the helix backbone is an unlikely explanation for the stability changes observed in this study. The so-called 'charge–helix dipole' effect employed above to account for these observations could result from interaction of the substituted charged side chain with either the classical helix macro-dipole that results from alignment of peptide bond dipoles or the unfulfilled main-chain polar groups at either end of the helix. The results of the current study cannot distinguish between these possibilities.

CONCLUSIONS

This work has shown that the substitution of Glu for Gln residues in the **e** and **g** heptad repeat positions of a stable two-stranded α-helical coiled-coil can have a significant effect on the stability of the coiled-coil, the direction of which depends on the position along the coiled-coil. At the N-terminus, Glu substitution increases stability, which can be explained by the charge-helix dipole interaction theory. This stability increase is modulated by ionic strength and pH changes, serving as evidence that the effect is electrostatic in origin. The apparent magnitude of these interactions is approximately 0.35 kcal/mol per Glu substitution. Various other studies of charge–helix dipole interactions listed in the introduction have estimated magnitudes for the charge–helix dipole interaction in either isolated helical peptides or globular proteins in the range of 0.3–2.1 kcal/mol (see for example: [8, 19, 22, 23, 25–27]. The wide range of values suggests that the apparent charge–helix dipole interaction is highly dependent on the environment and the amino acid being studied. For example, while Lys appears to interact weakly with the helix dipole when present at the C-terminus of an α-helix, His appears to interact much more strongly at the C-terminus [22, 23, 61]. While the values obtained in the coiled-coil model are at the low end of this range, they are quite close to the results obtained by Eijsink *et al.* [26] in which replacement of a Lys at the N-terminus of a helix in *Bacillus subtilis* neutral protease by Ser increased stability of 0.3 kcal/mol and replacement of the Lys with Asp increased stability by 0.6 kcal/mol. The small effect of the substitution on the stability of the coiled-coil may be related to the inherent stability of the model. A less stable coiled-coil may exhibit a larger apparent charge–helix dipole interaction. In contrast to N-terminal substitution, Glu substitution in the middle of the helix leads to a loss of stability due to the lower helical propensity and hydrophobicity of Glu relative to Gln for which it is substituted. The intrinsic nature of this effect is confirmed by the inability of salt to screen it. At the C-terminus a larger destabilization occurs (compare E20 and E34, both **e** positions) due to both intrinsic destabilization and unfavourable charge–helix dipole interactions, the opposite of what occurs at the N-terminus.

This initial positional dependence study has concentrated only on the effects of Glu for Gln substitutions for two major reasons: Glu has been the focus of studies in our previous work on interhelical repulsions in coiled-coils [35, 36] from which the current work originated, as well as the fact that Glu is the most commonly occurring residue at the **e** and **g** positions of coiled-coils [76]. For example, 29 of 80 residues in the **e** and **g** positions of rabbit skeletal α-tropomyosin are Glu, the next most plentiful being Lys with 16 occurrences. The generality of charge–helix dipole effects

in coiled-coils should be further established through additional experiments incorporating other amino acids.

These results are important for coiled-coil (leucine zipper) design, which is potentially useful in transcription regulation and other biotechnological applications [32, 77–79]. In order to design coiled-coils successfully for various applications, the effects of substitutions at all positions of the coiled-coil on dimerization stability and specificity must be quantified. For example, the recent study of Krylov and colleagues [78], in which a scale for the interhelical **g–e′** interactions between various residues was presented, suggested that the calculated $\Delta\Delta G_u$ values per **g–e′** pair are nearly independent of the heptad in which they occur. However, while this approximation may be the case for many amino acid combinations in the **g–e′** pair, the results from our studies with Glu substitutions illustrate that this will not always hold true. Specifically, while a Glu-Glu pair is destabilizing in the central heptad of the 35 residue coiled-coil, it is actually stabilizing in the N-terminal heptad [36], illustrating that end effects due to charge–helix dipole interactions can in some cases significantly affect overall protein stability.

In addition, it is clear from this and other studies [80] that placement of charged residues near the ends of helices can be used advantageously to alter the stability of engineered proteins in general.

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