Disulfide Bond Contribution to Protein Stability: Positional Effects of Substitution in the Hydrophobic Core of the Two-Stranded α-Helical Coiled-Coil†

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ABSTRACT: To investigate the positional effect of the disulfide bond on the structure and stability of a two-stranded α-helical coiled-coil, an interchain disulfide bond was systemtically introduced into the hydrophobic core of a de novo designed model coiled-coil at the N-terminus (position 2), C-terminus (position 33), and nonterminal positions a (positions 9, 16, 23, and 30) and d (positions 5, 12, 19, and 26). The rate of formation of a disulfide bond is faster at position d compared to at the corresponding position a under nondenaturing conditions, suggesting that position d is more suitable for engineering a disulfide bond. The structure and stability of the reduced and oxidized coiled-coils were determined by circular dichroism studies in the absence and presence of guanidine hydrochloride. Our results demonstrate that the improvement of protein stability by introduction of a disulfide bond is very relevant to its location and the most effective disulfide bonds are those that can be introduced in the hydrophobic core without any disruption of the protein structure. The disulfide bond at position d with near-optimal geometry does not perturb the coiled-coil structure and makes the largest contribution to coiled-coil stability. In contrast, the inappropriate geometry of the disulfide bond at nonterminal position a introduces a high strain energy on the disulfide bond which disrupts the coiled-coil structure. At positions a, the closer the disulfide bridge is to the center of the coiled-coil, the larger the disruption on the coiled-coil structure and the smaller the contribution the disulfide bond makes to coiled-coil stability. The computer modeling results also suggest that an insertion of an interchain disulfide bond at position a in the GCN4 leucine zipper X-ray structure has a higher potential energy than insertion at position d. The energy-minimized coiled-coil structure with an interchain disulfide bond at position a has a larger root mean square difference from the X-ray structure of GCN4 than the coiled-coil with a disulfide bond at position d. Because interhelical interactions are common in globular proteins as well as coiled-coils, the results obtained in this study will have general utility for selecting the sites for engineering disulfide bonds between α-helices.

One of the major goals of protein engineering is to design proteins with enhanced stability and activity. It has been shown for many naturally occurring proteins that disulfide bonds can enhance protein stability considerably (Lin et al., 1984; Ueda et al., 1985; Creighton, 1986; Schwarz et al., 1987; Pace et al., 1988; Lin & Kim, 1989; Taniyama et al., 1990; Inaka, et al., 1991; Cooper et al., 1992). This stabilizing potential of disulfide bonds has made their engineering into proteins an attractive strategy for improving protein stability. Disulfide bonds have been successfully introduced into various proteins, such as dihydrofolate reductase (Villafranca et al., 1983, 1987), phage T4 lysozyme (Perry & Wetzel, 1984, 1986; Wetzel et al., 1988; Matsumura & Matthews, 1989; Matsumura et al., 1989a,b), subtilisin (Wells & Powers, 1986; Pantoliano et al., 1987; Mitchinson & Wells, 1989; Takagi et al., 1990), λ repressor (Sauer et al., 1986), ribonuclease H (Kanaya et al., 1991), LamB protein (Luckey et al., 1991), and β/α-barrel protein (Eder & Wilmanns, 1992). However, only in some cases did the addition of a disulfide bond increase protein stability relative to that of the wild type (Wetzel et al., 1988; Matsumura et al., 1989a,b; Kanaya et al., 1991; Luckey et al., 1991). In other cases, no increase in protein stability was observed (Creighton, 1988; Wells & Powers, 1986; Wetzel, 1987).

Matsumura and Matthews (1991) compared the effect of nonnative disulfide bonds located in different positions in T4 lysozyme on protein stability. The three disulfide bonds (3–97, 9–164, and 21–142) that increased the thermostability of the protein were located within flexible regions of the molecule. The two disulfide bonds (90–122 and 127–154) that did not increase stability were both located within the α-helices of the C-terminal domain, which are in the most rigid parts of the molecule. On the basis of these results, it was proposed that disulfide bonds should not be introduced in regions consisting of elements of closely packed secondary structure such as α-helices and β-sheets or in rigid parts of the molecule (Matsumura & Matthews, 1991; Matsumura et al., 1989a). However, both theoretical calculations (Flory, 1956; Poland & Scheraga, 1965; Chan & Dill, 1989) and thermodynamic studies (Cooper et al., 1992) have suggested that the stabilizing effect of a disulfide bond in a protein can be attributed predominantly to an increase in the entropy difference between the folded and unfolded states of the disulfide bonded protein versus the reduced protein. Thus, the disulfide bonds which provide the greatest stability should be those introduced into the most closely packed part of the protein interior (Creighton, 1988). This is consistent with the fact that most naturally occurring disulfide bonds in proteins are buried (Thoronton, 1981). This stabilizing effect can be offset by two destabilizing features: (1) the disruption of preexisting interactions in the wild-type structure due to introduction of cysteine residues

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for existing residues in the native protein and (2) the energetic constraints associated with the formation of the disulfide bond in the folded structure. In a closely packed interior of a protein, the cysteine mutations are more likely to be destabilizing since they are probably replacing larger hydrophobic residues and the geometry of the disulfide bond would be expected to be more critical. On the other hand, introducing Cys residues and disulfide bonds onto the protein surface should minimize such destabilizing effects, but the resulting disulfide bonds are likely to have a smaller stabilizing effect (Creighton, 1988). Therefore, the most critical and difficult problem is to find optimal sites that are compatible with the strict stereochemical requirements of the disulfide bond in the closely packed interior of a protein. Although it is thought that the location of the disulfide bridge in a protein is a principal determinant in its stabilizing effect, the criteria for selecting the appropriate locus for the disulfide bridge in a given protein have not been firmly established.

To explore these concerns, an interchain disulfide bond was systematically introduced into the hydrophobic core of a two-stranded α-helical coiled-coil at either position a or position d in the N-terminus, C-terminus, or the central region of the coiled-coil. The positional effects of the disulfide bond on the structure and stability of the coiled-coils were investigated by CD[1] studies in the absence and presence of guanidine hydrochloride. The α-helical coiled-coil structure, which consists of two amphipathic α-helices associated together via interchain hydrophobic interactions, is an important structural motif in a diverse group of proteins from the muscle proteins to the DNA-binding proteins (Cohen & Parry, 1986, 1990; Mcknight, 1991; O'Shea et al., 1989, 1991). Hodges and co-workers designed and synthesized the first model coiled-coil protein and demonstrated its utility as an ideal model system for studying de novo design principles involving both intra- and intermolecular interactions related to protein folding and stability (Hodges et al., 1981; Talbot & Hodges, 1982; Hodges, 1992; Lau et al., 1984a; Zhou et al., 1992a–c; Zhu et al., 1992, 1993). This coiled-coil model has also been used to determine the α-helical propensity of amino acids (O'Neil & DeGrado, 1990) and as part of a much larger de novo designed protein (Engel et al., 1991). It has been suggested that more than 200 proteins may contain this coiled-coil motif (Lupas et al., 1991). Thus, the information from this study should have general utility for selecting the site of engineering interhelical disulfide bonds in proteins.

MATERIALS AND METHODS

Peptide Synthesis and Purification. All peptides were synthesized by solid-phase peptide synthesis methodology using a benzhydrylamine hydrochloride resin on an Applied Biosystems peptide synthesizer Model 430A (Foster City, CA) with conventional *N-tert*-butyloxycarbonyl (*t*-Boc) chemistry as described previously (Hodges et al., 1988). The peptides were cleaved from the resin by reaction with HF (20 mL/g of resin) containing 10% anisole and 2% 1,2-ethanedithiol for 1 h at −5 to 0 °C. The crude reduced peptides were purified by reversed-phase high-performance liquid chromatography (RPC) on a SynChropak RP-P semipreparative C_{18} colum (250 × 10 mm i.d., 6.5-μm particle size, 300-Å pore size) (SynChrom, Lafayette, IN), with a linear AB gradient of 0.5% B/min and 2 mL/min, where solvent A is 0.05% trifluoroacetic acid (TFA) in water and solvent B is 0.05% TFA in acetonitrile. The purified peptides were homogeneous as determined by RPC and confirmed by amino acid analysis and mass spectroscopy. For amino acid analysis, purified peptides were hydrolyzed in 6 N HCl containing 0.1% phenol at 110 °C for 24 h or 1 h at 160 °C in evacuated sealed tubes. Amino acid analysis was performed on a Beckman Model 6300 amino acid analyzer (Beckman, San Ramon, CA). The correct primary ion molecular weights of the reduced peptides were confirmed by plasma desorption time of flight mass spectroscopy on a BIOION-20 Nordic (Uppsala, Sweden).

Preparation of Oxidized Peptides. For the peptides with cysteine residues at terminal positions (positions 2a and 33d), oxidized peptides with an interchain disulfide bond between the two α-helices were obtained by air oxidation of a solution of reduced peptides in 0.1 M NH_4HCO_3, pH 8.3, at room temperature with stirring (Lee et al., 1991; Zhou et al., 1992c). For the peptides with cysteine residues at nonterminal positions (positions 5d, 9a, 12d, 16a, 19d, 23a, 26d, and 30a), oxidized peptides were obtained by air oxidation of a solution of reduced peptides in 0.1 M NH_4HCO_3 containing 6 M guanidine hydrochloride (Gdn·HCl), pH 8.3, at room temperature with stirring. The reaction was monitored by analytical RPC. The oxidized peptides were purified by RPC on an analytical C_{18} column (SynChropak RP-PC_{18}, 250 × 4.6 mm i.d., 300-Å pore size; SynChrom, Inc.) with a linear AB gradient of 0.5% B/min and 1 mL/min, where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile.

Kinetics of Disulfide Bond Formation. The relative rate of oxidation or formation of the disulfide bond was monitored quantitatively by RPC on an analytical C8 column (Zorbax 300SB-C8, 2.1 × 150 mm, 5 μm particle size, 300 Å pore size, Rockland Technologies, Inc., Chadds Ford, PA) with a linear AB gradient of 2% B/min, at a flow rate of 0.25 mL/min, where solvent A is 0.05% TFA in water and solvent B is 0.05% TFA in acetonitrile. All the peptides were oxidized at similar peptide concentrations (0.5 mg/mL) under nondenaturing conditions (0.1 M Tris-HCl buffer, pH 8.3, at 25 °C) and denaturing conditions (0.1 M Tris-HCl buffer containing 6 M Gdn·HCl, pH 8.3, at 25 °C). The oxidation reaction was quenched by the addition of 25 μL of 0.1 M HCl to the 25 μL of peptide samples taken out of the reaction mixture at appropriate intervals. The quenched solution was kept at 4 °C prior to injection onto a reversed-phase column. The reduced and oxidized peptides can be readily separated. The oxidation rate in percent was expressed as the ratio of the peak area of the oxidized form to the sum of the peak area of the oxidized and reduced form.

Molecular Weight Determinations. The molecular weights of the peptides in aqueous solution were determined by size-exclusion chromatography (SEC) and sedimentation equilibrium experiments. SEC was carried out on a Spherogel TSK 2000SW column (300 × 7.5-mm i.d., 10-μm particle size, 125-Å pore size) (Beckman Instruments, Japan) at a flow rate of 0.2 mL/min at room temperature. The eluent was a 0.5 M KCl, 50 mM PO_4, pH 7, buffer. The 14-, 21-, 28-, 42-, and 56-residue peptides were used as molecular weight standards (Zhou et al., 1990; Hodges et al., 1990). Sedimentation equilibrium experiments were carried out in a Model E analytical ultracentrifuge with the interference optical system at a speed of 32 000 rpm. Peptide C12d(o) was dissolved in 0.5 M KCl, 50 mM PO_4, pH 7, buffer and dialyzed against 0.5 M KCl, 50 mM PO_4, pH 7, buffer with an initial peptide concentration of 1.05 mg/mL.

[1] Abbreviations: CD, circular dichroism; RPC, reversed-phase chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol; Gdn·HCl, guanidine hydrochloride; SEC, size-exclusion chromatography; RMS, root mean square.

Circular Dichroism Measurements. Circular dichrosim (CD) spectra were recorded at 20 °C on a Jasco J-500C spectropolarimeter (Jasco, Easton, MD) equipped with a Jasco DP-500N data processor and a Lauda (Model RMS) water bath (Brinkmann Instruments, Rexdale, Ontario, Canada), used to control the temperature of the cell. Constant N_2 flushing was employed. The instrument was routinely calibrated with an aqueous solution of recrystallized *d*-10-(+)-camphorsulfonic acid at 290 nm. Molar ellipticity at 220 nm is reported as mean residue molar ellipticity ($[\theta]_{220}$, deg·cm^2·dmol^{-1}) and calculated from the equation:

$$[\theta] = [\theta]_{obs}\text{mrw}/10lc$$

$[\theta]_{obs}$ is the ellipticity measured in degrees, mrw is the mean residue molecular weight (molecular weight of the peptide divided by the number of amino acid residues), c is the peptide concentration in grams per milliliter, and l is the optical path length of the cell in centimeters. CD spectra were the average of three scans obtained by collecting data at 0.25-nm intervals from 250 to 190 nm. Guanidine hydrochloride (Gdn·HCl) denaturation studies wer carried out by preparing mixtures of a stock solution of peptide in buffer (0.1 M KCl, 50 mM PO_4, pH 7), buffer alone, and a solution of 6 M Gdn·HCl in buffer where the ratios of buffer and 6 M Gdn·HCl solutions were varied to give the appropriate final Gdn·HCl concentrations. Peptide concentrations were determined by amino acid analysis.

Molecular Modeling of Disulfide Mutants. Two oxidized coiled-coils with an interchain disulfide bond either at the nonterminal position a (position 23) or at the nonterminal position d (position 19) were modeled on a Silicon Graphics Personal Iris with the Insight II and Discover programs (Biosym Technologies, Inc. San Diego, CA). The high-resolution X-ray structure of a two-stranded α-helical coiled-coil, GCN4 leucine-zipper (O'Shea et al., 1991), was used for the initial coordinates. Cysteines were placed either at positions 19 and 19′ or at positions 23 and 23′ in both chains and then an interchain disulfide bond was formed. The two modeled disulfide bridged structures were energy minimized using consistent valence force-field (CVFF) (Dauber-Osguthorpe et al., 1988) and a 12-Å interatomic nonbond cutoff for 100 steps of steepest descents and 1000 steps of conjugate gradients with all atoms in the polypeptide backbone fixed. The geometry and potential energy of the disulfide bond at the nonterminal position a and d were measured and compared. The next 100 steps of steepest descents and 1000 steps of conjugate gradients of energy minimization were performed for each structure, using the same parameterization without any constraints on the polypeptide backbone. These two energy minimized coiled-coil structures were compared to the X-ray structure of GCN4. The two structures were superimposed to minimize the root mean square (RMS) difference of the α-carbons of residues 1–31 in both chains of the coiled-coil. The RMS differences were calculated using programs written by T. Jellard, University of Alberta.

RESULTS

Design of Interhelical Disulfide Bonds. The de novo designed coiled-coil protein consists of two identical 35-residue polypeptide chains each containing 5 heptads (Table I). Leu residues at positions a and d create an amphipathic α-helix, which can dimerize in a parallel and in-register fashion to form a continuous hydrophobic core as a result of the "knobs-into-holes" packing as proposed by Crick in 1953. It is possible to form an interhelical disulfide bond between two cysteines at position a or d in the coiled-coil. The distance of Cα–Cα′ is in the range from 5.5 to 6.4 Å at these positions in the coiled-coil structure of the GCN4 leucine zipper (O'Shea et al., 1991), which falls in the effective range for Cα–Cα′ distances (range from 4.8 to 6.6 Å) of disulfide bonds in native proteins (Thoronton, 1981). To investigate the positional effect of the disulfide bond on the structure and stability of the coiled-coil, a leucine residue was systematically mutated to cysteine at each of the 10 a and d positions (Table I). The resulting coiled-coils would contain an interhelical disulfide bridge at one of five a positions (2, 9, 16, 23, and 30) and five d positions (positions 5, 12, 19, 26, and 33). The remaining nine a and d positions in each chain are occupied by leucine residues. The peptide with one cysteine at position 2 (position a) is designated as C2a. C2a(o) or C2a(r) represents the coiled-coil with or without a disulfide bridge (oxidized or reduced), respectively. Similarly, the other coiled-coils are designated as C5d(o), C5d(r), C9a(o), C9a(r), C12d(o), C12d(r), C16a(o), C16a(r), C19d(o), C19d(r), C23a(o), C23a(r), C26d(o), C26d(r), C30a(o), C30a(r), C33d(o), and C33d(r).

Table I: Synthetic Peptides Used To Form Two-Stranded α-Helical Coiled-Coils with an Interchain Disulfide Bond

peptide	sequence nomenclature
	a b c d e f g a d a d a d a d
	1 2 5 9 12 16 19 23 26 30 33 35
Native	Ac-KLEALEGKLEALEGKLEALEGKLEALEGKLEALEG-amide
C2a	Ac-KC▼EALEGKLEALEGKLEALEGKLEALEGKLEALEG-amide
C5d	Ac-KLEAC▼EGKLEALEGKLEALEGKLEALEGKLEALEG-amide
C9a	Ac-KLEALEGKC▼EALEGKLEALEGKLEALEGKLEALEG-amide
C12d	Ac-KLEALEGKLEAC▼EGKLEALEGKLEALEGKLEALEG-amide
C16a	Ac-KLEALEGKLEALEGKC▼EALEGKLEALEGKLEALEG-amide
C19d	Ac-KLEALEGKLEALEGKLEAC▼EGKLEALEGKLEALEG-amide
C23a	Ac-KLEALEGKLEALEGKLEALEGKC▼EALEGKLEALEG-amide
C26d	Ac-KLEALEGKLEALEGKLEALEGKLEAC▼EGKLEALEG-amide
C30a	Ac-KLEALEGKLEALEGKLEALEGKLEALEGKC▼EALEG-amide
C33d	Ac-KLEALEGKLEALEGKLEALEGKLEALEGKLEAC▼EG-amide

Rate of Formation of the Disulfide Bond. The formation of the disulfide bond was monitored quantitatively by RPC. All oxidized 70-residue peptides are eluted prior to their corresponding reduced 35-residue peptides, suggesting that the oxidized peptides retain a large portion of their coiled-coil structure during RPC (Hodges et al., 1988; Zhou, et al., 1992c; Zhu et al., 1992). Our previous studies (Hodges et al., 1990; Zhou et al., 1992c) showed that the interhelical disulfide bond could be readily formed by air oxidation of the reduced peptides in 0.1 M NH_4CO_3, pH 8.3, when a Cys residue is located at the ends of the coiled-coil (positions 2a and 33d). However, the oxidation rates are much slower when a Cys is located at a nonterminal position. The rate of formation of an interhelical disulfide bond in the coiled-coil under nondenaturing conditions is significantly dependent upon the position of the disulfide bond (Figure 1). Both local structure and flexibility of the region in which the Cys residue is located affect the rate of formation of the disulfide bond. It has been shown previously that the amino acids at positions a have similar local structure and are structurally distinct from the d positions which also have a similar local structure in the coiled-coil (O'Shea et al., 1991; Zhou et al., 1992c). The ends of the coiled-coil have been shown to be more flexible than the middle section (Goodman & Kim, 1991; Zhou et al., 1992a). As a

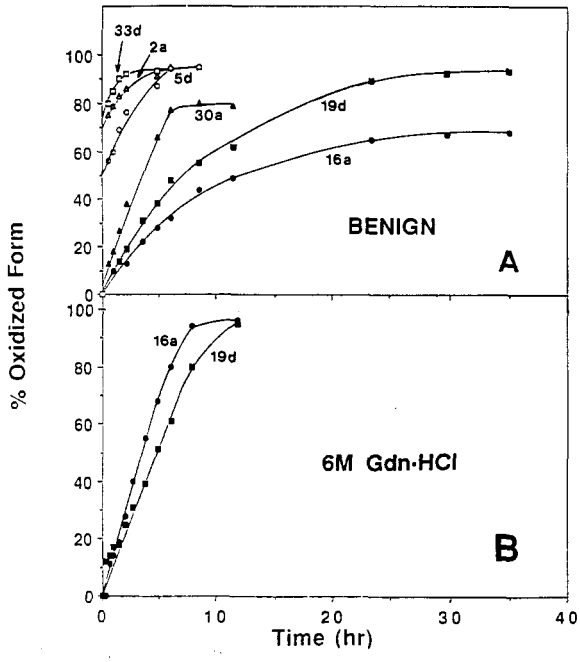

FIGURE 1: Kinetics of the formation of the interhelical disulfide bond of peptides C2a, C5d, C16a, C19d, C30a, and C33d under nondenaturing conditions (0.1 M Tris-HCl buffer, pH 8.3, at 25 °C) (panel A) and under denaturing conditions (0.1 M Tris-HCl buffer containing, 6 M Gdn·HCl, pH 8.3, at 25 °C) (panel B). All the peptides have been oxidized at the similar peptide concentrations (about 0.5 mg/mL). The percentage of the oxidized form was determined by RPC and expressed as the ratio of the peak area of the oxidized form to the sum of the peak areas of the oxidized and reduced forms.

disulfide bond is moved from the end to the central region of coiled-coil, the rate of disulfide bond formation is decreased (Figure 1). The oxidations were essentially completed within 4 h when the disulfide bond was formed at the end of the coiled-coil (positions 2a and 33d), whereas it required 6 or 24 h to reach the maximum oxidation for the peptide with a disulfide bond at positions 5d and 30a or positions 16a and 19d, respectively. These results indicate that a disulfide bond can be formed more easily in a flexible region than in a rigid region, even within a similar local structural environment. By comparison of the oxidation rate between position a and corresponding position d at the other end of the coiled-coil, the order of the oxidation rate was C33d > C2a, C5d > C30a and C19d > C16a as shown in Figure 1 under nondenaturing conditions. The formation of a disulfide bond is faster at position d compared to that at the corresponding position a. In addition, the formation of the disulfide bond could not be completed at nonterminal positions a under nondenaturing conditions (only 70% of C16a and 80% of C30a were oxidized), whereas under denaturing conditions (0.1 M Tris-HCl buffer containing 6 M Gdn·HCl, pH 8.3, at 25 °C), these peptides have fast and similar oxidation rates and are completely oxidized (Figure 1). These results suggest that position d is more suitable for engineering a disulfide bond.

Positional Effect of the Disulfide Bond on the Structure of the Coiled-Coil. The structures of the reduced and oxidized peptides were studied by circular dichroism. The CD spectra for all the reduced peptide analogues are very similar to each other and to the native coiled-coil protein. All showed the double minima absorptions at 207 and 222 nm in benign medium (0.1 M KCl, 50 mM PO_4, pH 7) with high ellipticity values (–28 160 ± 850 at 220 nm) (Table II) indicating high helical content. In principle, isolated single-stranded helices are unstable in aqueous solutions (Dyson et al., 1988; Saudek et al., 1991) and require the additional stabilization provided by the tertiary/quaternary structure or a less polar solvent, e.g. 1,1,1-trifluoroethanol (TFE) to induce α-helical structure. All these reduced peptides did not show any increase in helicity upon addition of the α-helix inducing solvent TFE, as measured by molar ellipticity at 220 nm (the values of $\Delta[\theta]_{220(H_2O-TFE)}$ are all negative for the native and reduded peptides as listed in Table II). These results suggest that the interhelical hydrophobic interactions in the coiled-coil can induce more α-helix than TFE can induce in the single-stranded form. The dimeric molecular weights of the native and the reduced peptide analogues (the two-stranded form of the peptides, 70 residues) in aqueous solution were determined by SEC using methodology described previously (Hodges et al., 1990; Zhou et al., 1990; Lau et al., 1984b) (Figure 2). These results demonstrate that a single mutation of Leu → Cys at positions a and d did not change the coiled-coil structure.

Once a disulfide bond is formed in the coiled-coil, the structures of these oxidized peptide analogues are significantly dependent upon the location of the disulfide bond. The proteins with the disulfide bridge at the N- or C-terminus of the coiled-coil or at nontermianl positions d have similar CD spectra and have essentially the same α-helical content ($[\theta]_{220}$ = –30 100 ± 1000 compared to the native coiled-coil $[\theta]_{220}$ = –29 100, Table II). These proteins did not show any significant increase in helicity upon addition of the α-helix inducing solvent TFE (Table II), suggesting that the interchain hydrophobic interactions have stabilized the amphiphathic α-helices so as to induce maximal α-helical structure. In contrast, the disulfide bridge at nonterminal positions a disrupts part of the coiled-coil structure as indicated by decreased ellipiticities in benign medium (–25 250, –20 300, –26 150, and –27 700 for the peptide C9a(o), C16a(o), C23a(o), and C30a(o), respectively) compared to the native coiled-coil (–29 100). Also, the addition of TFE significantly increases the helicity (Table II). The more helical structure of C30a(o) compared to C16a(o) in benign medium (Table II) implies that the closer the disulfide bridge is to the center of the coiled-coil, the larger the disruption or strain on the coiled-coil structure, when the disulfide bond is located at a nonterminal position a. The peptide with a disulfide bridge at central position a (position 16) has the least α-helical content (ellipticity value at 220 nm is –20 300 which corresponds to ~60% α-helical structure, Table II). These results, based upon α-helical content, suggest that the local stereochemical environment at the nonterminal positions d is suitable for engineering a disulfide bond. In fact, disulfide bonds at these positions induce slightly more α-helical structure than the corresponding reduced peptides (the values of $\Delta[\theta]_{220(ox-red)}$ in Table II are all negative for the peptides with a disulfide bond at position d). In contrast, the values of $\Delta[\theta]_{220(ox-red)}$ are are positive for the peptides with a disulfide bond at nonterminal position a (Table II). Disulfide bonds in the terminal positions a and d both increase the α-helical content over the reduced coiled-coil. It has been previously shown that there is more flexibility at the ends of the reduced coiled-coils (Zhou et al., 1992a) which may account for the increased helical content on insertion of a disulfide bond at the ends of the coiled-coil.

Size-exclusion chromatographic studies demonstrated that the oxidized peptides have the same apparent molecular weight as the native and the reduced peptides in aqueous solution (a 70-residue two-stranded form of the peptide), except for the oxidized peptide C2a(o) which showed the dimeric apparent molecular weight in aqueous solution; that is, a dimer of two 70-residue oxidized molecules (Figure 2). The four-stranded

Table II: Circular Dichroism Results of the Reduced and Oxidized Two-Stranded α-Helical Coiled-Coils

peptide[a]	$[\theta]_{220}$[b] (deg·cm²·dmol⁻¹) reduced	$[\theta]_{220}$[b] (deg·cm²·dmol⁻¹) oxidized	$\Delta[\theta]_{220(H_2O-TFE)}$[c] reduced	$\Delta[\theta]_{220(H_2O-TFE)}$[c] oxidized	$\Delta[\theta]_{220(ox-red)}$[d]	$[Gdn\cdot HCl]_{1/2}$[e] (M) reduced[f]	$[Gdn\cdot HCl]_{1/2}$[e] (M) oxidized
native	−29 100		−900			2.8 (113)	
			Terminal Cys Mutants				
position a							
C2a	−27 700	−29 800	−2250	0	−2100	2.2 (101)	5.1
position d							
C33d	−27 450	−30 800	−4350	+400	−3350	2.0 (107)	5.1
			Nonterminal Cys Mutants				
position a							
C9a	−28 400	−25 250	−6400	+2750	+3150	1.8 (135)	4.0
C16a	−27 800	−20 300	−3200	+5100	+7500	1.6 (108)	2.4
C23a	−27 300	−26 150	−3300	+2550	+1150	1.8 (123)	2.6
C30a	−29 000	−27 700	−6100	+2300	+1300	1.8 (119)	3.8
position d							
C5d	−27 850	−30 000	−6850	−2000	−2150	1.6 (128)	5.5
C12d	−28 200	−29 150	−5900	+650	−950	1.5 (108)	5.6
C19d	−27 950	−29 850	−6800	−700	−1900	1.6 (92)	5.1
C26d	−27 400	−31 150	−6350	−2600	−3750	1.5 (113)	5.7

[a] The sequences of peptides are shown in Table I. [b] The mean residue ellipticities at 220 nm were measured at 20 °C in benign buffer (0.1 M KCl, 0.05 M PO_4, pH 7.0). [c] $\Delta[\theta]_{220(H_2O-TFE)}$ is the difference between the ellipticity at 220 nm in benign buffer and in 50% TFE: $\Delta[\theta]_{220} = [\theta]_{220(benign)} - [\theta]_{220(50\%TFE)}$. [d] $\Delta[\theta]_{220(ox-red)}$ is the difference between the ellipticity at 220 nm in benign buffer between the oxidized and reduced peptides: $\Delta[\theta]_{220(ox-red)} = \Delta[\theta]_{220(ox)} - \Delta[\theta]_{220(red)}$. [e] $[Gdn\cdot HCl]_{1/2}$ is the transition midpoint: the concentration of guanidine hydrochloride (M) required to give a 50% decrease in ellipticity. [f] The values in parentheses are the concentrations (micromolar) of the reduced peptides used in Gdn·HCl denaturation experiments.

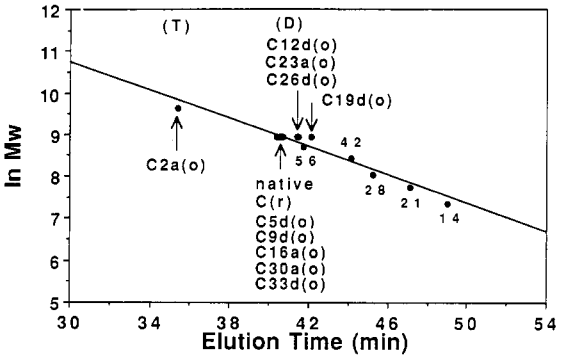

FIGURE 2: Standard plot of ln of the molecular weight versus the retention time of the synthetic reduced and oxidized peptides in SEC. The conditions for SEC are described in the Materials and Methods section. The samples were dissolved in 0.5 M KCl, 50 mM PO_4, pH 7, buffer. For the reduced peptides, 5 mM DTT was added to the solution to prevent the formation of an interchain disulfide bond. T or D represents the four-stranded or two-stranded form of the peptide, respectively. C(r) represents 10 single Cys-substituted peptides in their reduced forms, which were eluted at essentially the same retention time (range from 40.5 to 40.7 min). The nomenclature for the peptides is described in text. The numbers 28, 42, and 56 represent the number of residues in the molecular weight standard peptides (Hodges et al., 1990; Zhou et al., 1990) and are all two-stranded as a result of a disulfide bridge between peptides having the sequences Ac-K-C-A-E-L-E-G-[K-L-E-A-L-E-G-]$_n$-amide, where n = 1, 2, or 3. The 14- and 21-residue peptides of the above sequence are single-stranded.

form of C2a(o) (a dimer of two-stranded α-helical coiled-coils where the two α-helices of each coiled-coil are disulfide bridged) is not as stable as the two-stranded α-helical coiled-coil monomer, and the folded dimer can convert to the folded monomer on addition of low levels of Gdn·HCl without any change in α-helical content (Zhu et al., 1993). The transition in the Gdn·HCl denaturation profile between 4 and 6 M (top panel in Figure 3) corresponds to the conversion of the two-stranded α-helical coiled-coil to unfolded monomer. Sedimentation equilibrium study of the peptide C12(o) gave a molecular weight of 7330, which is in good agreement with that expected for the two-stranded structure (calculated molecular weight 7506).

Positional Effects of Disulfide Bonds on the Coiled-Coil Stability. The stabilities of the reduced and oxidized peptides were determined by measuring the ellipticities of the peptides at 220 nm as a function of Gdn·HCl concentration at 20 °C. As the peptides unfold, the ellipticity of the peptides decreases with increasing Gdn·HCl concentration. Figure 3 (top panel) shows representation Gdn·HCl denaturation curves of four of the disulfide bridged coiled-coils. The dramatic effect disulfide bridge location can have on protein stability is immediately apparent. Since the Gdn·HCl concentration at the midpoint ($[Gdn\cdot HCl]_{1/2}$) of the denaturation curve is the most accurate value for determining protein stability, the stabilities of the reduced and oxidized coiled-coils were expressed as $[Gdn\cdot HCl]_{1/2}$ (Table II).

It has been shown previously that protein stability is related to the hydrophobicity of the residues in the hydrophobic core of coiled-coils (Hodges et al., 1990; Zhou et al., 1992a,c) and globular proteins (Kellis et al., 1988, 1989; Dill, 1990; Matsumura et al., 1988; Lim & Sauer, 1989; Pace, 1992). The mutation of Leu residues at positions a and/or d to less hydrophobic amino acids normally decreases the stability of the coiled-coil (Hodges et al., 1990; Zhou et al., 1992a,c). In this study, all the reduced coiled-coils with a single Leu → Cys mutation were less stable than the native coiled-coil as shown by the transition midpoints (Table II and the bottom panel in Figure 3). However, the decrease in stability caused by a single Leu → Cys mutation is dependent on the position of the mutation and the destablizing effect is smaller at the ends of the coiled-coil (positions 2a and 33d) than in the middle section (positions 5d, 9a, 12d, 16a, 19d, 23a, 26d, and 30a) (bottom panel in Figure 3). These observations are consistent with our previous study (Zhou et al., 1992a) on coiled-coils with Leu → Ala mutations and demonstrates again that the Leu-Leu hydrophobic interactions are less important at the ends of the coiled-coil due to the increased flexibility.

As shown in Figure 3, the effect of an interchain disulfide bond on coiled-coil stability is considerably dependent upon its location. The peptides with a disulfide bridge at the ends of the coiled-coil (positions 2a and 33d) have the stability with a $[Gdn\cdot HCl]_{1/2}$ value of 5.1 M (Table II), implying that the insertion of disulfide bonds at a flexible region of a protein can significantly increase protein stability, whereas, the contribution of the insertion of disulfide bonds in the less

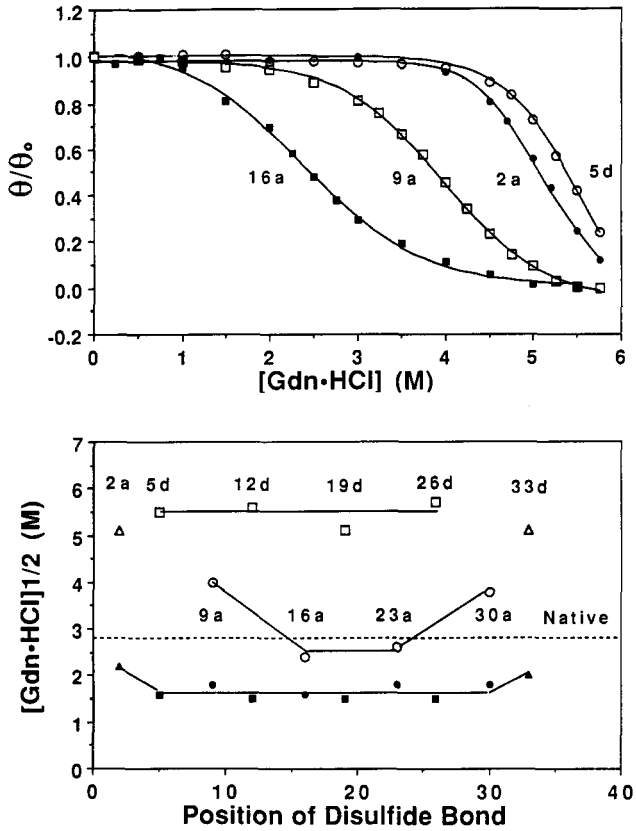

FIGURE 3: Top Panel: Guanidine hydrochloride (Gdn•HCl) denaturation profiles of the oxidized coiled-coils C2a(0), C5d(0), C9a-(0), and C16a(0) in 0.1 M KCl, 50 mM PO_4 buffer, pH 7, θ/θ_0 represents the ratio of molar ellipticity at 220 nm at the indicated molarity of guanidine hydrochloride concentration to the ellipticity without guanidine hydrochloride. Bottom Panel: Plot of the transition midpoint of the guanidine hydrochloride denaturation profiles (Table II) vs the position of an interhelical disulfide bond (5, 9, 12, 16, 19, 23, 26, 30, or 33). Open triangles denote the coiled-coils with a terminal disulfide bond at position 2 or 33. Open squares denote the coiled-coils with a nonterminal disulfide bond at positions d (position 5, 12, 19, or 26). Open circles denote the coiled-coils with a nonterminal disulfide bond at position a (position 9, 16, 23, or 30). Closed symbols denote the coiled-coils without a disulfide bridge. The dashed line indicates the $[Gdn•HCl]_{1/2}$ of the native coiled-coil.

flexible hydrophobic core is very dependent on the local stereochemical environment and the degree of disruption of the structure. The peptides with a disulfide bond at nonterminal positions "d" have similar stabilities with an average $[Gdn•HCl]_{1/2}$ value of 5.5 M and are more stable than those with a disulfide bonds at nonterminal positions a (Table II and bottom panel in Figure 3). The inappropriate local stereochemical environment of the disulfide bonds at positions a forces the disulfide bonds either to adopt an unfavorable geometry without disturbing the coiled-coiled backbone structure or to adopt the appropriate geometry of the disulfide bond and distort the coiled-coil structure. In either case, the strain energy introduced by this disulfide bond or distortion of the coiled-coil structure will destabilize the protein. For a disulfide bond at positions a in the coiled-coil structure, the stabilizing effect of the disulfide bond is decreased as the disulfide bond is moved from the ends of the coiled-coil to the central region of the coiled-coil. To evaluate the net effect of the disulfide bond on the protein stability, one needs to compare the stability of the oxidized form with the corresponding reduced form (Matsumura & Matthews, 1991). Figure 4 (top panel) plots the difference in the $[Gdn•HCl]_{1/2}$ between the oxidized and reduced form of a single Cys mutant coiled-coil. The order of the stability difference between the

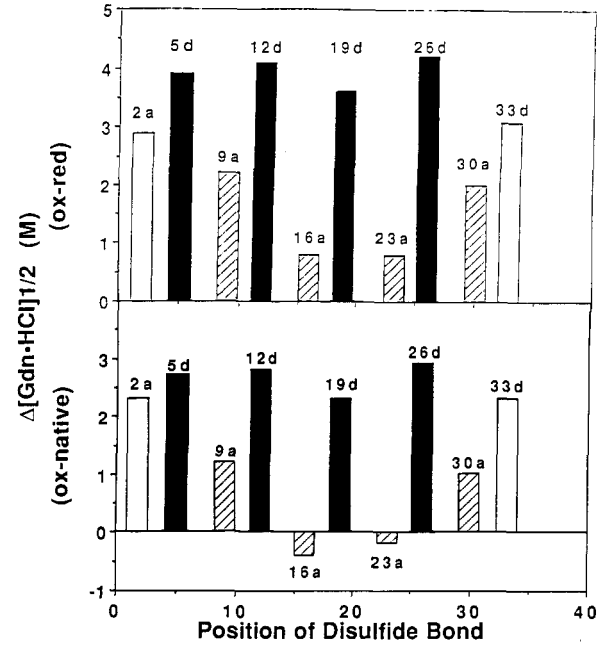

FIGURE 4: Plots of the difference in the $[Gdn•HCl]_{1/2}$ values between the oxidized and reduced form of the mutant coiled-coils (top panel) and the differences between the oxidized coiled-coils and the native coiled-coil (bottom panel). Open bars, solid bars, and shaded bars represent the mutation at the terminal positions, at the nonterminal positions d, and at the nonterminal positions a, respectively.

oxidized peptide with a disulfide bond at position a and the corresponding reduced form is C2a > C9a ~ C30a > C16a ~ C23a (the $\Delta[Gdn•HCl]_{1/2(ox-red)}$ values are 2.9, 2.2, 2.0, 0.8, and 0.8 M, respectively, (top panel in Figure 4)). These results, in agreement with Matsumura and Matthews (1991), imply that a flexible region of a protein may be a better site for engineering a disulfide bond with near-optimal geometry without the introduction of excessive strain. However, for a disulfide bond located in the appropriate stereochemical environment, such as those at positions d in the coiled-coil structure, the stabilizing effect of the disulfide bridge is greater in a rigid part of the molecule than in a flexible region. The average $\Delta[Gdn•HCl]_{1/2(ox-red)}$ value of 3.9 M (range from 3.5 to 4.2 M) for the mutation at the nonterminal positions d (positions 5d, 12d, 19d, and 26d) is larger than the average value of 3.0 M for the mutation at either terminal position a or d (position 2a or 33d) (top panel in Figure 4). It should be noted that when comparing the stabilities of the reduced and oxidized coiled-coils, we are comparing bimolecular and unimolecular unfolding transitions, where the $[Gdn•HCl]_{1/2}$ values of the reduced coiled-coils are dependent upon peptide concentration (Zhou et al., 1992a,b). Thus, in this study, the $[Gdn•HCl]_{1/2}$ values were determined at similar peptide concentrations.

In evaluating the overall effect of a disulfide bond on protein stability, the result of introducing a Cys residue into the wild-type protein must be considered. For this reason, the stabilities of the disulfide bond linked coiled-coils were compared with that of the native coiled-coil as shown in the plot of the difference in the $[Gdn•HCl]_{1/2}$ between the oxidized peptides and the native peptide (bottom panel in Figure 4). The three most effective disulfide bonds for improving the stability of the coiled-coil are formed at the nonterminal positions d (positions 5d, 12d, and 26d). In contrast, the two disulfide bonds which are formed at the central positions a (positions 16a and 23a) destabilize the coiled-coil compared to the native structure.

Table III: Comparison of the Geometry and Potential Energy of the Disulfide Bond at Non-Terminal Positions a (Position 23) and d (Position 19)

residue	$d(C_\beta - C_{\beta'})^a$ (Å)	$\chi_3{}^b$	τ^c	potential energy/cystine[d] (kcal·mol^{-1}) bond	angle	torsion	total
C23–C23′ (fix backbone)[e]	5.0 (6.1)	−138° (−90°)	120° (103°)	6.12	31.4	8.18	45.7
C19–C19′ (fix backbone)[e]	4.0 (3.9)	104° (+90°)	107° (103°)	2.63	6.89	3.86	13.4

[a] Distance between C_β and $C_{\beta'}$ in angstroms (Å). The values in parentheses are the distances in the X-ray structure of the GCN4 leucine zipper. [b] χ_3 is the dihedral angle about the disulfide bond. The values of −90° and +90° in parentheses correspond to a left handed and right handed disulfide. [c] τ is the bond angle between two γ-sulfur atoms and a β-carbon atom of Cys residue; 103° in parentheses is a ideal bond angle in the optimal disulfide. [d] Potential energy (kilocalories per mole) in one cystine due to the deformation of bond lengths, valence angles, and torsional angles, respectively.[e] The geometry and energy of the disulfide bond were measured in the coiled-coil structure after energy minimization with all atoms in the polypeptide backbone fixed. See text for details.

Molecular Modeling Study. As a covalent bond, the disulfide bond has strict stereochemical requirements for the relative positions and orientations of the two participating Cys residues. Satisfying the geometrical requirement seems to be most important in building a new disulfide bond in proteins. The interhelical disulfides bond at either the nonterminal position a (position 23) or nonterminal position d (position 19) were modeled on the GCN4 structure of a two-stranded α-helical coiled-coil (O'Shea et al., 1991), which is generally representative of all coiled-coils and can be superimposed on an ideal coiled-coil Cα backbone (generated using Crick's parameterization (Crick, 1953)) with a RMS deviation of 0.25 Å. The geometry and potential energy of the disulfide bond at position 23 or 19, which are representative of a disulfide bond at nonterminal position a or at nonterminal position d in the coiled-coil, were measured after energy minimization with all atoms in the polypeptide backbone fixed (Table III). A right-hand disulfide bond can be modeled at position d with near-optimal geometry (the dihedral angle about the disulfide bond is 104° and the bond angle between two γ-sulfur atoms and a β-carbon atom of cystine is 107° which are very close to the ideal values in the right-hand disulfide bond (Table III)) without changing the backbone structure and even the position of Cβ atoms in the coiled-coil (Figure 5). The distance between the two Cβ atoms of the cystine at position d is 4.0 Å which is almost the same as that observed in the GCN4 coiled-coil structure (3.9 Å), whereas only a disulfide bond with inappropriate geometry can be built at position a without changing the backbone structure of the coiled-coil (Table III and Figure 5). The inappropriate geometry of the disulfide bond at position a tends to introduce high strain energy (Table III) and changes the Cα–Cβ vectors as shown in Figure 5. The distance between the two Cβ atoms of the cystine at position a is 5.0 Å which is substantially shorter than the 6.1 Å observed in the GCN4 coiled-coil structure. It has been shown that a mutation which alters the positions of buried main-chain atoms or β-carbons is likely to have a greater effect on structure and stability than mutations that perturb only positions of the distal atoms of side chains (Daopin et al., 1991). The energy minimized coiled-coil structure with an interchain disulfide bond either at position a or at position d was compared to the X-ray structure of GCN4 (Figure 6). As shown in Figure 6, formation of a disulfide bond at position d in the coiled-coil only induces a small local structural change at the mutation site (right panel). In contrast, the formation of a disulfide bond at a nonterminal position a disrupts the polypeptide backbone along the entire coiled-coil structure (left panel in Figure 6). The energy minimized coiled-coil structure with an interchain disulfide bond at position a has a larger RMS difference from the X-ray structure of GCN4 than the coiled-coil with a disulfide bond at position d (Figure 7). The average RMS difference is 0.89 Å for the coiled-coil with a disulfide bond at position a compared to 0.48 Å for the coiled-coil with a disulfide bond at position d. These results suggest that an insertion of an interchain disulfide bond at position a in the coiled-coil structure has a higher potential energy and induces larger structural changes than an insertion at position d.

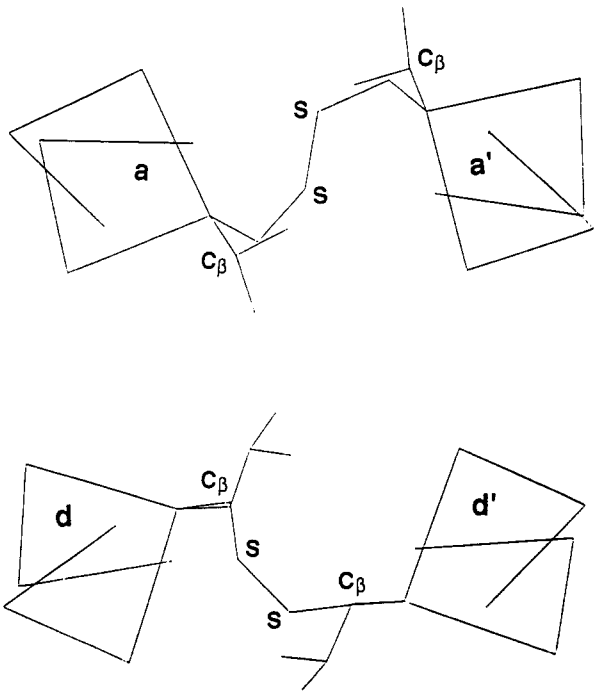

FIGURE 5: Computer modeling of an interhelical disulfide bond at position a or position d. The GCN4 leucine-zipper X-ray structure was used as a representation of the coiled-coil structure (O'Shea et al., 1991). Only side-chain heavy atoms of the residues at position 23 (a and a′) and position 19 (d and d′) as well as seven main chain α-carbon around this residue are displayed. The view is along the coiled-coil axis from the N-terminus.

DISCUSSION

The stabilizing effect of a disulfide cross-link is usually assumed to arise mainly from its effect on the unfolded state by decreasing the conformation entropy of the unfolded state (Flory, 1956; Poland & Scheraga, 1965; Chan & Dill, 1989). However, in many instances the disulfide cross-link can have an important effect not only on the unfolded state but also on the folded state as well. A complete analysis of the effect of a disulfide cross-link on protein stability needs to consider its effect on both folded and unfolded states. In principle, the insertion of a disulfide bond at a particular site in a protein will also involve alterations in structure which may produce additional destabilizing or stabilizing interactions. Doig and Williams (1991) have recently shown that when disulfide bonds stabilize proteins, they destabilize the folded structures

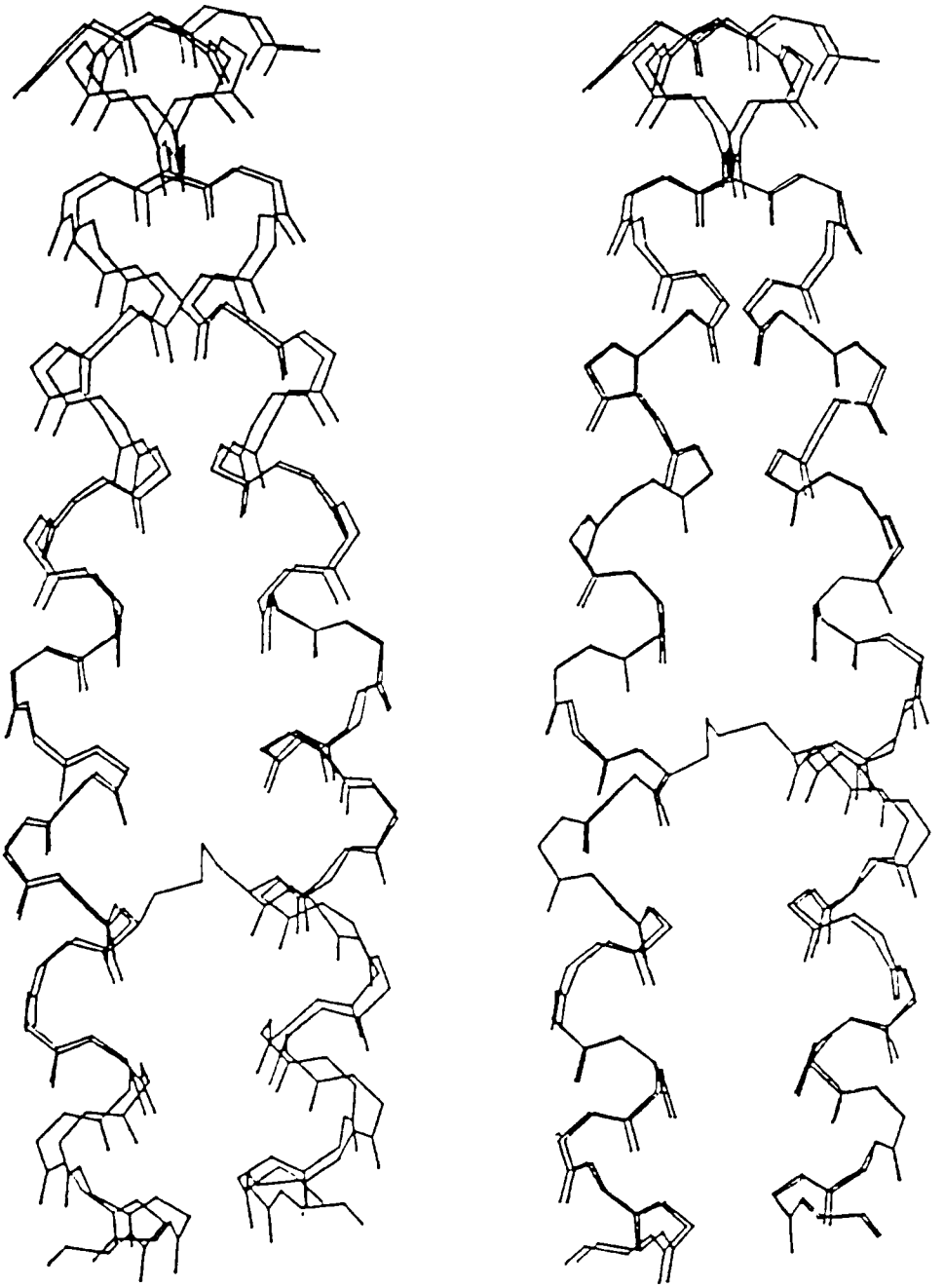

FIGURE 6: Comparison of the GCN4 leucine-zipper X-ray structure with the energy minimized structure of the oxidized coiled-coil where the disulfide bond is either at the nonterminal position a (position 23) (left panel) or at the nonterminal position d (position 19) (right panel). The structures are best fitted to minimize the root mean square deviation of α-carbons of residues 1–31 in the two chains of the coiled-coil. Only backbone atoms and cystine side-chain atoms are displayed.

entropically but stabilize them enthapically to a greater extent. In our recent study (Zhou et al., 1992c), we have demonstrated that the disulfide bridge between two helical chains at position 2 increased the hydrophobic interactions around the Leu residue at position 5 and decreased the flexibility of the N-terminal region of the peptide chains, resulting in more stable coiled-coils and more fixed side chain packing in the hydrophobic core.

In particular, the disulfide bridge can destabilize the folded protein by geometry constraints due to the covalent linkage. The strain energy associated with engineering a disulfide bond can be analyzed by comparing its structural features with the structure of the optimal disulfide bond. For the strict stereochemical requirements of a disulfide bond, the most important parameters are the distance between two sulfur atoms which must be close to 2.0 Å, the angle (τ) between the sulfide and β-carbon of each Cys residue which must be close to 103°, and the two β-carbon atoms which must be oriented to correspond to either +90° (right-handed) or −90° (left-handed) for the dihedral angle about the disulfide bond (χ_3) (Creighton, 1988). Considerable variations are possible with the other atoms of the two Cys residues, since at least 13 different conformations of the disulfide-bonded Cys side chains have been found in folded proteins (Creighton, 1988; Thornton, 1981; Richardson, 1981; Katz & Kossiakoff, 1986). The disulfide bond at position 19d can be built with the near-optimal geometry, while the disulfide bond at position 23a has inappropriate geometry with 2.07 Å for the disulfide bond length, 120° for the bond angle (τ), and −138° for the dihedral angle of χ_3 (Table III). Katz and Kossiakoff (1986, 1990) have analyzed the strain energies of disulfide bonds in proteins using an energy expression taken from Weiner et al. (1984).

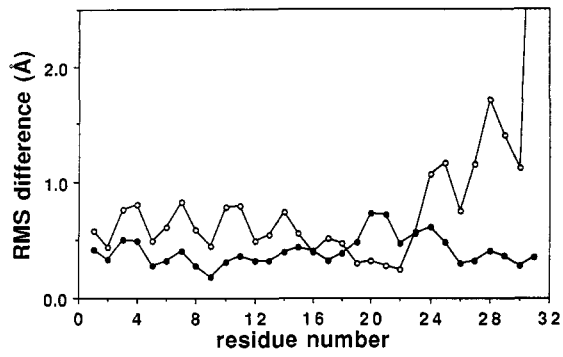

FIGURE 7: RMS difference between the polypeptide backbone atoms of the oxidized coiled-coil and the reduced coiled-coil. The two structures are best fitted to the α-carbons of residues 1–31 in the two chains of the coiled-coil. The energy minimized X-ray structure of GCN4 leucine-zipper was used as the reduced coiled-coil structure. The value plotted for each residue is the average difference of the backbone atoms N, CA, C, and O in the two chains of the coiled-coil. The open circles represent the average difference of each residue in the oxidized coiled-coil with the disulfide bond at nonterminal position a (position 23). The closed circles represent the average difference of each residue in the oxidized coiled-coil with the disulfide bond at nonterminal position "d" (position 19).

However, their calculations considered only the dihedral angles of the disulfide bond and did not include other factors, such as bond length and bond angle. In this study, the strain energies of the disulfide bonds were estimated by comparing the total potential energy in the cystine arising from the deformation of bond lengths, bond angles, and dihedral angles (Table III). In addition, the distances between both the Cα and Cα' atoms and Cβ and Cβ' atoms in cysteine pairs seem to be critical for the formation of a disulfide bond. For the cystines in native proteins, the Cα–Cα' distance range from 3.95 to 6.98 Å and the Cβ–Cβ' distance range from 3.37 to 5.21 Å (Kanaya et al., 1991). In the coiled-coil structure of the GCN4 leucine zipper, the distances of Cα–Cα' and Cβ–Cβ' are 5.5–5.9 Å and 5.7–5.8 Å at positions a and 6.1–6.4 Å and 3.6–4.1 Å at positions d. Only at positions d do the Cα–Cα' and Cβ–Cβ' distances fall in the effective distance range observed in native disulfide bonds. The Cβ–Cβ' distances at positions a are outside the effective distance range observed in native disulfide bonds. The observation that the disulfide bond induces less strain energy and causes less structural change at position d than position a, suggests that comparison of the potential energy in the cystine residue from the deformation of bond lengths, bond angles, and dihedral angles can give useful insights into the relative amount of strain in a disulfide bond.

On the basis of the observations with T4 lysozyme that the most effective disulfide bonds for improving protein stability were located at a flexible region of the molecule and that disulfide bonds which do not increase stability were located in rigid parts of the molecule, Matthews and co-workers (Matsumura & Matthews, 1991; Matsumura et al., 1989a) have suggested that the disulfide bonds should not be introduced in regions consisting of closely packed secondary structure such as α-helices and β-sheets or other rigid parts of the molecule. Studies on the X-ray structures of four genetically engineered disulfide variants of subtilisin have also indicated that the disulfides located in buried regions induced larger structural changes than those located on the surface of the molecule and these disulfide bonds exhibited atypical structures compared with known natural disulfide bonds (Katz & Kossiakoff, 1990). According to the survey by Thornton (1981), the naturally occurring disulfide bonds are rarely found within the secondary structure (α-helix and β-sheet) of proteins. However, the present study and the studies on the N-terminal domain of λ repressor (Sauer et al., 1986) and on the β/α-barrel protein (Eder et al., 1992) have clearly demonstrated that a disulfide bond can be introduced into the hydrophobic interface between two helices with considerable increase in protein stability without a change in protein structure. It has been observed that the cysteine residue at position 190 in the tropomyosin coiled-coil structure can be oxidized to form an interchain disulfide cross-link between two parallel helices (Johnson & Smillie, 1975; Lehrer, 1975) and the thermal denaturation of this cross-linked coiled-coil is different from that of a non-cross-linked tropomyosin coiled-coil (Lehrer, 1978; Holtzer et al., 1986). This disulfide cross-link, which is in the interior position a of the coiled-coil (Hodges et al., 1972; Sodek et al., 1972), introduced a considerable steric strain and destabilized coiled-coil structure resulting in the pretransition of a local unfolding of the helix in the vicinity of Cys-190, where the cross-link is located (Lehrer, 1978; Skolnick & Holtzer, 1986). This observation is also in agreement with the results reported in this study.

The results from this study have shown that the three most effective disulfide bonds (5d, 12d, and 26d) and the two most destabilizing disulfide bonds (16a and 23a) are all located in the closely packed interior or rigid part of the coiled-coil. These results demonstrate that whether a new disulfide bond should be engineered at a flexible or a rigid region of molecule is significantly dependent on the geometry or the local stereochemical environment of the disulfide bond. A disulfide bond, which can be built with optimal geometry in a closely packed hydrophobic core, will provide the greatest stabilizing effect. However, if the disulfide bond introduces strain it will disrupt the protein structure and may be less effective than a disulfide bond introduced into a more flexible region of the protein. Because the interhelical interactions are common in globular proteins as well as coiled-coils, the results obtained in this study will have general utility for selecting the sites for engineering interhelical disulfide bonds.

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