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Design and characterization of the anion-sensitive coiled-coil peptide

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Abstract

As a model for analyzing the role of charge repulsion in proteins and its shielding by the solvent, we designed a peptide of 27 amino acid residues that formed a homodimeric coiled-coil. The interface between the coils consisted of hydrophobic Leu and Val residues, and 10 Lys residues per monomer were incorporated into the positions exposed to solvent. During the preparation of a disulfide-linked dimer in which the two peptides were linked in parallel by the two disulfide bonds located at the N and C terminals, a cyclic monomer with an intramolecular disulfide bond was also obtained. On the basis of CD and ^{1}H-NMR, the conformational stabilities of these isomers and several reference peptides were examined. Whereas all these peptides were unfolded in the absence of salt at pH 4.7 and 20 °C, the addition of $NaClO_4$ cooperatively stabilized the α-helical conformation. The crosslinking of the peptides by disulfide bonds significantly decreased the midpoint salt concentration of the transition. The ^{1}H-NMR spectra in the presence of $NaClO_4$ suggested that, whereas the disulfide-bonded dimer assumed a native-like conformation, the cyclic monomer assumed a molten globule-like conformation with disordered side chains. However, the cyclic monomer exhibited cooperative transitions against temperature and Gdn-HCl that were only slightly less cooperative than those of the disulfide-bonded parallel dimer. These results indicate that the charge repulsion critically destabilizes the native-like state as well as the molten globule-like state, and that the solvent-dependent charge repulsion may be useful for controlling the conformation of designed peptides.

Keywords: α-helix; anion binding; leucine zipper; molten globule; protein folding

The stability of the folded state of proteins is determined by a balance of various forces that favor the compactly folded conformation or the extended unfolded conformation (Dill et al., 1995; Makhatadze & Privalov, 1995). Whereas the major forces favoring the folded state are hydrophobic interactions, specific polar interactions, and van der Waals interactions, those favoring the unfolded state are conformational entropy, hydration of polar groups, and charge repulsion. Among these forces, electrostatic repulsion is the most classical factor proposed to be important in determining the stability of proteins (Linderstrom-Lang, 1924; Tanford & Kirkwood, 1957). However, the exact role of electrostatic repulsion is ambiguous because it is determined not only by the properties of the protein itself, but also by complex interactions with ions in solution (Yang & Honig, 1993; Nakamura, 1996).

A series of studies conducted by Goto and coworkers (Goto et al., 1990a, 1990b, 1991, 1993; Goto & Nishikiori, 1991; Goto & Fink, 1994) indicated that the net charge repulsion critically destabilizes intermediate conformational states such as the molten globule state, which was defined originally as a compact denatured state with significant native-like secondary structures but with largely disordered side chains. It should be noted that the exact picture of the molten globule state may be very different from the original definition, and that there is some controversy with continued use of the term "molten globule state" (see Discussion). These considerations notwithstanding, although the conformation and stability of the molten globule state do differ from the original definition, some of the properties are still similar, and therefore we use the term molten globule in this paper.

Whereas several proteins, including cytochrome *c* and apomyoglobin, are unfolded at pH 2 in the absence of salt by charge repulsion between the positive groups, addition of salt stabilizes the molten globule state. Added anions, either from salt or acid, bind to the positive charges, shielding the charge repulsion, and this results in manifestation of the hydrophobic interactions stabilizing the molten globule state. The order of effectiveness of var-

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Abbreviations: KL26, a model peptide consisting of 26 residues; mKL26red, reduced form of KL26; dKL26ox, dimeric oxidized form of KL26 in which two peptides are linked in parallel by a disulfide bond; KL27, a model peptide consisting of 27 residues; mKL27red, reduced form of KL27; dKL27ox, dimeric oxidized form of KL27 in which two peptides are linked in parallel by two disulfide bonds; mKL27ox, monomeric oxidized form of KL27 in which two Cys residues at the N and C terminals form a disulfide bond; Gdn-HCl, guanidine hydrochloride.

ious anions is consistent with the electroselectivity series of anions toward anion-exchange resins (Goto et al., 1990b; Goto & Hagihara, 1992), indicating that direct anion binding, not the general ionic strength effect, causes the conformational transition. However, because the folded states examined so far are the fluctuating molten globule or related states (see Discussion), it is not clear if such effects are common to the rigid native states.

Leucine zipper peptides form a tightly packed parallel coiled-coil structure and provide an excellent model for studying the conformation and stability of the native state of proteins (O'Shea et al., 1991; Zitzewitz et al., 1995). Many studies have addressed the factors stabilizing the specific coiled-coil structure. The role of electrostatic interactions, i.e., charge repulsion and salt bridges, is one of the most important topics covered by such studies. The repulsion between charges of the same kind destabilizes the homodimeric coiled-coil and is important for specific formation of the heterodimeric coiled-coil (O'Shea et al., 1992; Kohn et al., 1995a, 1995b; Lavigne et al., 1995). On the other hand, it is a point of argument whether the salt bridge contributes to stabilizing the coiled-coil (Zhou et al., 1994a; Kenar et al., 1995; Kohn et al., 1995a, 1995b; Lumb & Kim, 1995; Lavigne et al., 1996; Yu et al., 1996). In these studies, the salt effects generally have been treated in terms of ionic strength.

We considered that the native-like characteristics of the leucine zipper coiled-coil would be useful for examining the role of charge repulsion and its response to salt conditions. Therefore, we designed a model peptide of 27 amino acid residues in which the basic motif of the coiled-coil interface mimicked the leucine zipper, but several Lys residues were incorporated so as to introduce salt-dependent charge repulsion. The dimeric parallel peptide linked by two disulfide bonds, i.e., dKL27ox, was unfolded in the absence of salt, but, with addition of $NaClO_4$ at concentrations as low as 5 mM, it folded cooperatively into a native-like coiled-coil conformation. These results indicate that, as observed with several molten globule states, charge repulsion can unfold the native-like rigid state and that the conformation can be controlled critically by a very low concentration of salt through the direct binding mechanism. During the preparation of the parallel dimer linked by two disulfide bonds (dKL27ox), we obtained a monomeric circular species with an intramolecular disulfide bond (mKL27ox). Interestingly, this species assumed a molten globule-like helical state in the presence of salt, exhibiting salt-dependence similar to that of dKL27ox.

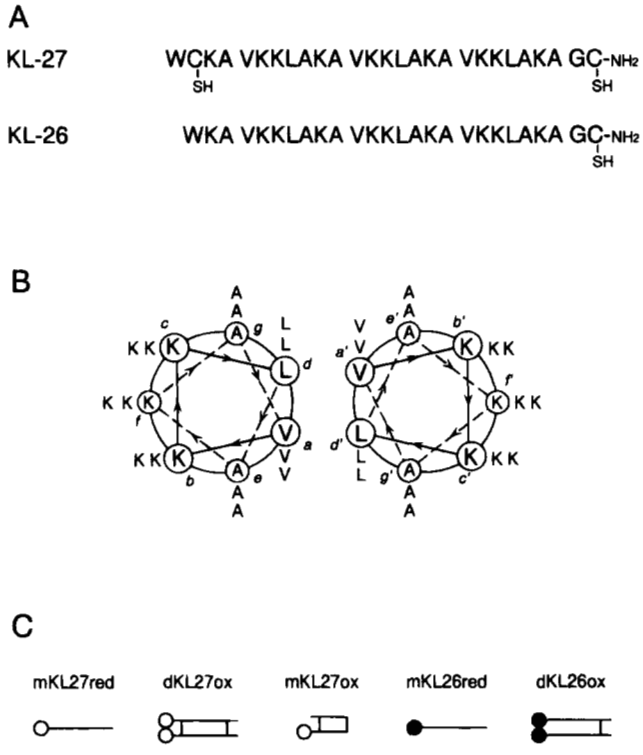

Fig. 1. A: Amino acid sequences of the model peptides, KL26 and KL27. **B:** Helical wheel representation of the region of the three-heptad repeat. **C:** Schematic representation of various conformers of peptides. Circles in C represent Trp residues at the N terminal.

Results

Design and preparation of the model peptides

The peptides KL26 and KL27 were designed, on the basis of a leucine zipper motif, e.g., GCN4, to form a parallel coiled-coil structure stabilized mainly by the hydrophobic interactions between Val and Leu at the helical interface (Fig. 1A,B). Peptides KL26 and KL27 consist of 26 and 27 residues, respectively, and have three internal heptad repeats (*abcdefg*). In both peptides, Val and Leu occupy the *a* and *d* positions, respectively, as in the leucine zipper motif (O'Shea et al., 1989, 1991). The positions *e* and *g* are occupied by Ala, which has a high helical preference (Zhou et al., 1994b; Chakrabartty & Baldwin, 1995). The positions not involved in the dimer interface, *b*, *c*, and *f*, are occupied by Lys in order to introduce anion-dependent control of the electrostatic repulsion (Goto & Aimoto, 1991). Accordingly, the specific intrachain repulsions occur between charged residues *i* and *i* + 3 or *i* and *i* + 4. In addition, under the low-salt conditions, the high net charge destabilizes the folded states nonspecifically by the long-range repulsive forces (Hagihara et al., 1992). To stabilize the coiled-coil, we introduced two disulfide bonds at the N and C terminals. As a reference, we prepared KL26, in which one Cys residue was incorporated into the C terminal.

The oxidization of the Cys residues of KL26 resulted in one peak on HPLC, whose retention time was longer than that of the reduced form (mKL26red). The mass spectrum showed that the peak contained the disulfide-bonded dimer, i.e., a parallel dimer connected at the C terminus (dKL26ox). In the case of KL27, which has two cysteine residues at the N and C terminals, the oxidation produced two peaks on HPLC (Fig. 2). At first we thought that the two peaks corresponded to the parallel and antiparallel dimers, respectively. However, the molecular masses of the two peaks indicated that, whereas peak a was a dimer, peak b was a monomer. Therefore, it became evident that peak b was a monomeric circular species (mKL27ox) in which a disulfide bond was formed intramolecularly between the N-terminal and C-terminal Cys residues (Fig. 1C).

On the other hand, peak a was either a parallel dimer or an antiparallel dimer. Because KL27 has only one Trp at its N terminal and no Tyr, tryptic fragments with absorption at 280 nm are derived from the N-terminal portion. For the parallel dimer, the fragment with absorption at 280 nm should consist of only the N-terminal fragment, whereas for the antiparallel species, the fragment with absorption at 280 nm consists of the N- and C-terminal fragments. To distinguish the parallel and antiparallel dimers, the

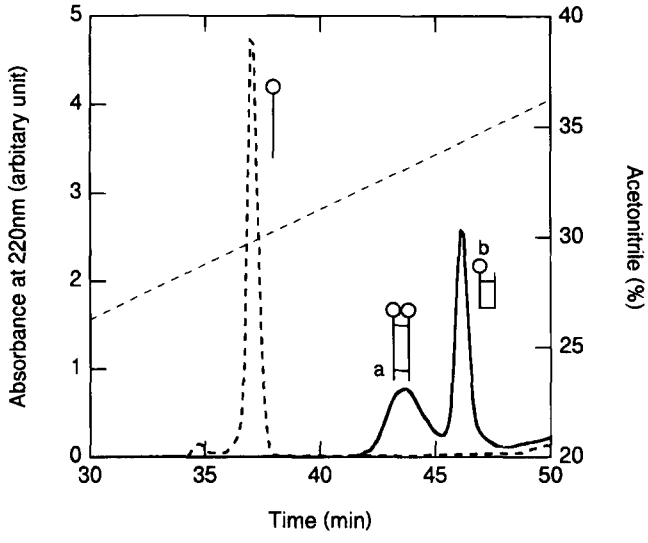

Fig. 2. Reversed-phase HPLC patterns of the reduced (dashed line) and oxidized (solid line) forms of KL27, detected by the absorbance at 220 nm. The reduced sample contained mKL27red and the oxidized sample contained the parallel dimer (dKL27ox) and the cyclic monomer (mKL27ox). Acetonitrile concentration was also shown (dashed line).

peptide prepared from peak a was digested with trypsin at pH 8.6 for 24 h and the digests were applied to a C_{18} HPLC column. Only one peak detected by the absorption at 280 nm was collected, and its amino acid sequence was determined. It was found that the fragment had a unique sequence of Trp-Cys-Lys, indicating that the peptide from peak a is a parallel dimer, i.e., dKL27ox.

The proportion of the parallel dimer (dKL27ox) and circular monomer (mKL27ox) depended on the conditions used for oxidation. The increase in peptide concentration increased the proportion of the parallel dimer, although the cyclic monomer always appeared to some extent. On the other hand, we did not observe any other peaks, indicating that the formation of antiparallel dimer and other oligomeric species was unfavorable.

With these oxidized isomers (dKL27ox, mKL27ox, and dKL26ox) and their reduced forms (mKL27red and mKL26red), we studied their conformation and response to salt conditions.

$NaClO_4$-induced conformational change

Figure 3A and B shows the far-UV CD spectra of dKL27ox and mKL27ox, respectively, in the presence of various concentrations of $NaClO_4$ at pH 4.7. We used $NaClO_4$ to examine the salt-induced conformational transition, because its effect in inducing the anion-dependent conformational transition is stronger than other salts such as NaCl, and its absorption in the far-UV region is very low (Goto et al., 1990b; Goto & Aimoto, 1991; Goto & Fink, 1994). At this pH value, all Lys residues are expected to be charged positively. Whereas the spectrum in the absence of salt showed that the peptide has little ordered secondary structure, the addition of millimolar concentrations of $NaClO_4$ transformed the spectrum to one with minima at 222 and 208 nm, showing that the α-helix was stabilized. Other species also showed similar spectral changes (data not shown). These findings indicate that, although the peptides were unfolded in the absence of salt because of the charge repulsion, the addition of salt shielded the repulsion and, consequently, the α-helix was stabilized. The spectra at different salt concentrations showed an isodichroic point at 204 nm, being consistent with a two-state transition between the unfolded and α-helical forms.

Effects of disulfide bonds

Figure 4A shows the dependence on $NaClO_4$ concentration of the ellipticity at 222 nm of various KL26 and KL27 derivatives at a peptide concentration of 0.1 mg/mL. Conformational transitions similar to those of dKL27ox and mKL27ox were also observed for mKL27red, mKL26red, and dKL26ox. For all species, the transi-

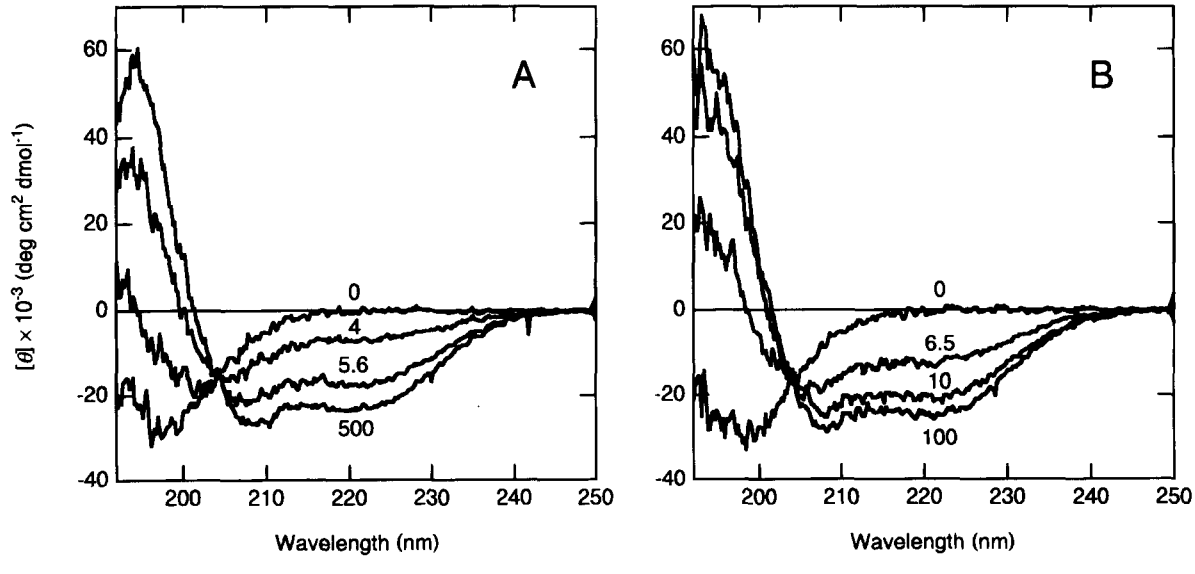

Fig. 3. Far-UV CD spectra of dKL27ox (**A**) and mKL27ox (**B**) in the presence of various concentrations of $NaClO_4$ at pH 4.7 and 20 °C. Numbers show concentrations of $NaClO_4$ at millimolar. Peptide concentrations were 0.1 mg/mL.

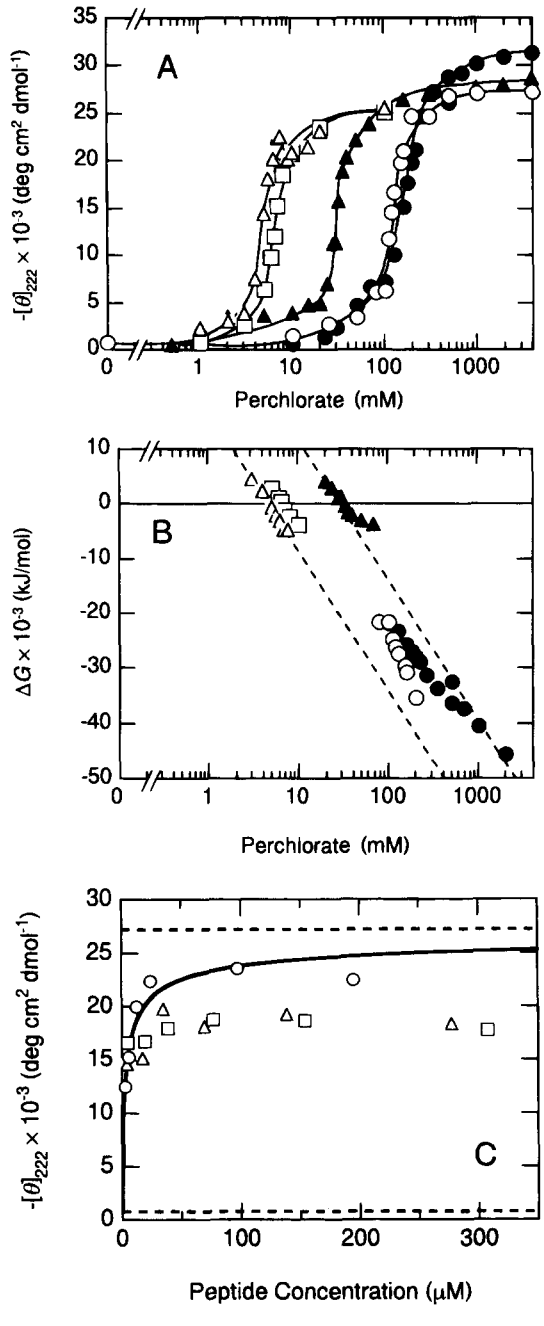

Fig. 4. Dependence of the ellipticity at 222 nm (**A**) and ΔG_F (**B**) on $NaClO_4$ concentration, and dependence of the ellipticity at 222 nm on peptide concentration (**C**), of the model peptides at pH 4.7 and 20 °C. The peptides are mKL27red (○), mKL27ox (□), dKL27ox (△), mKL26red (●), and dKL26ox (▲). In A and B, peptide concentrations were 0.1 mg/mL. ΔG_F was calculated from the transition curves shown in A with Equations 3 or 4. Values for the unfolded and folded states were assumed to be the lowest and highest values for the respective transitions. In C, the $NaClO_4$ concentrations were 200 mM for mKL27red and 10 mM for both mKL27ox and dKL27ox. The solid line indicates the theoretical curve on the basis of Equation 1, $K_F = 2.6 \times 10^5$ M, and assuming that the ellipticity values for the unfolded and helical states to be −800 and −27,200, respectively.

tions were cooperative and the maximal ellipticities were similar, although there was a tendency for the reduced isomers to have higher values. The slight decrease in the maximal ellipticity may be due to the disruption of the terminal helical structures by the introduction of unfavorable disulfide bond(s) at position *b* for dKL26ox and at positions *b* and *e* for dKL27ox (see Zhou et al., 1993, for disulfide bond contribution to helix stability). The maximal ellipticity value for dKL27ox was −25,600, and the helical content was estimated to be 77% by the method of Chen et al. (1972). On the other hand, the concentration of salt required to induce the transition differed considerably among the different species. The apparent C_m value was 120 mM for both mKL27red and mKL26red, 30 mM for dKL26ox, 6.5 mM for mKL27ox, and 4.5 mM for dKL27ox. These findings indicate that the stability of the helical state was increased markedly by the introduction of disulfide bond(s). C_m values for dKL27ox and mKL27ox were about 25 times greater than those for the corresponding reduced form.

Various oligomerization states of the coiled-coils are known (Ogihara et al., 1997). In order to consider the oligomerization state, the dependency on peptide concentration of the CD spectra was measured for mKL27red, mKL27ox, and dKL27ox in the presence of $NaClO_4$ (Fig. 4C). The CD intensity at 222 nm of mKL27red increased and then saturated with the increase in $NaClO_4$ concentration, indicating that the conformational transition includes the intermolecular interaction. Assuming a bimolecular two-state transition between the monomeric unfolded state (U) and the dimeric helical state (H_2) (Materials and methods, Equation 1), we estimated the equilibrium constant for formation of the helical state (K_F) to be 2.6×10^5 M^{-1} using a least-squares fitting program. On the other hand, both the CD spectra of dKL27ox and mKL27ox showed only slight dependence on the peptide concentration, consistent with a monomeric conformational transition. Although the exact oligomerization states are unknown, the results suggest that, whereas the conformational change of mKL27red involves a dimeric association, those of dKL27ox and mKL27ox are predominantly a monomeric intramolecular transition.

The decrease in entropy resulting from introduction of a crosslink in the disordered conformation is calculated by $\Delta S = -(3/2)R \ln n - 8.78$ (J/mol/K), where R is the gas constant, and n is the number of amino acids in the loop created by the disulfide linkage (Pace, 1990). In the present study, the difference in free energy of folding (ΔG_F) between dKL26ox and dKL27ox was −23.6 kJ/mol, assuming a two-state transition between the unfolded and helical states and a linear dependence of ΔG_F against logarithm of the salt concentration (Fig. 4B). It is noted that the linear extrapolation is a tentative attempt to compare the ΔG_F values of different species and its validity is unknown. The calculated value assuming $n = 48$ was −13.5 kJ/mol, slightly smaller in magnitude than the observed value. On the other hand, although the apparent stability of dimeric dKL26ox was higher than that of monomeric mKL26red (Fig. 4A), ΔG_F of mKL26red calculated by including the peptide concentration (Equation 3) indicated that the stability of the helical state of dKL26ox is less than that of mKL26red. However, because the validity of the linear extrapolation is not clear, we cannot conclude at present if these differences in stability of various species are explained exactly by the chain entropy and mixing entropy effects.

Conformation measured by 1D ^{1}H-NMR spectrometry

To further characterize the helical structures of dKL27ox and mKL27ox, 1D ^{1}H-NMR measurements were conducted. Figure 5A and B shows the 1D ^{1}H-NMR spectra of dKL27ox and

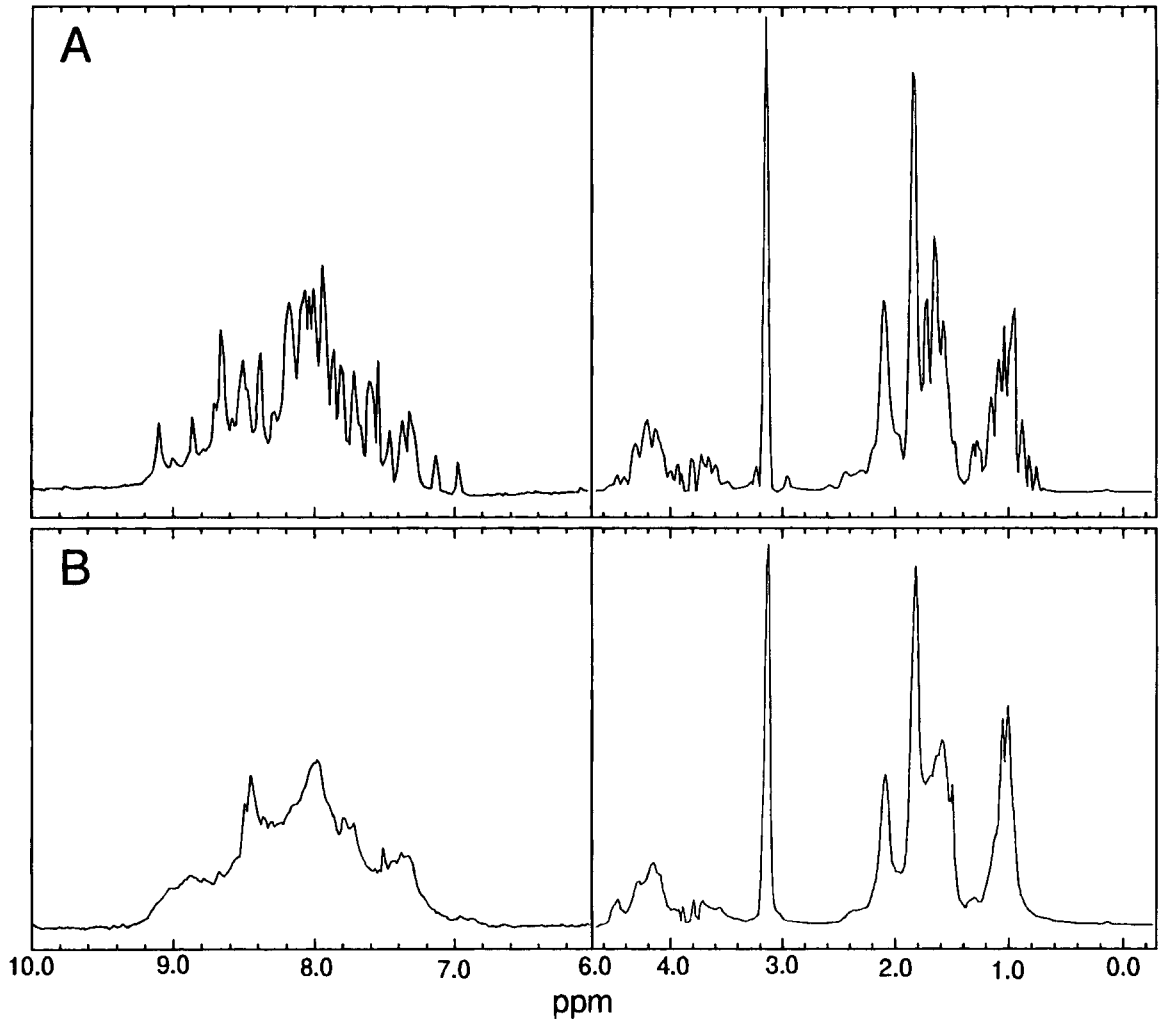

Fig. 5. Proton NMR spectra of dKL27ox (**A**) and mKL27ox (**B**) in 30 mM $NaClO_4$ at pH 3.8 and 20 °C. Peptide concentrations were 12 mg/mL.

mKL27ox, respectively, at a peptide concentration of 12 mg/mL in 30 mM $NaClO_4$ at pH 3.8. The spectra of the two isomers were clearly distinct. Although the peaks in the spectrum of dKL27ox were very sharp and diverged well in both the aromatic and aliphatic regions, those in the spectrum of mKL27ox were broad and did not diverge. This indicates that, under the experimental conditions employed, the structure of the backbone and side chain of dKL27ox is unique and native-like, whereas that of mKL27ox is slowly fluctuating. Consistent with these observations, the 2D DQF-COSY spectrum of dKL27ox showed many cross peaks (data not shown). However, because of the significant overlapping of the chemical shifts of the peaks, their assignments still have not been made. On the other hand, we could not observe the cross peaks for mKL27ox.

Thermal stability of the oxidized KL27 isomers

We measured the heat-induced unfolding of dKL27ox and mKL27ox (Fig. 6). In the presence of 20 mM $NaClO_4$, the peptides were fairly stable against heat, and the midpoint temperature (T_m) of heat-induced unfolding was 75 °C for both peptides (Table 1). The unfolding transitions were completely reversible, judging from the ellipticity value after cooling. The cooperativity of unfolding of dKL27ox was higher than that of mKL27ox. Assuming a two-state transition, the ΔH_U and ΔC_p values of heat denaturation at 20 °C were estimated to be 46.8 kJ/mol and 2.8 kJ/mol/K, respectively, for dKL27ox and 72.4 kJ/mol and 0.6 kJ/mol/K, respectively, for mKL27ox (Table 1). It is noted that "mol" in the units represents mole of monomer, consisting of 26 or 27 residues. For comparison, the ΔC_p value for the leucine zipper peptide GCN4 was reported to be 1.481 kJ/K/mol of monomer, consisting of 33 residues (Tompson et al., 1993).

Gdn-HCl-induced conformational change

The effects of Gdn-HCl on positively charged peptides and proteins are often unusual because the chloride anion of Gdn-HCl interacts with the positive charges of peptides at a low concentration of Gdn-HCl before Gdn-HCl acts as a denaturant (Hagihara et al., 1993, 1994b; Kohn et al., 1995a). Several acid-unfolded proteins and an amphiphilic model peptide refold to the helical conformation upon addition of low concentrations of Gdn-HCl

Table 1. *Comparison of thermodynamic parameters of mKL27ox and dKL27ox*

	$NaClO_4$-induced folding	Thermal unfolding				Gdn-HCl-induced transition	
	C_m[a] (mM)	T_m (°C)	ΔG_U at 20°C (kJ/mol[b])	ΔH_U at 20°C (kJ/mol[b])	ΔC_p (kJ/mol[b]/K)	C_m for folding (mM)	C_m for unfolding (M)[c]
dKL27ox	4.5	75.1 ± 0.04	19.1 ± 0.9	46.8 ± 1.1	2.8 ± 0.22	100	6.0
mKL27ox	6.5	75.5 ± 0.06	14.2 ± 0.2	72.4 ± 0.6	0.6 ± 0.07	150	7.0

[a] C_m values of mKL26red, dKL26ox, and mKL27red were 120, 30, and 120 mM, respectively.
[b] Mol of monomer consisting of 27 residues.
[c] Ellipticity of the denatured state was assumed to be 0.

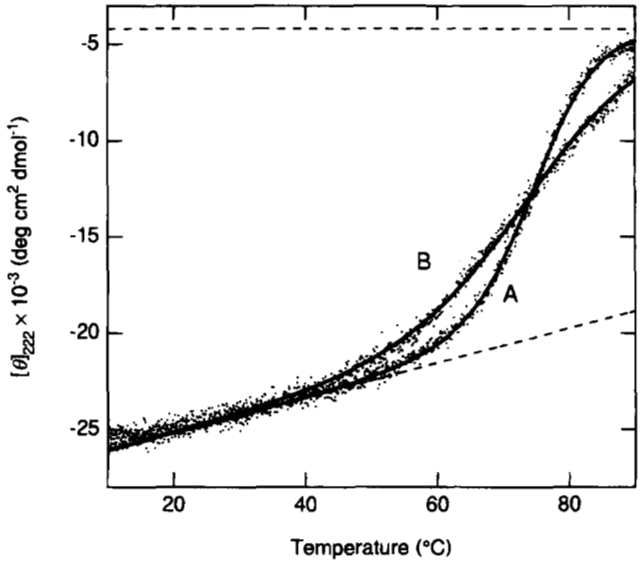

Fig. 6. Thermal unfolding of dKL27ox (**A**) and mKL27ox (**B**) in the presence of 20 mM $NaClO_4$ at pH 4.7 detected by the ellipticity at 222 nm. Peptide concentrations were 0.1 mg/mL. Dots indicate raw data. Lines are the theoretical curves drawn on the basis of Equation 6 and the parameters shown in Table 1 using the indicated baselines (dashed line).

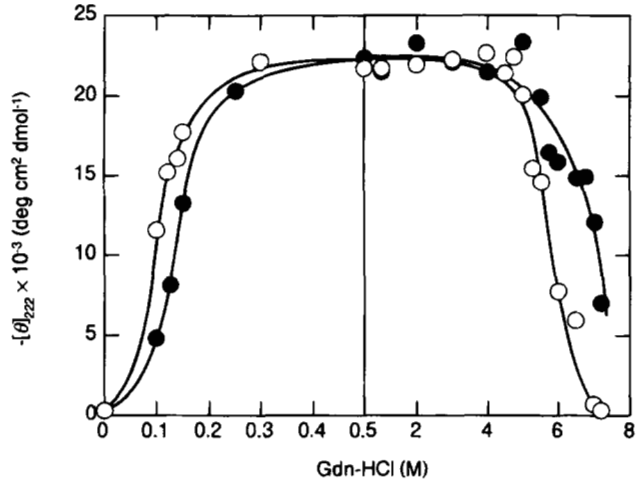

Fig. 7. Dependence on Gdn-HCl concentration of the ellipticity at 222 nm of dKL27ox (○) and mKL27ox (●) at pH 4.7 and 20 °C. Peptide concentrations were 0.1 mg/mL.

and, at high concentrations, they again unfold to a random coil conformation. The effects of Gdn-HCl at low concentrations are explained by the same mechanism as that used for the anion-dependent stabilization of the molten globule states (Hagihara et al., 1993, 1994b).

Because KL27 has 10 positive charges, this peptide is anticipated to show folding and unfolding transitions upon addition of Gdn-HCl. Figure 7 shows the dependencies on Gdn-HCl concentration of the ellipticity at 222 nm of dKL27ox and mKL27ox, showing the refolding and unfolding transitions. The C_m values for the refolding transition were 100 mM and 150 mM for dKL27ox and mKL27ox, respectively, being much higher than that of the $NaClO_4$-induced folding transition (Table 1). The C_m value for the unfolding transition of dKL27ox was 6 M Gdn-HCl, and complete unfolding was observed at 8 M Gdn-HCl. On the other hand, mKL27ox was apparently more stable against Gdn-HCl than dKL27ox, and significant ellipticity remained even in the presence of 8 M Gdn-HCl.

Discussion

Charge repulsion and its modulation by anion binding

To address the role of charge repulsion in the native-like state and its modulation by the ions in a solvent, we designed a coiled-coil peptide of 27 amino acid residues in which anion-dependent conformational control was introduced. The dimeric parallel peptide (dKL27ox) exhibited the expected conformational properties. Whereas it was unfolded in the absence of salt, the addition of a low concentration of perchlorate cooperatively stabilized the helical state. The effect of NaCl was much less than that of $NaClO_4$ (data not shown, see the effect of Gdn-HCl in Table 1), indicating that anion biding is responsible for the transition. Judging from the NMR spectrum, the helical state was a native-like coiled-coil, as observed for other leucine zipper peptides.

A series of studies by Goto and coworkers (Goto et al., 1990a, 1990b, 1991, 1993; Goto & Nishikiori, 1991; Goto & Fink, 1994) indicated that the charge repulsion and its modulation by counterion binding are the important factors determining the conformational stability of the molten globule state. They further showed that the anion-dependent conformational transition is not limited to acid-denatured proteins, but can occur at neutral pH.

Melittin is a basic peptide of honeybee venom consisting of 26 amino acid residues, 5 of which are basic and none of which are acidic (Habermann, 1972). Whereas melittin was unfolded under low-salt conditions, a tetrameric helical state was stabilized by addition of salt (Talbot et al., 1979). Goto and coworkers (Goto & Hagihara, 1992; Hagihara et al., 1992) showed that the conformational transition of melittin is also explained by the same mechanism. Similar conformational transition has also been observed with mastoparan, a basic tetradecapeptide from wasp venom (Hoshino & Goto, 1994). As a model of the anion-dependent conformational transition of proteins, Goto and Aimoto (1991) prepared an amphiphilic model peptide of 51 amino acid residues consisting of tandem repeats of a Lys-Lys-Leu-Leu sequence and containing a turn sequence at the center of the molecule. Whereas the model peptide showed no significant conformation under low-salt conditions at neutral pH, addition of anion induced the folding transition to a helical state. Comparison of various anions showed that anion binding is responsible for the transition.

All these results emphasize the importance of charge repulsion and its shielding by the direct binding of counter ions in determining the stability of proteins. However, because the folded states examined so far are the fluctuating molten globule or related states, it has not yet been confirmed whether such effects are common to the rigid native states. It is noteworthy that, although the crystallographic structure of melittin, a helical tetramer, has been reported (Terwilliger & Eisenberg, 1982), Hagihara et al. (1994a) have indicated that the tetrameric state of melittin in an aqueous environment is more disordered than the crystal structure. The present results indicate that the anion-dependent folding transition can occur even for the native-like conformation when the molecule is highly charged.

Because the model peptides used here have many Lys residues, the nature of electrostatics may be substantially different from that of natural leucine zipper peptides such as GCN4, where the specific interchain attraction across the hydrophobic interface is important. Nevertheless, it might be useful to compare the observed effects of salt on model peptides with those on leucine zipper peptides. One of the arguments about leucine zipper peptides is the role of electrostatic interactions in stabilizing the coiled-coil. Kenar et al. (1995) studied the effects of different salts on the stability of a 33-residue peptide corresponding to the leucine zipper region of GCN4 at pH 7. They showed that salt below 0.5 M always destabilized the coiled-coil, and that this effect could be interpreted in terms of an ionic strength effect destabilizing the specific salt bridges between Lys^+ and Glu^-, which stabilize the coiled-coil. They also showed that high concentrations of salt stabilize the coiled-coil, and that this effect depends on the salt species, following a Hofmeister series of anions ($F^- > Cl^- > Br^-$).

If the salt bridges do contribute to stabilization of the coiled-coil, it is expected that the coiled-coil would be destabilized under acidic conditions. However, it has been reported that this does not occur and, consequently, the contribution of salt bridges has been questioned (Lumb & Kim, 1995, 1996). Recently, Yu et al. (1996) showed that acid-destabilization of the coiled-coil occurs under conditions of low salt (10 mM), but that, under physiological salt conditions (100–150 mM NaCl), this destabilization was shielded completely by counter anion binding. This shows that counter anion binding is an important factor, at least under acidic conditions, modulating the conformational stability of the natural leucine zipper peptide.

Conformation of cyclic peptide

Of particular interest is the conformation of the cyclic monomer. It was surprising that a cyclic peptide exhibited a conformational transition similar to the designed parallel dimeric peptide. The protein concentration dependence of the perchlorate-induced transition suggests that this peptide assumes a monomeric helical state under the conditions used. Assuming that the antiparallel coiled-coil of mKL27ox is formed intramolecularly, each helix consists of 10 amino acid residues or so (i.e., three turns). Such a short helix may be difficult to stabilize by the tight packing, as observed for leucine zipper peptides, and the structure would be very flexible. Indeed, the NMR spectrum was that of the disordered state. Nevertheless, it still exhibits significant cooperativity upon unfolding. The linkage of the N and C terminals by the disulfide bond increases the effective concentration of the interacting residues, thereby stabilizing the molten globule-like helical structure.

Various oligomerization states of the coiled-coils are known (Ogihara et al., 1997). Because we did not detect directly the oligomerization state, we cannot exclude the possibility that the oligomers such as tightly associated dimer or trimer were formed and the oligomerization equilibrium was apparently insensitive to the peptide concentration in the range that we examined.

Apart from the oligomerization state, the conformational transition of the cyclic monomer, mKL27ox, indicates that the formation of a dynamic helical structure requires no rigid packing of side chains. Recently, the validity of the molten globule hypothesis has been questioned (Privalov, 1996). In the original molten globule hypothesis, melting of the side-chain packing (i.e., tertiary structure) while maintaining the secondary structures is emphasized. The tertiary chain fold in the structured region is assumed to be similar to that of the native state. The present picture of the molten globule state is very different from the original definition, and depends on the protein species (Kataoka et al., 1997). The molten globule state does not necessarily include as much secondary structure as that in the native state. Probably, the significant melting of the side-chain packing is an important criterion defining the molten globule state. The conformational properties of mKL27ox are consistent with the molten globule hypothesis and support the possibility that such a conformational state can be formed even by natural proteins.

Concluding remarks

Previous studies have suggested that anion-dependent formation of the helical structure might be useful for controlling the conformation of peptides. However, all of the folded states stabilized by anion binding are the molten globule or related conformational states. The present results obtained with our designed coiled-coil peptides indicate that such anion-dependent conformational control is useful even for a native-like conformational state. These results also emphasize the importance of the solvent-dependent charge repulsion in determining the conformation and stability of proteins and peptides.

Materials and methods

Materials

All peptides were synthesized on an ABI 430A peptide synthesizer (Applied Biosystems) using solid-phase methodology with Fmoc chemistry. The crude material was applied to a preparative C_{18}

HPLC column, and the major sharp peak, eluted with a gradient of acetonitrile in 0.05% trifluoroacetic acid, was collected and stored after lyophilization. The purity and identity of the synthesized products were checked by analytical HPLC, amino acid analysis, amino acid sequencing, and time of flight mass spectrometry.

To prepare the disulfide-bonded dimers, peptides were oxidized by air in 10 mM Tris-HCl at pH 8.6 for three days. Complete conversion to the oxidized forms was confirmed by the titration of free thiol groups with 5,5′-dithiobis(2-nitrobenzoic acid) and by analytical HPLC, in which the oxidized form was eluted more slowly than the corresponding reduced form.

Methods

CD measurements were performed with a Jasco spectropolarimeter, model J-500A, equipped with an interface and a personal computer. The instrument was calibrated with ammonium *d*-10-camphorsulfonic acid. The results were expressed as the mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue moles per liter, and l is the length of the light path in centimeters. Far-UV CD spectra were measured using a cell with a 1-mm light path. The temperature was controlled at 20 °C with a thermostatically controlled cell holder.

Typically, 30 μL of peptide solution at a peptide concentration of 1 mg mL^{-1}, dissolved in deionized water, was mixed with 270 μL of salt solution. Sodium acetate buffer (10 mM) at pH 4.7 was used to control the pH of the solution. The pH of the sample was measured using a Radiometer pH meter, model PHM83, at 20 °C. Concentration of peptides was determined from the absorption at 280 nm assuming a molar extinction coefficient of 5,500 for Trp.

For the heat-denaturation measurements, the temperatures were increased from 10 °C at a rate of 1 °C min^{-1}. The changes in temperature were monitored with a thermocouple (Senserteck, BAT-12, and a flexible probe) inserted directly into a cell.

1D ^{1}H-NMR spectra were recorded with an AM500 Bruker spectrometer. The spectra were recorded at 20 °C and pH 3.8 in 30 mM $NaClO_4$ at a peptide concentration of 12 mg/mL.

Analysis of conformational transition

For the reduced peptides, we assumed a bimolecular two-state transition between the monomeric unfolded state (U) and the dimeric helical state (H_2):

$$2U \rightleftarrows H_2. \tag{1}$$

Based on Equation 1, the equilibrium constant for the formation of the helical structure (K_F) is defined by $K_F = [H_2]/[U]^2$, where $[H_2]$ and [U] are the concentrations of H_2 and U. For the oxidized peptides, we assumed a monomeric two-state transition between the unfolded state (U_2) and the helical state (H_2):

$$U_2 \rightleftarrows H_2. \tag{2}$$

K_F for Equation 2 is defined by $K_F = [H_2]/[U_2]$. The free energy change ($\Delta G_F = -RT \ln K_F$) of formation of the helical structure of the reduced peptides can be calculated by

$$\Delta G_F = -RT \ln \frac{f_H}{2p_0(1 - f_H)^2}, \tag{3}$$

and for the oxidized forms:

$$\Delta G_F = -RT \ln \frac{f_H}{1 - f_H}, \tag{4}$$

where p_0 is the total concentration of peptide, R is the gas constant, T is the temperature in Kelvin, and f_H is the fraction of the species in a helical state calculated by

$$f_H = \frac{[\theta] - [\theta]_U}{[\theta]_H - [\theta]_U}, \tag{5}$$

where $[\theta]_U$, $[\theta]_H$ are the ellipticities of the unfolded and helical states, respectively, and $[\theta]$ is the ellipticity of the peptides at given salt concentrations.

The free energy change of heat-induced unfolding (ΔG_U) of dKL27ox and mKL27ox can be represented by the standard thermodynamic equations:

$$\Delta G_U = (-\Delta G_F) = \Delta H - T\Delta S$$
$$= \Delta H_m + \Delta C_p(T - T_m) - T\left\{\frac{\Delta H_m}{T_m} + \Delta C_p \ln\left(\frac{T}{T_m}\right)\right\}, \tag{6}$$

where T is the temperature in Kelvin, T_m is the midpoint temperature for transition, ΔH_m is the enthalpy change at T_m, and ΔC_p is the heat capacity change.

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References

Chakrabartty R, Baldwin RL. 1995. Stability of α-helices. *Adv Protein Chem 46*:143–176.

Chen YH, Yang JT, Martinez HM. 1972. Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry 11*:4120–4131.

Dill KA, Bromberg S, Yue K, Fiebig KM, Yee DP, Thomas PD, Chan HS. 1995. Principles of protein folding—A perspective from simple exact models. *Protein Sci 4*:561–602.

Goto Y, Aimoto S. 1991. Anion and pH-dependent conformational transition of an amphiphilic polypeptide. *J Mol Biol 218*:387–396.

Goto Y, Calciano LJ, Fink AL. 1990a. Acid-induced folding of proteins. *Proc Natl Acad Sci USA 87*:573–577.

Goto Y, Fink AL. 1994. Acid-induced folding of heme proteins. *Methods Enzymol 232*:3–15.

Goto Y, Hagihara Y. 1992. Mechanism of the conformational transition of melittin. *Biochemistry 31*:732–738.

Goto Y, Hagihara Y, Hamada D, Hoshino M, Nishii I. 1993. Acid-induced unfolding and refolding transitions of cytochrome *c*: A three-state mechanism in H_2O and D_2O. *Biochemistry 32*:11878–11885.

Goto Y, Nishikiori S. 1991. Role of electrostatic repulsion in the acidic molten globule of cytochrome *c*. *J Mol Biol 222*:679–686.

Goto Y, Okamura N, Aimoto S. 1991. ATP-induced conformational transition of denatured proteins. *J Biochem Tokyo 109*:746–750.

Goto Y, Takahashi N, Fink AL. 1990b. Mechanism of acid-induced folding of proteins. *Biochemistry 29*:3480–3488.

Habermann E. 1972. Bee and wasp venoms. *Science 177*:314–322.

Hagihara Y, Aimoto S, Fink AL, Goto Y. 1993. Guanidine hydrochloride-induced folding of proteins. *J Mol Biol 231*:180–184.

Hagihara Y, Kataoka M, Aimoto S, Goto Y. 1992. Charge repulsion in the conformational stability of melittin. *Biochemistry 31*:11908–11914.

Hagihara Y, Oobatake M, Goto Y. 1994a. Thermal unfolding of tetrameric melittin: Comparison with the molten globule state of cytochrome *c*. *Protein Sci 3*:1418–1429.

Hagihara Y, Tan Y, Goto Y. 1994b. Comparison of the conformational stability of the molten globule and native states of horse cytochrome *c*. Effects of acetylation, heat, urea and guanidine-hydrochloride. *J Mol Biol 237*:336–348.

Hoshino M, Goto Y. 1994. Perchlorate-induced formation of the alpha-helical structure of mastoparan. *J Biochem Tokyo 116*:910–915.

Kataoka M, Kuwajima K, Tokunaga F, Goto Y. 1997. Structural characterization of the molten globule of α-lactalbumin and a novel approach for discriminating the molten globule by solution X-ray scattering. *Protein Sci 6*:422–430.

Kenar KT, Garcia Moreno B, Freire E. 1995. A calorimetric characterization of the salt dependence of the stability of the GCN4 leucine zipper. *Protein Sci 4*:1934–1938.

Kohn WD, Kay CM, Hodges RS. 1995a. Protein destabilization by electrostatic repulsions in the two-stranded alpha-helical coiled-coil/leucine zipper. *Protein Sci 4*:237–250.

Kohn WD, Monera OD, Kay CM, Hodges RS. 1995b. The effects of interhelical electrostatic repulsions between glutamic acid residues in controlling the dimerization and stability of two-stranded α-helical coiled-coils. *J Biol Chem 270*:25495–25506.

Lavigne P, Kondejewski LH, Houston ME Jr, Sonnichsen FD, Lix B, Skyes BD, Hodges RS, Kay CM. 1995. Preferential heterodimeric parallel coiled-coil formation by synthetic Max and c-Myc leucine zippers: A description of putative electrostatic interactions responsible for the specificity of heterodimerization. *J Mol Biol 254*:505–520.

Lavigne P, Sonnichsen FD, Kay CM, Hodges RS. 1996. Interhelical salt bridges, coiled-coil stability, and specificity of dimerization. *Science 271*:1136–1137.

Linderstrom-Lang K. 1924. On the ionization of proteins. *Compt Rend Trav Lab Carlsberg, Ser Chim 15*:1–29.

Lumb KJ, Kim PS. 1995. Measurement of interhelical electrostatic interactions in the GCN4 leucine zipper. *Science 268*:436–439.

Lumb KJ, Kim PS. 1996. Interhelical salt bridges, coiled-coil stability, and specificity of dimerization. *Science 271*:1137–1138.

Makhatadze GI, Privalov PL. 1995. Energetics of protein structure. *Adv Protein Chem 47*:307–425.

Nakamura H. 1996. Roles of electrostatic interaction in proteins. *Quart Rev Biophys 29*:1–90.

Ogihara NL, Weiss MS, Degrado WS, Eisenberg D. 1997. The crystal structure of the designed trimeric coiled coil-V_aL_d: Implications for engineering crystals and supramolecular assemblies. *Protein Sci 6*:80–88.

O'Shea EK, Klemm JD, Kim PS, Alber T. 1991. X-ray structure of the GCN4 leucine zipper, a two-stranded, parallel coiled coil. *Science 254*:539–544.

O'Shea EK, Rutkowski R, Kim PS. 1989. Evidence that the leucine zipper is a coiled coil. *Science 243*:538–542.

O'Shea EK, Rutkowski R, Kim PS. 1992. Mechanism of specificity in the Fos-Jun oncoprotein heterodimer. *Cell 68*:699–708.

Pace CN. 1990. Measuring and increasing protein stability. *Trends Biotechnol 8*:93–98.

Privalov PL. 1996. Intermediate states in protein folding. *J Mol Biol 258*:707–725.

Talbot JC, Dufourcq J, de Bony J, Faucon JF, Lussan C. 1979. Conformational change and self association of monomeric melittin. *FEBS Lett 102*:191–193.

Tanford C, Kirkwood J. 1957. Theory of protein transition curves. I. General equations for impenetrable spheres. *J Am Chem Soc 79*:5333–5339.

Terwilliger TC, Eisenberg D. 1982. The structure of melittin. I. Structure determination and partial refinement. *J Biol Chem 257*:6010–6015.

Tompson KS, Vinson CR, Freire E. 1993. Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transition factor GCN4. *Biochemistry 32*:5491–5496.

Yang AS, Honig B. 1993. On the pH dependence of protein stability. *J Mol Biol 231*:459–474.

Yu Y, Monera OD, Hodges RS, Privalov PL. 1996. Ion pairs significantly stabilize coiled-coils in the absence of electrolyte. *J Mol Biol 255*: 367–372.

Zhou NE, Kay CM, Hodges RS. 1994a. The role of interhelical ionic interactions in controlling protein folding and stability. De novo designed synthetic two-stranded alpha-helical coiled-coils. *J Mol Biol 237*:500–512.

Zhou NE, Monera OD, Kay CM, Hodges RS. 1993. Disulfide bond contribution to protein stability: Positional effects of substitution in the hydrophobic core of the two-stranded α-helical coiled-coil. *Biochemistry 32*:3178–3187.

Zhou NE, Monera OD, Kay CM, Hodges RS. 1994b. α-Helical propensities of amino acids in the hydrophobic face of an amphipathic α-helix. *Protein Peptide Lett 1*:114–119.

Zitzewitz JA, Bilsel O, Luo J, Jones BE, Matthews CR. 1995. Probing the folding mechanism of a leucine zipper peptide by stopped-flow circular dichroism spectroscopy. *Biochemistry 34*:12812–12819.