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STABILIZATION OF β-RIBBON STRUCTURES IN PEPTIDES USING DISULFIDE BONDS

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Summary: The effect of a disulfide crosslink between two peptide chains on the stability of β-ribbon secondary structures formed by these peptides has been investigated. Based on structural principles, we hypothesized that introduction of an unstrained disulfide crosslink at appropriate locations on two peptide chains should have a stabilizing effect on the β-ribbon structure formed by these two peptide chains. To test this hypothesis, we designed and synthesized two sets of 9-residue peptides incorporating cysteine in one and (S)-α-amino-ε-mercaptohexanoic acid in the other. Comparison of the CD data clearly show that the dimer containing a disulfide bond between the longer sidechains of (S)-α-amino-ε-mercaptohexanoic acid shows dramatically higher β-ribbon character as compared to the dimer with cystine disulfide bond, thus validating our structural hypothesis.

Designing peptides that will adopt predetermined secondary structures is the goal of several active laboratories (1-4). α-Helix (5, 6) and β-ribbon (7) are two common secondary structures found in proteins. Although substantial progress has been achieved in designing α-helices (8, 9), very little progress has been made in designing β-ribbon structures. Molecular dynamics simulations (10), host guest studies (11-13) study of amphiphilic structures formed by leucine-rich peptides (14), copolymers containing alternating hydrophobic and hydrophilic residues (15-19) and switch peptides that undergo conformational change as a function of pH (20) or side chain polarity in methionine rich peptides (21), use of planar hydrogen bonding templates (22-23) and use of benzofuran derivatives to anchor two peptide chains at a suitable distance from each other (24-27), and use of cystine disulfide linkages to hold two strands together (28-30) represent major advances in the field. However, designing water-soluble β-ribbon peptides is still a challenging problem. NMR studies on short cystine peptides show the presence of some β-ribbon like hydrogen bonds in organic solvents (28-30). Unfortunately, cystine disulfide bonds do not have the perfect geometry to hold two adjacent strands in β-ribbon conformation (31). We hypothesized that if we cross link two independent peptide chains via a single disulfide bond at appropriate locations without causing any strain, we should be able to stabilize β-ribbon secondary structures in aqueous solution.

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We designed two sets of 9-residue peptides containing different mercaptoamino acid residues to test our structural hypothesis (Figure 1). One peptide incorporates a cysteine residue and the other contains (S)-α-amino-ε-mercaptohexanoic acid. Model building revealed that the cystine disulfide bridge introduces some structural strain as previously suggested (31), whereas the disulfide linkage between two longer side chains of α-amino-ε-mercaptohexanoic acid appropriately positioned would be strain free. Our system is modelled after the β-ribbon segment of the Met J repressor responsible for its sequence specific DNA-binding (32). The peptides were synthesized as carboxamides using Fmoc chemistry and the amino group at the amino terminus was kept unprotected. The peptides contain two lysine residues near the amino end. We hoped that the presence of three positive charges at one end of the chain would orient the two chains to be antiparallel after oxidation to form the dimer. The secondary structural studies were done using CD spectroscopy.

MATERIALS AND METHODS

Peptide synthesis. The peptides were synthesized as carboxamides by using PAL resin on a Milligen/Biosearch 9050 peptide synthesizer using 9-fluorenylmethyloxycarbonyl (Fmoc) chemistry and BOP / HOBT activation. The peptides were cleaved from the resin with trifluoroacetic acid-thioanisole-ethane dithiol-anisole (90:5:3:2) at room temperature for 6 hours and precipitated in diethyl ether at -20° C. Enantiomerically pure N-Fmoc-S-Mob-(S)-α-amino-ε-mercaptohexanoic acid (Amh) was synthesized in a three step process starting from (L)-lysine, via nucleophilic displacement of the pyridinium salt (33). The unnatural amino acid was incorporated into the peptides using standard coupling procedures described above. The Mob protecting group was cleaved along with the other side chain protecting groups using trifluoroacetic acid in the presence of scavengers described above.

Purification. Peptides were purified to homogeneity on a reversed-phase C_{18} HPLC column (Rainin, Woburn, MA) using water-trifluoroacetic acid (0.1 %) and acetonitrile-trifluoroacetic acid

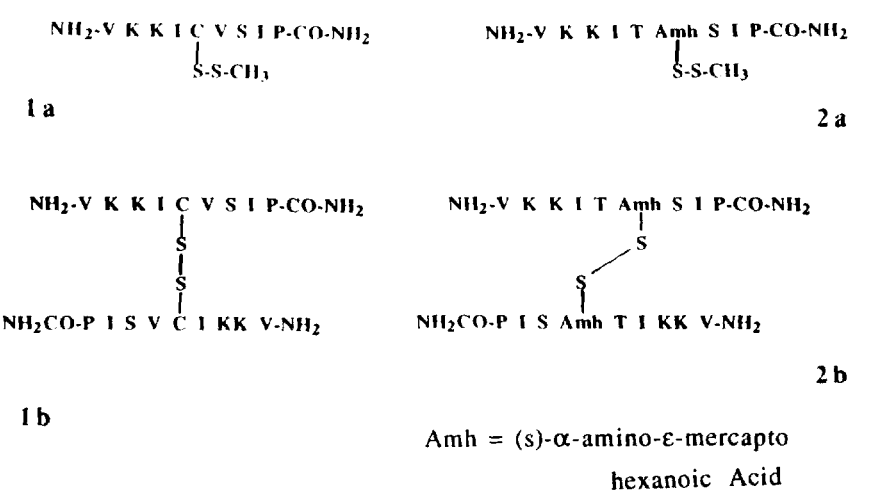

Figure 1. The sequences of the two sets of peptides β-cys and β-Amh.

(0.1 %) mixtures. The detector was set at 215 nm. The peptide purity was checked by analytical HPLC and the primary structure confirmed by quantitative amino acid analysis done on a Beckman 6300 high-performance amino acid analyzer at the Protein Structure Laboratory at University of California, Davis and the molecular weight determined using FAB-MS done at the Advanced Instrumentation Lab at University of California, Davis.

Blocking the sulfhydryl groups of the monomers. The thiol monomer in water was reacted with a solution of methyl methanethiosulfonate in ethanol solution at 0° C for 3 hours (34). The reaction mixture was directly injected into a preparative reversed phase HPLC column and the major peak was isolated.

Oxidation of the sulfhydryl groups to the disulfide. The thiol peptide in 80% aqueous acetic acid solution was reacted with a solution of iodine (2 eq.) at 0° C for 45 min (35). The reaction mixture was injected into a preparative reversed phase HPLC column and the major peak was isolated.

Circular dichroism (CD) measurements. CD spectra were recorded on a Jasco 600 spectropolarimeter. A total of 15 scans were averaged to obtain each spectrum. All CD studies were carried out in water containing micromolar concentrations of sodium dodecyl sulfate (SDS) at pH 5.5, using 10 mm path length cell at room temperature. The presence of low concentrations of SDS is known to favor β-ribbon structure formation (36-38). The peptide concentration in CD experiments were 5 μM. The peptide concentrations were determined by quantitative amino acid analysis done on a Beckman 6300 high-performance amino acid analyzer, with an error estimate of ± 2%.

RESULTS AND DISCUSSION

The Cysteine Model. In the cysteine based system, the thiol protected monomer showed a CD spectrum characteristic of a random coil while the disulfide bridged dimer showed some β-ribbon character (Figure 2) (39). For three different SDS concentrations, the -SCH_3 protected monomer

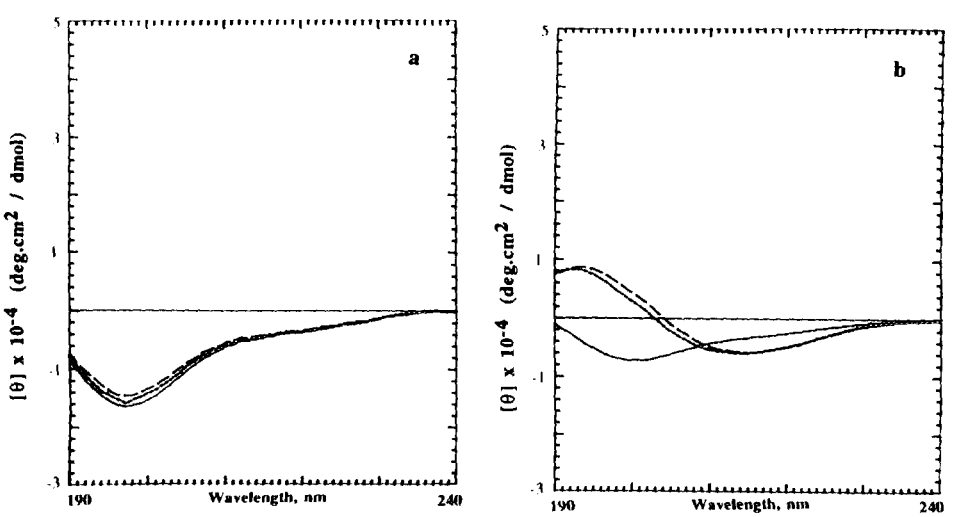

Figure 2. CD spectra of 5 μM solution of peptides in aqueous solution containing 20 (——), 60 (·····), and 100 (------) μM SDS at room temperature. (a) methylthio (CH_3S-) protected monomer (**1a**). (b) Disulfide bridged dimer (**1b**).

(**1a**) showed the characteristic random coil pattern, having a large negative mean residue ellipticity at 197 nm. The disulfide bridged peptide dimer showed distinct β-ribbon properties at 60 μM SDS, with a minima around 217 nm and its maxima around 195 nm.

(S)-α-Amino-ε-Mercaptohexanoic Acid (Amh) Model. The "Amh" model attempts to address the "Cys" peptide's shortcomings. The length and conformation of a cystine disulfide bond does not perfectly match the spacing between two adjacent strands in a β-ribbon structure (31). The "Amh" design places the sulfhydryl residue in a staggered fashion with respect to the C_2-axis of symmetry upon oxidation. The disulfide bridge between the longer side chains of Amh enjoys greater flexibility and imposes no strain on the peptide chains. CD studies substantiate these structural considerations, as shown in Figure 3.

The β-Amh -SCH_3 protected monomer showed almost similar random coil spectra as the cysteine-SCH_3 protected monomer. However, the "Amh" dimer showed typical β-ribbon CD spectrum. At 100 μM SDS, the "Amh" system's β-ribbon character is much more pronounced. Its maxima and minima are approximately three times that of its cystine analog. Its $[\theta]_{217}$ was -18, 200 deg.cm^2.dmol^{-1}, which is very similar to the ellipticity of polylysine in β-ribbon form (39). Upon heating the solution, β-ribbon character of the peptide decreases with increasing temperature as expected (Figure 4).

CONCLUSION

We have demonstrated that it is possible to design water-soluble β-ribbon secondary structures by the introduction of a disulfide bridge between appropriately positioned amino acid

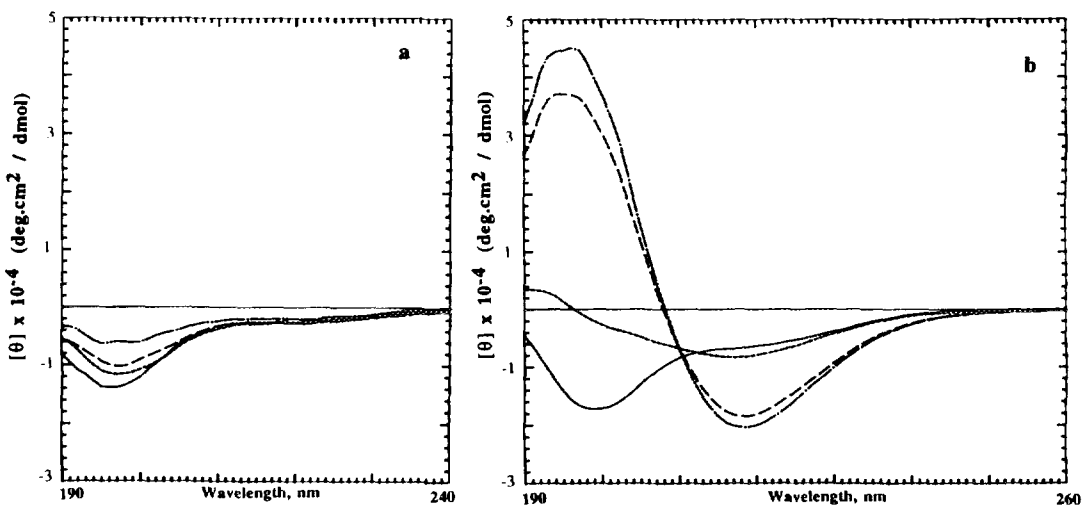

Figure 3. CD spectra of 5 μM solution of peptides in aqueous solution containing 20 (——), 60 (·····), 100 (------) and 130 (——) μM SDS at room temperature. (a) methylthio (CH_3S-) protected monomer (**2a**). (b) Disulfide bridged dimer (**2b**).

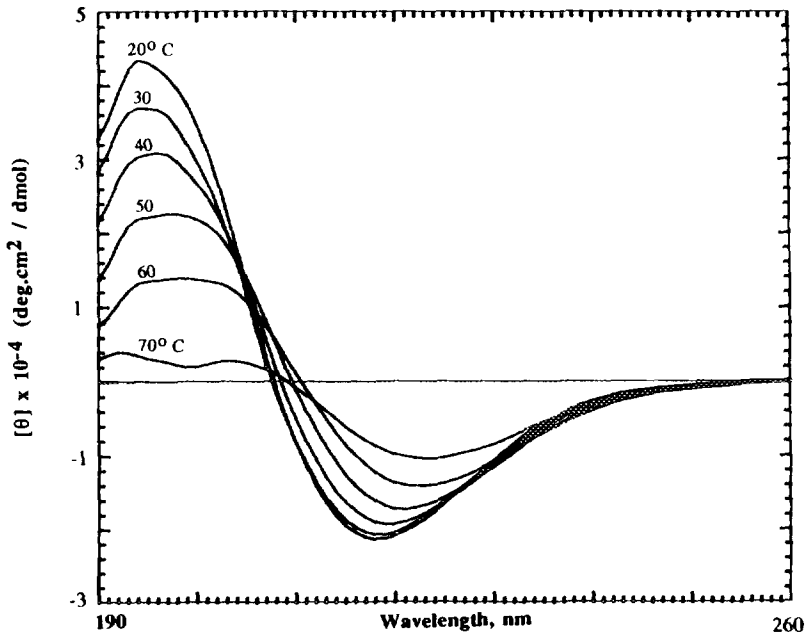

Figure 4. Changes in CD spectra of 5 μM solution of Amh peptide dimer (**2b**) as a function of temperature (20-70° C).

residues on two peptide chains. Comparison of our "Cys" and "Amh" models clearly shows that β-ribbon formation and stability is enhanced by the introduction of a strain-free disulfide bridge between the two chains. While the "Cys" and "Amh" thiol protected monomers exhibit very similar random coil behavior, the contrast between the respective dimer systems is striking.

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