Wetterau, J. R., Combs, K. A., Spinner, S. N., & Joiner, B. J. (1990) *J. Biol. Chem. 265*, 9800–9807.
Wiech, H., Stuart, R., & Zimmermann, R. (1990) *Semin. Cell Biol. 1*, 55–63.
Wodak, S. J., de Crombrugghe, M., & Janin, J. (1987) *Prog. Biophys. Mol. Biol. 49*, 29–63.
Wong, K.-P., & Tanford, C. (1973) *J. Biol. Chem. 248*, 8518–8523.
Wrba, A., Jaenicke, R., Huber, R., & Stetter, K. O. (1990a) *Eur. J. Biochem. 188*, 195–201.
Wrba, A., Schweiger, A., Schultes, V., Jaenicke, R., & Závodszky, P. (1990b) *Biochemistry 29*, 7584–7592.
Wright, P. E., Dyson, H.-J., & Lerner, R. A. (1988) *Biochemistry 27*, 7167–7175.
Yu, M. H., & King, J. (1988) *J. Biol. Chem. 263*, 1424–1431.
Yutani, K. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4441–4444.
Zettlmeissl, G., Rudolph, R., & Jaenicke, R. (1979) *Biochemistry 18*, 5567–5571.
Zettlmeissl, G., Teschner, W., Rudolph, R., Jaenicke, R., & Gäde, G. (1984) *Eur. J. Biochem. 143*, 401–407.

Articles

Designed Coiled-Coil Proteins: Synthesis and Spectroscopy of Two 78-Residue α-Helical Dimers†

Marisa Engel,‡ Robert W. Williams,§ and Bruce W. Erickson*,‡
Department of Chemistry, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3290, and Department of Biochemistry, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814
Received September 6, 1990; Revised Manuscript Received December 5, 1990

ABSTRACT: Receptor-adhesive modular proteins are nongenetic proteins designed to contain ligand, spacer, coil, and linker modules and to interact strongly with integrins or other types of cell-surface receptors. We have designed, chemically synthesized, and characterized a 39-residue peptide chain having a 6-residue ligand module (Gly-Arg-Gly-Asp-Ser-Pro-) for adherence to Arg-Gly-Asp-binding integrin receptors, a 3-residue spacer module (-Gly-Tyr-Gly-) for flexibility, and a 30-residue coil module [-(Arg-Ile-Glu-Ala-Ile-Glu-Ala)$_4$-Arg-Cys-NH_2] containing four 7-residue repeats for dimerization. This chain was designed to form a 78-residue noncovalent dimer (P39) by folding the coils of two chains into an α-helical coiled coil through hydrophobic interaction of eight pairs of Ile residues. Air oxidation of P39 gave P78, a 78-residue covalent dimer having a disulfide bridge linking its C termini. Raman spectroscopy indicated that both synthetic proteins have high α-helical content. Ultraviolet circular dichroic spectroscopy indicated that both dimers contain stable α-helical coiled coils. Its C-terminal disulfide bridge renders P78 significantly more stable than P39 to thermal denaturation or denaturation by urea. The coiled coil of P39 was 30% unfolded near 55 °C and half-unfolded in 8 M urea, while that of P78 was 30% unfolded only near 85 °C. These studies have demonstrated the feasibility of using these ligand, spacer, and coil modules to construct the designed coiled-coil proteins P39 and P78, a stage in the nanometric engineering of receptor-adhesive modular proteins.

Integrins are cell-surface receptors present on cells such as fibroblasts (Ruoslahti et al., 1985; Ruoslahti & Pierschbacher, 1986, 1987) or platelets (Hynes, 1987; Phillips et al., 1988; Giltay & van Mourik, 1988) that interact with fibronectin, vitronectin, fibrinogen, or other extracellular matrix glycoproteins. Several integrins function through binding to regions containing the tripeptide segment -Arg-Gly-Asp-. These protein/cell interactions are important for the growth, differentiation, proliferation, and functional regulation of cells (Juliano, 1987). Development of synthetic molecules that could specifically and strongly inhibit an extracellular protein/cell-surface receptor interaction or promote cell/cell adhesion would allow diagnostic agents or drugs to be targeted to specific types of cells.

Receptor-Adhesive Modular Proteins. A common and fruitful approach to cell-surface targeting uses an antibody (a soluble dimeric receptor) to bind bivalently to two copies of an epitope (a cell-surface ligand). We are exploring a complementary approach that uses a *soluble dimeric ligand* to bind bivalently to two copies of a *cell-surface receptor*. A receptor-adhesive modular protein (RAMP)[1] is a nongenetic protein designed to function as a soluble dimeric ligand through the presence of specific peptide segments that serve as ligand, spacer, coil, and linker modules. For example, the 82-residue structure of the monomeric RAMP P82 (Figure 1) has two

† This work was supported by a graduate fellowship (to M.E.) during 1987–1989 from La Caxia (Spain), by Grant CO71A1 (to R.W.W.) from the Uniformed Services University, and by U.S. Public Health Service Grant GM 42031 (to B.W.E.) from the National Institute of General Medical Sciences.
‡ University of North Carolina.
§ Uniformed Services University.

[1] Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butoxycarbonyl; BAW, 4:1:5 (v/v) 1-butanol/acetic acid/water; BSA, bovine serum albumin; CHO, chinese hamster ovary; CD, circular dichroism; DCM, dichloromethane; Dmb, dimethylbenzoyl; DIEA, *N*,*N*-diisopropylethylamine; DTT, dithiothreitol; *E*, volume percentage of TFE; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; M_r, mass ratio; NMP, *N*-methyl-2-pyrrolidinone; RAMP, receptor-adhesive modular protein; RGD, Arg-Gly-Asp; *T*, temperature; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; t_r, retention time; *U*, molar concentration of urea.

0006-2960/91/0430-3161$02.50/0

P33, A Noncovalent Spacer-Coil Dimer

```
GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S(Acm)
  :  :    :  :    :  :    :  :
GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S(Acm)
```

P39, A Noncovalent Ligand-Spacer-Coil Dimer

```
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-SH
             :  :    :  :    :  :    :  :
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-SH
```

P78, A Covalent Ligand-Spacer-Coil Dimer

```
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S
             :  :    :  :    :  :    :  :     |
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S
```

P82, A Monomeric RAMP

```
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S
             :  :    :  :    :  :    :  :      >Dmb-GKC-SH
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S
```

P164, A Homodimeric RAMP

```
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S
             :  :    :  :    :  :    :  :      >Dmb-GKC-S
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S          |
                                                         |
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S          |
             :  :    :  :    :  :    :  :      >Dmb-GKC-S
GRGDSP-GYG-RIEAIEA-RIEAIEA-RIEAIEA-RIEAIEA-RC-S
______ ___ _______________________________   ________
ligand |              coil                       |
   spacer                                     linker
          <-- α-helical coiled coil -->
```

FIGURE 1: Two receptor-adhesive modular proteins and three related dimeric proteins. Key: A = Ala; Acm = acetamidomethyl; Dmb = 3,5-dimethylbenzoyl; C = Cys-NH_2; D = Asp; E = Glu; G = Gly; H = hydrogen; I = Ile; K = Lys; P = Pro; R = Arg; s = sulfur; S = Ser; Y = Tyr; – = covalent bond; | = disulfide bond; : = hydrophobic interaction.

copies of a two-chain α-helical coiled coil joined by a linker module. Its ligand modules are RGD-containing peptide segments for interacting with an RGD-binding integrin receptor. Joining two such monomeric RAMP molecules by forming a disulfide bond between their C-terminal CysNH_2 residues would give the 164-residue homodimeric RAMP P164. The cross-linking dimethylbenzoyl (Dmb) moiety could be introduced through succinimido 3,5-bis(bromomethyl)-benzoate (Albrecht et al., 1990). Similarly, linking two monomeric RAMPs bearing different ligand modules would give a heterodimeric RAMP.

Monomeric RAMP P82 should bind only weakly to one integrin receptor on the surface of a target cell (Figure 2). In contrast, homodimeric RAMP P164 should be a potent inhibitor of receptor binding by competitively binding to two copies of the targeted receptor on the surface of the same cell at the same time. A heterodimeric RAMP should be not only a potent but also a highly selective inhibitor of receptor binding because it should bind strongly and selectively to a cell bearing *two* different types of targeted receptors on its surface but should bind only weakly to a cell bearing just one of the two types of targeted receptors. Such dimeric RAMPs would be a new class of potent and selective reagents for cell-surface targeting. They should be useful in receptor phenotyping; as high-affinity inhibitors of specific cell/cell, cell/matrix, or cell/ligand interactions; or for delivering reporter groups (chromophores or radiolabels) or cytotoxins (drugs or radionuclides) to specific cells.

RAMP Design. RAMPs are synthetic macromolecules designed to interact strongly with specific types of cell-surface

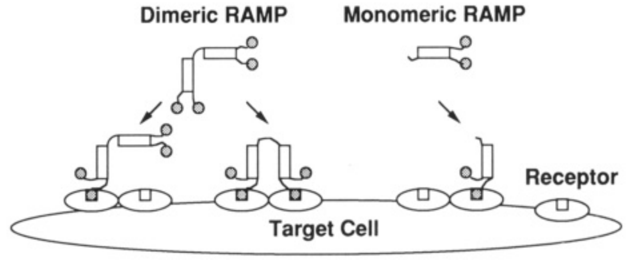

FIGURE 2: Proposed interactions of monomeric and dimeric RAMP molecules with cell-surface receptors.

receptors. The design of RAMP P164 required information about the size and spacing of the targeted cell-surface receptors. Electron microscopic images of the GPIIb/IIIa complex, a typical integrin receptor isolated from human platelets (Phillips et al., 1988), reveal an ellipsoidal structure with axial dimensions of about 80 × 80 × 100 Å (Carrell et al., 1985). This result is consistent with a calculated value of 120 Å for the mean center-to-center distance between these receptors on the surface of a single platelet on the basis of 44 000 receptors/cell (Shiba et al., 1988). Thus, the edge-to-edge distance between GPIIb/IIIa complexes on the platelet surface is estimated to be about 30 Å. Addition of ADP and fibrinogen, however, induces these GPIIb/IIIa complexes to cluster on the platelet surface to a mean center-to-center distance of 50 Å or less, on the basis of nuclear magnetic resonance line-broadening experiments (Shiba et al., 1988). This distance is much less than the minor axial diameter (80 Å) of an isolated GPIIb/IIIa complex, which suggests that substantial conformational changes are induced in cell-bound GPIIb/IIIa receptors by the action of ADP and fibrinogen. These results indicate that bivalent binding of a dimeric RAMP to a blood platelet should be possible by using a flexible structure that can position two ligand modules at distances between 50 and 120 Å.

Monomeric RAMPs are designed to contain a rigid two-stranded coiled coil with each coil bearing a ligand on a flexible spacer. The four structural modules (ligand, spacer, coil, linker) are peptide segments assembled from amino acids or other chemical units by peptide synthesis. Two α helices can wrap around one another parallel and in register to form a stable coiled-coil structure. Two natural examples are tropomyosin (Hodges et al., 1972) and streptococcal M protein (Hollingshead et al., 1986). The structural motif required for formation of a coiled coil is repetition of a pattern of seven residues (XHXXHXX) with the second and fifth being hydrophobic (H), especially the large aliphatic hydrophobes leucine or isoleucine (Talbot et al., 1982). These pattern places these hydrophobic residues on the same side of the α helix. Two such amphiphilic α helices can interact noncovalently so that the hydrophobic residues at the second positions of two juxtaposed repeats pack against one another, as do those at the fifth positions.

Robert Hodges and co-workers designed a synthetic peptide consisting of five adjacent repeats of Lys-Leu-Glu-Ala-Leu-Glu-Gly (KLEALEG) and found that it dimerized to form a two-stranded α-helical coiled-coil protein (Hodges et al., 1981, 1988, 1990; Lau et al., 1984a). The synthetic peptide having only four such repeats also gave a stable coiled-coil structure (Lau et al., 1984a,b). We designed a coil module having four repeats of the segment Arg-Ile-Glu-Ala-Ile-Glu-Ala (RIEAIEA) plus the beginning of a fifth repeat (Arg-Cys-NH_2), which should dimerize to form a coiled coil. The Lys, Leu, and Gly residues of the Hodges repeat were replaced by the chemically similar residues Arg, Ile, and Ala, respec-

tively. The resulting coiled coil would be stabilized by the helix-forming tendencies of Ala, Arg, Glu, and Ile (Padmanabhan et al., 1990; Merutka et al., 1990) and by interstrand bonding of eight Ile/Ile hydrophobic interactions, eight Arg/Glu ion pairs, and one covalent Cys/Cys disulfide bridge (Hodges et al., 1981, 1988, 1990).

RAMP Valency. Dimeric RAMP P164 could be prepared by cross-linking the C-terminal Cys-NH_2 residues of two monomeric RAMPs through formation of a disulfide bond. Since a monomeric RAMP contains two identical peptide chains, it is economical to extend each chain at the same time to incorporate a ligand (A or B) and a flexible spacer. A dimeric RAMP bears four ligands (A,A/B,B), two on each monomer. It is a homodimer if A = B and a heterodimer if A ≠ B. Two ligands (A,A or B,B) on the same monomer are functionally equivalent but probably too close to one another to be able to bind to two receptors on the same cell at the same time. But two ligands (A/B) attached to different monomers can easily be 100 Å apart. The length of each 30-residue coiled coil is estimated to be 30 × 1.5 Å = 45 Å. If the flexible linker were fully extended (40 Å) between two coiled coils (45 Å each), the outer ends of the coiled coils could be as much as 130 Å apart. Adding two fully extended flexible spacers (10 Å each) yields a maximum distance of 150 Å between ligands A and B. If this extended, "I"-shaped structure is bent into an "L"-shaped structure by rotating about the central disulfide bond until the coiled coils are at right angles to one another, the distance between ligands A and B becomes about 105 Å. In practice, this distance should vary dynamically over a wide range of values as the spacer and linker modules flex. More likely values fall in the range of 55–135 Å. Any of these values should be adequate to allow ligand A of one monomer of a dimeric RAMP to bind to one receptor and ligand B of the other monomer to bind to another receptor on the same cell at the same time. Even though a dimeric RAMP contains four ligand modules (A,A/B,B), it is effectively only *bivalent* (A/B).

This line of reasoning is supported by results of Danilov and Juliano (1989) on the ability of Gly-Arg-Gly-Asp-Ser-Pro-Cys (GRGDSPC) ligand modules covalently bound to BSA to adhere to vitronectin integrin receptors on CHO cells. Radiolabeled CHO cells were allowed to adhere to a plastic well coated with a $(GRGDSPC)_n$BSA conjugate. For the *same* amount of peptide ligand per well, cell adhesion increased dramatically with increasing n, the mean number of peptide chains per conjugate. Adhesion measured about 5% of maximal for $n = 2.5$, 35% for $n = 5$, 65% for $n = 9.5$, and 95% for $n = 19$. It was not the number of peptide ligands per well but the number of peptide ligands per *molecule* of BSA carrier protein that governed the efficiency of the conjugate/cell adhesion. Even more revealing, doubling n from 5 to 9.5 (or from 9.5 to 19) decreased the concentration of peptide ligand needed for half-maximal adhesion by about a factor of 4. One explanation for this apparent dependency of adhesion on n^2 is that for effective adhesion a CHO cell needs to bind to *two* peptide ligands on the *same* BSA molecule, behaving as a bivalent RGD/RGD homodimer. The maximal effective distance between two ligands in this conjugate is 60 Å, the diameter of the globular BSA protein. This separation could be long enough to allow to GRGDSPC ligands on the same BSA molecule to bind to two vitronectin integrin receptors on the same CHO cell at the same time. By analogy, a flexible dimeric RAMP could also bind through two nonadjacent ligands (A/B) to two receptors on the same cell at the same time.

We report here the chemical synthesis and characterization of proteins P33, P39, and P78 (Figure 1). Raman and ultraviolet CD spectroscopy indicate that they mainly contain two-stranded α-helical coiled coils, that P33 and P39 are noncovalent dimers, and that P78 is a relatively stable covalent dimer.

Experimental Procedures

Solid-Phase Assembly of P39. The 39-residue linear peptide chain Gly-Arg-Gly-Asp-Ser-Pro-Gly-Tyr-Gly-(Arg-Ile-Glu-Ala-Ile-Glu-Ala)$_4$-Arg-Cys-NH_2 was assembled by the stepwise solid-phase method (Erickson & Merrifield, 1976) with an Applied Biosystems Model 430 automatic peptide synthesizer. The solid support was 4-methylbenzhydrylamine–copoly(styrene–1% divinylbenzene) (0.5 mmol; 0.62 mmol of amino groups/g) (Matsueda & Stewart, 1981). All reagents, solvents, and protected amino acids were obtained from Applied Biosystems. The N^{α}-protecting group was Boc, and the side-chain-protecting groups were 4-toluenesulfonyl for Arg; benzyl for Asp, Glu, and Ser; 4-methylbenzyl for Cys; and 2-bromobenzyloxycarbonyl for Tyr. Each Boc-amino acid was converted to the 1-benzotriazolo ester by reaction with 1-hydroxybenzotriazole and *N,N'*-dicyclohexylcarbodiimide just before coupling. A typical synthetic cycle involved N^{α} deprotection of the Boc-peptidyl resin with 1:1 (v/v) TFA/DCM, neutralization with 1:19 (v/v) DIEA/DCM, coupling of 4 equiv of the Boc-aminoacyl-1-benzotriazolo ester in NMP for 30 min, neutralization with 1:19 (v/v) DIEA/DCM, and coupling of 4 equiv of this ester in 1:4 (v/v) dimethyl sulfoxide/NMP for 16 min followed by the addition of 3.8 equiv of DIEA and further coupling for 7 min. After the second coupling, a portion (5–10 mg) of the peptidyl resin was removed, and the completeness of coupling was monitored by a ninhydrin spot test (Kaiser et al. 1970). After the second coupling, unreacted groups were capped by two acetylations with 2:1:17 (v/v) acetic anhydride/DIEA/DCM for 6 min. The peptidyl resin was cleaved with the low HF/high HF method (Tam et al., 1983) by reaction with a mixture of HF (2.5 mL), 4-methylphenol (0.75 mL), 4-methylbenzenethiol (0.25 mL), and dimethyl sulfide (6.5 mL) for 2 h at 0 °C, evaporation of the HF and dimethyl sulfide, addition of more HF (10 mL) followed by stirring for 1 h at 0 °C, evaporation of the HF and extraction of nonpeptide byproducts with cold anhydrous diethyl ether, and extraction of the crude synthetic peptide from the resin beads with 1:9 (v/v) acetic acid/water.

Air Oxidation of P39. Thiol P39 (15 mg) was dissolved in a solution (1 mL) containing 6 M guanidine hydrochloride and 0.1 M sodium bicarbonate at pH 7.42. The solution was stirred in an open vessel at room temperature. Formation of the disulfide-bridged dimer P78 was monitored by analytical reversed-phase HPLC (Figure 3).

Reversed-Phase Chromatography. Synthetic protein was purified by preparative reversed-phase HPLC on an octyl–silica column (C8, Applied Biosystems) with a Waters Associates HPLC system. The column (100 mm × 10 mm (i.d.), 300-Å pore size) was eluted over 40 min at 3 mL/min with a linear gradient of 21–49% (v/v) acetonitrile/water (both containing 0.1% TFA). Eluate absorbance was monitored at 230 nm. The homogeneity of the purified protein was determined by analytical reversed-phase HPLC (Figure 3) using a microbore octyl–silica column (C8, Applied Biosystems) with an Applied Biosystems HPLC system. The column (50 mm × 1 mm (i.d.), 300-Å pore size) was eluted over 45 min at 0.1 mL/min with a linear gradient of 0–70% (v/v) acetonitrile/water (both containing 0.1% TFA). Eluate absorbance was monitored at 230 nm.

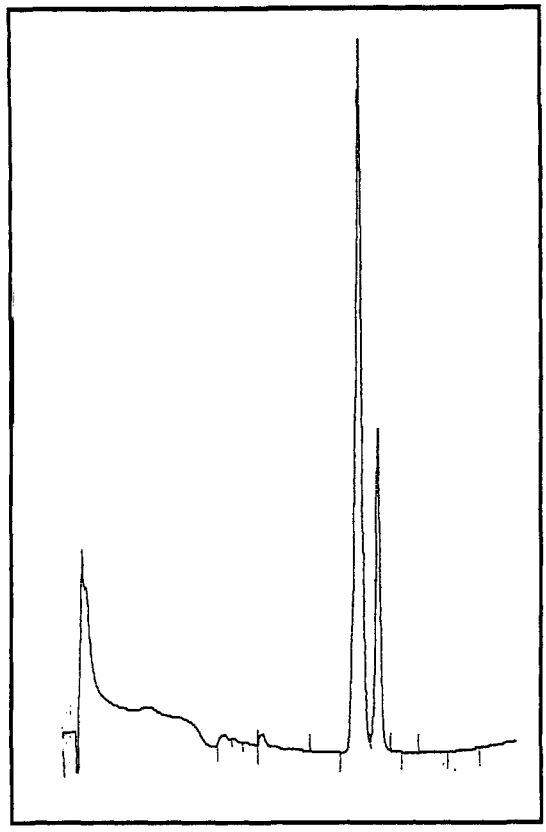

FIGURE 3: Analytical separation of proteins P39 (33.4 min) and P78 (35.5 min) by reversed-phase HPLC monitored at 230 nm. A microbore octyl–silica column was eluted with a linear gradient of 0–70% acetonitrile over 45 min (see Experimental Procedures).

Size-Exclusion Chromatography. Protein (0.2 g) was chromatographed on an agarose column (Superose GF HR 10/30, Pharmacia) and a Pharmacia FPLC system. The column (300 mm × 10 mm (i.d.), 100-Å pore size) was eluted at 0.2 mL/min with 50 mM phosphate buffer at pH 6.5. Eluate absorbance was monitored at 280 nm. DTT was added to keep P39 reduced. The globular protein standards were alcohol dehydrogenase (M_r 150 000), albumin (M_r 66 000), carbonic anhydrase (M_r 29 000), cytochrome *c* (M_r 12 400), and aprotinin (M_r 6 500).

Mass Spectrometry. Molecular mass of the covalent protein component was determined by fast atom bombardment in a VG Model 70/SEQ hybrid tandem mass spectrometer (P33, P39) or by fission fragmentation in a time-of-flight instrument (P78).

Amino Acid Analysis. Dry protein (0.5–1 nmol) was dissolved in a solution (10 μL) containing the internal standard norleucine (0.1 mM) and the metal ion scavenger EDTA (0.25 mg/L). This solution was hydrolyzed in the vapor phase with 6 N HCl at 165 °C for 2 h. The phenyl thiocarbamate derivatives of the resulting amino acids were formed, separated, and quantitated with an Applied Biosystems derivatizer/amino acid analyzer.

Peptide Sequencing. Purified protein was subjected to *N*-terminal Edman degradation, and the resulting phenylthiohydantoin derivatives were separated and quantitated with an Applied Biosystems protein/peptide sequencer.

Partition Ratio Determination. Protein was dissolved by shaking with equal volumes of the two phases produced by mixing 1-butanol, acetic acid, and water in the volume ratio of 4:1:5. The partition ratio (protein dissolved in the upper, organic phase divided by that in the lower, aqueous phase) in BAW was determined by quantitative analytical HPLC of equal volumes of the separated phases.

Raman Spectroscopy. The laser Raman spectrophotometer (Williams, 1986) was operated at 514.5 nm with 200 mW of light power focused at the sample to a beam diameter of about 0.1 mm. The sample holder was thermostated at 14 °C. Raman spectra were analyzed for secondary-structure information according to Williams (1983, 1986).

Ultraviolet Circular Dichroic Spectroscopy. CD spectra were recorded on an Aviv Associates Model 60DS spectropolarimeter controlled by an AT&T computer. The digitized data were averaged, subjected to a five-point smoothing algorithm, and plotted with the Cricket Graph program on a Macintosh II computer. CD spectra are presented as a plot of the mean molar ellipticity per residue ($[\theta]$, deg cm^2 dmol^{-1}) versus the wavelength in 0.5-nm steps. Protein concentration was determined by quantitative amino acid analysis of a stock solution or by quantitative dilution of the stock solution. Solvents were flushed with nitrogen to minimize oxidation of dithiol P39 to disulfide P78.

Thermal denaturation studies were performed on solutions of P39 (10 μM) or P78 (5 μM) in 0.1% TFA/water. Each solution was cooled from room temperature to 5 °C, allowed to equilibrate for 2 min at that temperature, and spectrally scanned three times. The temperature was raised about 10 °C, the solution was allowed to equilibrate for 2 min, and its CD spectrum was recorded three times over 25 min. This procedure was repeated at 10-deg intervals up to 85 °C. The three spectra recorded at each temperature were essentially identical, which confirmed that the protein solution had reached thermal equilibrium. The reported result is the smoothed average of these three measurements.

Denaturation studies in urea or TFE used a stock solution of P39 or P78 in water containing 0.1% TFA and 250 ppm EDTA. Part of the stock solution (10 μL) was diluted to 2 mL with water containing 0.1% TFA, 250 ppm EDTA, and the desired concentration of urea or TFE. The CD spectrum was scanned twice at 25 °C, and the result shown is the smoothed average.

Estimation of Helicity. The percentage of theoretical helicity (% helix) of a protein in solution is estimated by eq 1, where $[\theta]_{222}$ is the observed ellipticity at 222 nm and $[\theta]_{max}$ is the maximal theoretical ellipticity at 222 nm calculated by eq 2 from *n*, the number of peptide residues per chain (Chen et al., 1974).

$$\% \text{ helix} = [\theta]_{222}/[\theta]_{max} \quad (1)$$

$$[\theta]_{max} = -39\,500[1 - (2.57/n)] \text{ (deg cm}^2 \text{ dmol}^{-1}) \quad (2)$$

Results and Discussion

Protein P39. The 39-residue ligand–spacer–coil chain Gly-Arg-Gly-Asp-Ser-Pro-Gly-Tyr-Gly-(Arg-Ile-Glu-Ala-Ile-Glu-Ala)$_4$-Arg-Cys-NH_2 was assembled on 4-methylbenzhydrylamine–polystyrene beads with use of the Boc/benzyl strategy of the solid-phase method (Erickson & Merrifield, 1976). Completeness of coupling for residues 7–39 was monitored by ninhydrin detection of uncoupled amino groups. After the second coupling, each coupling yield was 99.5% or better except for coupling of Ile21 to Glu22 (98.7% yield) and Arg10 to Ile11 (99.3% yield). Analytical HPLC after HF cleavage showed P39 to be the major component. After purification by preparative reversed-phase HPLC, it was homogeneous by analytical HPLC (t_r 33.4 min, Figure 3). Mass spectrometry of P39 gave M_r 4255 corresponding to the molecular ion of composition $C_{180}H_{302}N_{58}O_{59}S$. Amino acid analysis gave acceptable amino acid ratios [found (expected):

Ala, 8.05 (8); Arg, 5.92 (6); Asp, 1.22 (1); Cys, not determined (1); Glu, 8.20 (8); Gly, 4.00 (4); Ile, 8.03 (8); Pro, 0.82 (1); Ser, 0.89 (1); Tyr, 0.88 (1)]. The amino acid sequence of P39 was confirmed by 38 cycles of automatic Edman sequencing. On size-exclusion chromatography, the retention volume of P39 was 14.81 ± 0.2 mL corresponding to an apparent M_r 23 300 ± 1400. The Raman spectrum of P39 showed a characteristic thiol band near 2585 cm^{-1}.

Protein P78. The C-terminal disulfide bridge of P78 was formed by air oxidation of P39 in 6 M guanidine hydrochloride and 0.1 M sodium bicarbonate. The reaction was followed by analytical HPLC and was essentially complete after 24 h. P39 was also slowly oxidized to P78 in 0.1% TFA/water at pH 2. After purification by preparative reversed-phase HPLC, P78 was homogeneous by analytical HPLC (t_r 35.5 min, Figure 3). Mass spectrometry of P78 gave M_r 8508 corresponding to the molecular ion of the covalent dimer of composition $C_{360}H_{602}N_{116}O_{118}S_2$. Amino acid analysis gave acceptable amino acid ratios [found (expected): Ala, 7.64 (8); Arg, 6.42 (6); Asp, 1.19 (1); Cys, not determined (1); Glu, 8.21 (8); Gly, 3.91 (4); Ile, 7.66 (8); Pro, 0.93 (1); Ser, 1.02 (1); Tyr, 0.94 (1)]. On size-exclusion chromatography, the retention volume of P78 was 14.85 ± 0.2 mL corresponding to an apparent M_r 22 700 ± 1400. The Raman spectrum of P78 showed the absence of the thiol band near 2585 cm^{-1}.

Protein P33. The 33-residue spacer–coil chain Gly-Tyr-Gly-(Arg-Ile-Glu-Ala-Ile-Glu-Ala)$_4$-Arg-Cys(Acm)-NH_2 was assembled by the same procedures used for P39, except that the side-chain protecting group for Cys was Acm. After purification by preparative reversed-phase HPLC, it was homogeneous by analytical HPLC (t_r 32.5 min). Mass spectrometry gave M_r 3757 corresponding to the protonated molecular ion of composition $C_{161}H_{272}N_{50}O_{51}S$. Amino acid analysis gave acceptable amino acid ratios [found (expected): Ala, 8.00 (8); Arg, 5.36 (5); Cys, not determined (1); Glu, 7.99 (8); Gly, 2.01 (2); Ile, 7.54 (8); Tyr, 1.04 (1)].

Size-Exclusion Chromatography. When compared with globular protein standards, proteins P39 and P78 eluted from agarose with retention volumes corresponding to apparent mass ratios of 23 300 ± 1400 and 22 700 ± 1400, respectively, which represent the same apparent M_r within experimental error. These apparent values were 2.7 times the actual M_r of 8508 observed for the disulfide-bridged dimer P78 by mass spectrometry. This anomalous behavior on size-exclusion chromatography is characteristic of the rodlike shape of coiled-coil proteins. For example, the two-stranded coiled-coil protein tropomyosin gave an apparent M_r that was 2.1 times the actual M_r of 66 000 (Hodges et al., 1981), and several synthetic 70-residue proteins with a disulfide-bridged coiled coil gave an apparent M_r about 2.5 times the actual value (Hodges et al., 1990).

Partition Ratio. The partition ratios in BAW for P33 (<0.03), P39 (0.008), and P78 (<0.0006) indicate that all three proteins are hydrophilic. Evidently, their hydrophobic residues are mainly buried in the interior of the structure and relatively inaccessible to solvation by butanol, which is consistent with formation of a coiled coil.

Raman Spectroscopy. The 300–1800-cm^{-1} regions of the Raman spectra of P39 and P78 in acidic solution are practically superimposable (Figure 4). Since they are also very similar to the Raman spectra of solid samples of these two molecules, both proteins have very similar structures in the solid state and in aqueous solution.

The intensity of the amide-I Raman band has a maximum near 1650 cm^{-1} for proteins with predominantly α-helical

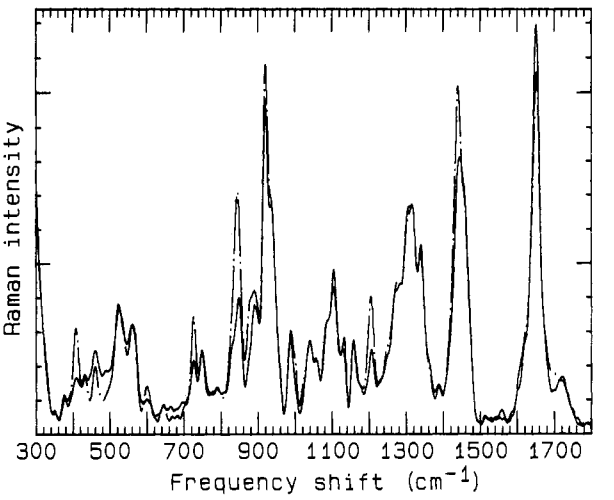

FIGURE 4: Raman spectra of proteins P39 and P78. Plot of the Raman intensity (arbitrary units) for frequency shifts in the range of 300–1800 cm^{-1} for P39 at pH 2.4 (—) and P78 at pH 1.0 (-·-).

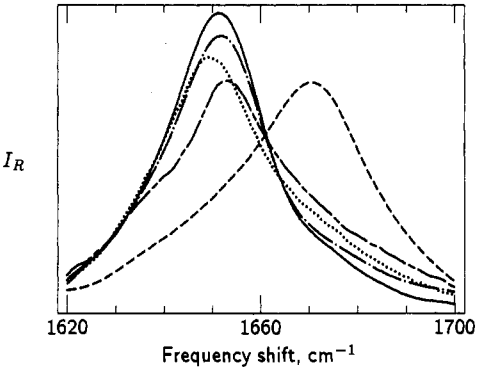

FIGURE 5: Position of the Raman amide-I band for two designed coiled-coil proteins and three reference proteins. Plot of the Raman intensity (arbitrary units) for frequency shifts in the range of 1620–1700 cm^{-1} for noncovalent dimer P39 at pH 2.4 (—) and covalent dimer P78 at pH 1.0 (-·-). Two predominantly α-helical proteins, deoxyhemerythrin (- - -) and capsid protein from bacteriophage fd (···), and a predominantly β-sheet protein, concanavalin A (– –), are included for comparison.

structure and near 1670 cm^{-1} for proteins with predominantly β-sheet structure (Williams, 1983, 1986). As shown in Figure 5, the maxima of the amide-I bands of α-helical reference proteins, such as deoxyhemerythrin (1653 cm^{-1}; about 80% helix) and the capsid protein of the bacterial virus fd (1649 cm^{-1}; about 90% helix), are easily distinguished from that of a predominantly β-sheet protein, such as concanavalin A (1670 cm^{-1}). The maximum of the amide-I band of P39 at pH 2.4 is at 1652 cm^{-1}, and that of P78 at pH 1.0 (or pH 7.7) is at 1652 cm^{-1} (Figure 5). Since both maxima lie between those of deoxyhemerythrin and the fd phage protein, both P39 and P78 have high helical content in aqueous solution.

The amide-III regions of the Raman spectrum (1220–1320 cm^{-1}) of both synthetic proteins (Figure 4) were also similar to those of both model helical proteins. Estimates of secondary structure based on the amide-I and amide-III bands indicated that both P39 and P78 are greater than 80% helix. The amide-I band of P39 and P78 was more intense at low pH (2.4 and 1.0, respectively) than at pH 7.7, which is typical of helical proteins that do not unfold at low pH (Williams et al., 1984).

Circular Dichroic Spectroscopy. The solution conformations of synthetic peptides P33, P39, and P78 were studied by ultraviolet CD methods. The CD spectra of dithiol P39 and disulfide P78 were similar at pH 2.00 and 25 °C (Figure 6A).

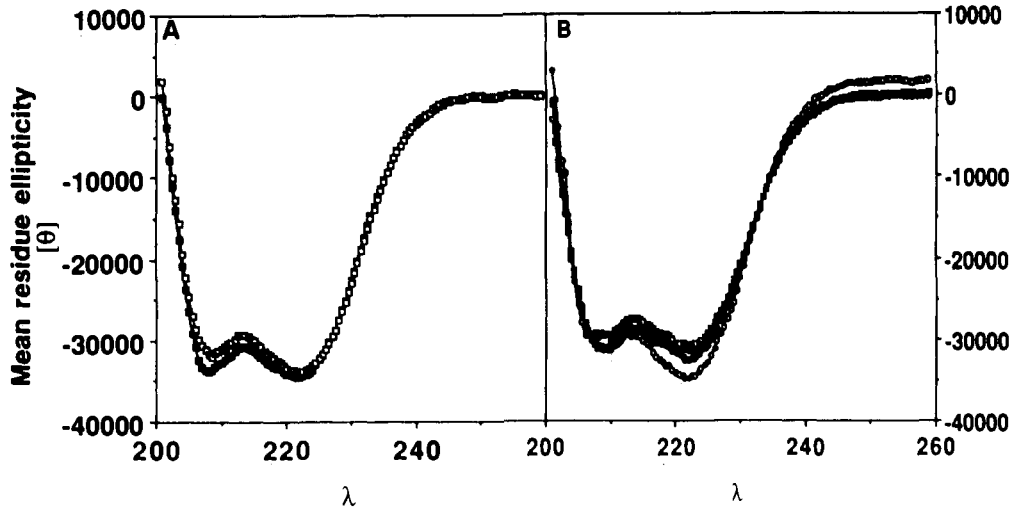

FIGURE 6: CD spectra of proteins P39 and P78 in 0.1% TFA or phosphate buffer: (A) P39 (□) and P78 (■) at pH 2.00 and 25 °C in water containing 0.1% TFA and 250 ppm EDTA. (B) P39 (□) and P78 (■) at pH 2.00 and 37 °C in water containing 0.1% TFA and 250 ppm EDTA; P39 (○) and P78 (●) at pH 7.06 and 37 °C in water containing 20 mM phosphate and 250 ppm EDTA.

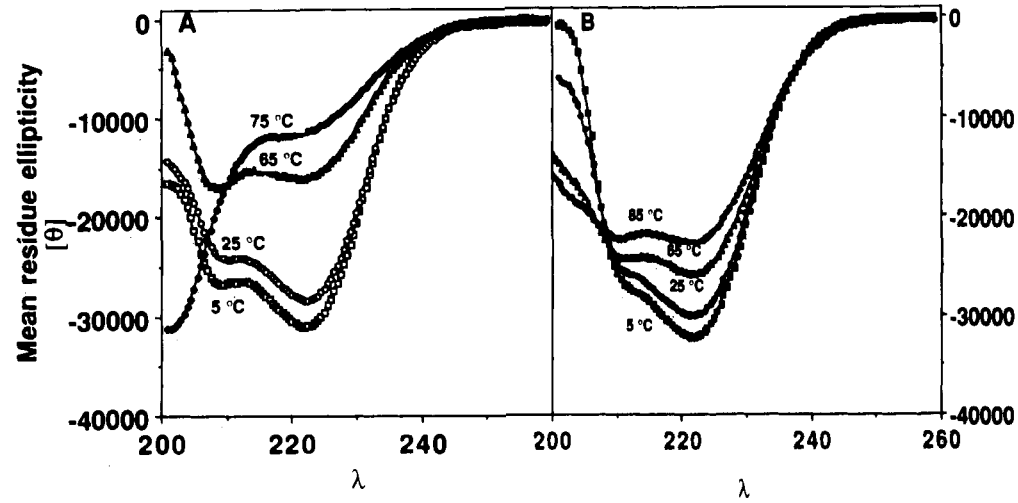

FIGURE 7: Temperature dependence of the CD spectra of proteins P39 and P78 at pH 2.00 in water containing 0.1% TFA and 250 ppm EDTA: (A) noncovalent dimer P39 at 5 °C (□), 25 °C (○), 65 °C (△), and 75 °C (◇); (B) covalent dimer P78 at 5 °C (■), 25 °C (●), 65 °C (▲), and 85 °C (◆).

Both spectra were bimodal with large minima at 207–208 and 222 nm, indicating a significant proportion of the residues in the α-helical conformation (Holzwarth & Doty, 1965). $[\theta]_{222}/[\theta]_{208}$ is about 0.8 for a single-stranded α helix and about 1.0 for a two-stranded α-helical coiled coil (Lau et al., 1984a). By this criterion, P33, P39, and P78 each contain a two-stranded α-helical coiled coil at pH 2.00 and 25 °C (Table I). Since they have peptide chains with specific lengths, sequences, and conformations, dimers P33, P39, and P78 are proteins (Creighton, 1984).

The CD spectra of both RGD-containing proteins were also examined at a physiologically relevant pH and/or temperature. A concentrated solution of P39 or P78 in 0.1% TFA was diluted 200-fold with phosphate buffer (20 mM KH_2PO_4, 250 ppm EDTA, adjusted to pH 7.13 with 1 N NaOH) to pH 7.06. The shapes of the CD spectra of both proteins at pH 7.06 and 37 °C were similar to those measured at pH 2.00 and 37 °C (Figure 6B). The similar values of $[\theta]_{208}$ and $[\theta]_{222}$ at pH 2.00 or 7.06 and at 25 or 37 °C indicate that P39 and P78 have similar solution conformations (Table I). $[\theta]_{222}/[\theta]_{208}$ for these proteins is >1.0 at all four of these combinations of pH and temperature (Table I). Thus, P39 is a dimeric protein consisting of two 39-residue peptide chains held together by noncovalent interstrand forces (Figure 1).

Table I: CD Data for Three Synthetic Coiled-Coil Proteins

protein	pH	T (°C)	$[\theta]_{208}$	$[\theta]_{222}$	$[\theta]_{222}/[\theta]_{208}$
P33	2.00	25	−26 700	−26 200	0.98
P39	2.00	25	−31 700	−33 800	1.07
P39	2.00	37	−29 700	−30 700	1.03
P39	7.06	25	−32 800	−37 100	1.13
P39	7.06	37	−30 800	−34 900	1.13
P78	2.00	25	−33 800	−34 600	1.02
P78	2.00	37	−31 100	−31 300	1.01
P78	7.06	25	−29 300	−33 700	1.15
P78	7.06	37	−29 200	−32 500	1.11

The protein helicity estimated from $[\theta]_{222}$ under these conditions ranges from 83% to 101% for P39 and 85% to 94% for P78 (Table II). It consistently decreased by 3–9% when the temperature was increased from 25 to 37 °C. The percentage of helix was essentially unchanged on reduction of disulfide P78 to dithiol P39 at pH 2 but increased by 7–10% on reduction at pH 7. The helicity of P33, estimated to be 72% helix at pH 2.00 and 25 °C, may be lower than that of P39 or P78 under these conditions because of the destabilizing effect of the nearby α-NH_3^+ of Gly1 (Fairman et al., 1989).

Thermal Unfolding. CD studies of P39 and P78 were conducted at pH 2.00 over the range of 5–85 °C. For both dimers, $[\theta]_{222}$ decreased with increasing temperature (Figure

Table II: Estimated Percent Helix for Coiled-Coil Proteins P39 and P78[a]

	pH 2.00		pH 7.06	
protein	25 °C	37 °C	25 °C	37 °C
P39	92	83	101	95
P78	94	85	91	88

[a] From eq 2 with $n = 39$, $[\theta]_{max}$ is 36900 deg cm^2 dmol^{-1}.

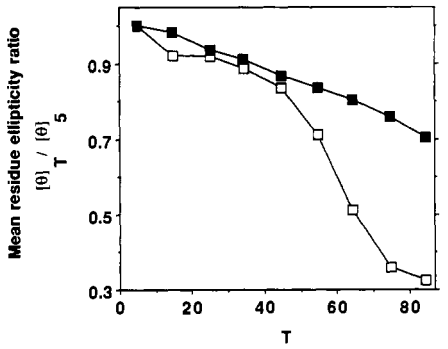

FIGURE 8: Influence of temperature T on the ellipticity minimum at 222 nm for proteins P39 and P78. Plot of $[\theta]_T/[\theta]_5$, $[\theta]_{222}$ at T vs $[\theta]_{222}$ at 5 °C, vs T for P39 (□) and P78 (■) at pH 2.00 in water containing 0.1% TFA and 250 ppm EDTA.

7). The relatively featureless spectrum seen for P39 at 75 °C is similar to that reported for proteins (Rudolph et al., 1986) and model α-helical peptides (Merukta et al., 1990) in the unfolded state. The absence of an isodichroic point for P39 indicates that its thermal denaturation cannot be simply explained by a two-state structural transition. A plot of $[\theta]_T/[\theta]_5$, $[\theta]_{222}$ at temperature T vs that at 5 °C, against T (Figure 8) shows that for the noncovalent dimer P39 this value gradually decreased to about 0.85 at 45 °C. On further heating, however, it abruptly decreased to 0.51 at 65 °C and to 0.37 at 75 °C (Figure 8). The shape of the CD spectrum of P39 at 75 °C and the absence of a minimum at 222 nm (Figure 7A) indicate that both chains of noncovalent dimer P39 are mainly unfolded at 75 °C.

In contrast, the presence of an isodichroic point at 208 nm for thermal denaturation of the covalent dimer P78 (Figure 7B) indicates the existence of a two-state structural transition, presumably conversion of an α-helical coiled coil into an unfolded disulfide-bridged dimer. A plot of $[\theta]_T/[\theta]_5$ against T for P78 (Figure 8) shows that this value gradually decreased to about 0.85 at 45 °C, as it did for P39. For P78, however, this value continued to decrease in a linear fashion. At 85 °C $[\theta]_T/[\theta]_5 = 0.7$, so P78 is approximately 30% unfolded. P39 is comparably denaturated at 55 °C, a temperature 30 °C lower than for P78. These results show that covalent dimer P78 is significantly more stable to heating than noncovalent dimer P39. Similar effects have been described for synthetic five-repeat coiled-coil proteins with and without an N-terminal disulfide bridge (Hodges et al., 1988, 1990). This increase in thermal stability is due to the presence in P78 of the disulfide bridge between the C-terminal Cys residues, which efficiently cross-links the pair of α helices and thus decreases the entropy gained during unfolding of the coiled-coil structure.

The greater thermal stability of the coiled coil of P78 may explain why a simple two-state transition was detected for P78 but not for P39 by CD spectroscopy. At the lower temperatures needed to disrupt the hydrophobic and ionic interchain interactions of the coiled coils of P39, the separated coils may still have partial α-helical character. But at the higher temperatures needed to disrupt the interchain interactions of the coiled coils of P78, the separated coils may be unstable. In

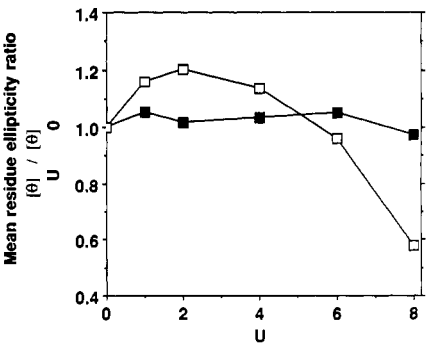

FIGURE 9: Influence of U, the molar concentration of urea, on the ellipticity minimum at 222 nm for proteins P39 and P78. Plot of $[\theta]_U/[\theta]_0$, $[\theta]_{222}$ at U vs that without urea ($U = 0$ M), vs U for P39 (□) and P78 (■) in aqueous urea at pH 2.0–3.5 and 25 °C.

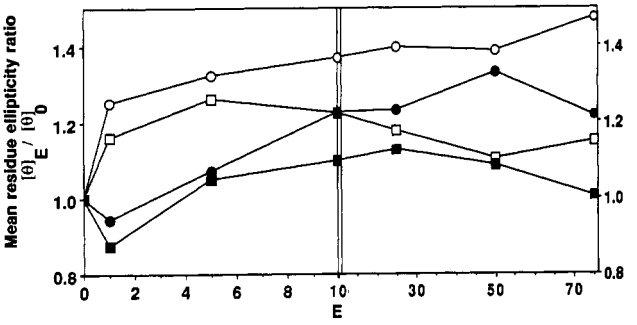

FIGURE 10: Influence of E, the volume percentage of TFE, on the ellipticity minima at 208 and 222 nm for proteins P39 and P78 at 25 °C in water containing 0.1% TFA and 250 ppm EDTA. Plot of $[\theta]_E/[\theta]_0$, $[\theta]$ at E vs $[\theta]$ in the absence of TFE ($E = 0\%$), vs E for P39 at 208 nm (○) and at 222 nm (□) and P78 at 208 nm (●) and at 222 nm (■).

other words, at the lower temperatures at least one partly folded state may intervene in detectable amounts between the folded state of noncovalent dimer P39 and its unfolded state. At the higher temperatures, the corresponding partly folded state of covalent dimer P78 either may not exist or may not be present in detectable amounts.

Stability in Urea Solutions. Urea can perturb the structure of α-helical coiled coils (Lau et al., 1984a), so CD studies of P39 and P78 were conducted at several urea concentrations. Part (10 μL) of a stock solution of protein P39 or P78 was diluted to 2 mL with water containing TFA (0.1%), EDTA (250 ppm), and urea (0, 1, 2, 4, 6, or 8 M). The CD spectrum of each peptide solution was recorded at 25 °C. For covalent dimer P78, $[\theta]_U/[\theta]_0$, $[\theta]_{222}$ at urea concentration U vs that in the absence of urea, did not change with increasing U (Figure 9). For noncovalent dimer P39, however, $[\theta]_U/[\theta]_0$ increased 20% at 2 M urea and thereafter steadily decreased to half to the maximal value at 8 M urea. Thus, the bridged coiled coil of P78 is more stable in urea than the unbridged coiled coil of P39, which contains only 50% helix in 8 M urea.

Stability of Trifluoroethanol Solutions. 2,2,2-Trifluoroethanol can increase the helicity of single-stranded peptides (Lau et al., 1984a,b; Lu et al., 1984; Lehrman et al., 1990). As the polarity of the medium decreases, the strength of the α-helical hydrogen bond between the carbonyl oxygen of residue i and the amide hydrogen of residue $i + 4$ increases, which stabilizes the α helix. Peptide P39 or P78 was dissolved in water containing TFA (0.1%), EDTA (250 ppm), and TFE (0, 1, 10, 25, 50, or 75 vol %). $[\theta]_E/[\theta]_0$, the observed ellipticity at E (volume percentage of TFE) vs that in the absence of TFE, changed less at 222 nm than at 208 nm with increasing TFE (Figure 10). At 222 nm and 5% TFE, this value increased only 5% for covalent dimer P78, indicating that its

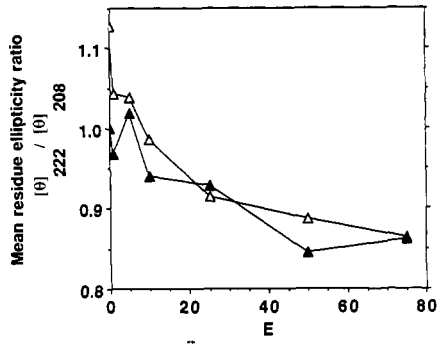

FIGURE 11: Dependence of $[\theta]_{222}/[\theta]_{208}$ on E, the volume percentage of TFE, in water (containing 0.1% TFA and 250 ppm EDTA) and TFE at 25 °C for proteins P39 (△) and P78 (▲).

coil modules were nearly fully helical even without TFE. In contrast, it increased 26% for noncovalent dimer P39, showing that its coil modules were induced to fold into significantly more helical conformations by this inert solvent. More revealing, the ellipticity ratio of $[\theta]_{222}$ to $[\theta]_{208}$ decreased with increasing TFE (Figure 11). It decreased steadily from 1.10 at 5% TFE to 0.86 at 75% TFE for P39 and from 1.00 at 5% TFE to 0.86 at 75% TFE for P78. Both proteins are estimated to contain about 80% helix in 75% TFE. Synthetic five-repeat coiled-coil proteins are single-stranded α helices in 50% TFE (Lau et al., 1984b), whereas synthetic five-repeat coiled-coil proteins with an N-terminal disulfide bridge are believed to be undissociated coiled coils in 50% TFE (Hodges et al., 1988, 1990).

Designed Coiled-Coil Proteins. These biophysical studies indicate that both P39 and P78 form stable α-helical coiled coils at pH 2 or 7 and at 25 or 37 °C. The coiled coil of the two-stranded protein P78 with a C-terminal disulfide bridge is particularly stable under denaturing conditions. The disulfide bridge of this modular protein structurally resembles in part the linker module of monomeric RAMP P82.

Conservative substitutions of Ile for Leu, Ala for Gly, and Arg for Lys in the coiled-coil repeat of the Hodges group did not disrupt the coiled coil. For comparison, substitution of Ile for the three Leu residues in the monomeric helical peptide CH_3CO-Tyr-(Lys-Ala-Ala-*Leu*-Ala-)$_3$-Lys-NH_2 decreased the helicity about 50% (Padmanabhan et al., 1990). In contrast, substitution of Ile for the single Leu in the monomeric helical peptide CH_3CO-Tyr-Glu-Ala_3-Lys-Glu-Ala-*Leu*-Ala-Lys-Glu-Ala_3-Lys-Ala-NH_2 increased the helicity by about 40% (Merutka et al., 1990).

The synthetic modular proteins P39 and P78 fold in benign media into stable two-stranded α-helical coiled-coil structures. This spontaneous dimerization of the designed 30-residue coil module has produced a useful component for building new structures on the molecular scale. This is a significant step toward the nanometric engineering of receptor-adhesive modular proteins, such as P82 and P164 (Figure 1). These novel designed proteins should adhere to cell-surface integrin receptors through their RGD-containing ligands (Figure 2). Indeed, fluorescein-labeled P39 has recently been shown to bind to substrate-adsorbed human platelet vesicles by total internal reflection fluorescence microscopy (Pisarchick, Engel, Erickson, and Thompson, unpublished results).

ACKNOWLEDGMENTS

We thank Russ Henry and Kitty Brostrom of the UNC/NIEHS Protein Chemistry Laboratory for assistance with the automated peptide chemistry, Dean Marbury and Dr. Judith Charles of the UNC Mass Spectrometry Laboratory for the mass spectral data for P33 and P39, Ron Davis and Dr. Brian Chait of the Rockefeller Mass Spectrometry Biotechnology Resource for the mass spectral data for P78, and Dr. Mary Donlan and Dr. Peter Jeffs of Glaxo Inc. for assistance in using their CD spectrophotometer.

REFERENCES

Albrecht, E., Engel, M., Melton, L. G., Peek, B. M., & Erickson, B. W. (1990) in *Peptides: Chemistry, Structure, and Biology* (Rivier, J. E., & Marshall, G. R., Eds.) pp 718–720, ESCOM, Leiden.

Carrell, N. A., Fitzgerald, L. A., Steiner, B., Erickson, H. P., & Phillips, D. R. (1985) *J. Biol. Chem. 260*, 1743–1749.

Chen, Y. H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350–3359.

Creighton, T. E. (1984) *Proteins: Structures and Molecular Properties*, p 4, Freeman, New York.

Danilov, Y. N., & Juliano, R. L. (1989) *Exp. Cell Res.* 182, 186–196.

Erickson, B. W., & Merrifield, R. B. (1976) in *Proteins (3rd Ed.)* 2, 255–527.

Fairman, R., Shoemaker, K. R., York, E. J., Stewart, J. M., & Baldwin, R. L. (1989) *Proteins: Struct., Funct., Genet. 5*, 1–7.

Giltay, J. C., & van Mourik, J. A. (1988) *Haemostasis 18*, 376–389.

Hodges, R. S., Sodek, J., Smillie, L. B., & Jurasek, L. (1972) *Cold Spring Harbor Symp. Quant. Biol. 37*, 299–310.

Hodges, R. S., Saund, A. K., Chong, P. C. S., St.-Pierre, S. A., & Reid, R. E. (1981) *J. Biol. Chem. 256*, 1214–1224.

Hodges, R. S., Semchuk, P. D., Taneja, A. K., Kay, C. M., Parker, J. M. R., & Mant, C. T. (1988) *Pept. Res. 1*, 19–30.

Hodges, R. S., Zhou, N. Z., Kay, C. M., & Semchuk, P. D. (1990) *Pept. Res. 3*, 123–137.

Hollingshead, S. K., Fischetti, V. A., & Scott, J. R. (1986) *J. Biol. Chem. 261*, 1677–1686.

Holzwarth, G., & Doty, P. (1965) *J. Am. Chem. Soc. 87*, 218–228.

Hynes, R. H. (1987) *Cell 48*, 549–554.

Juliano, R. L. (1987) *Biochim. Biophys. Acta 907*, 261–278.

Kaiser, E., Colescott, R. L., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595–598.

Lau, S. Y. M., Taneja, A. K., & Hodges, R. S. (1984a) *J. Biol. Chem. 259*, 13253–13261.

Lau, S. Y. M., Taneja, A. K., & Hodges, R. S. (1984b) *J. Chromatogr. 317*, 129–140.

Lehrman, S. R., Tuls, J. L., & Lund, M. (1990) *Biochemistry 29*, 5590–5596.

Lu, Z. X., Fok, K. F., Erickson, B. W., & Hugli, T. E. (1984) *J. Biol. Chem. 259*, 7367–7370.

Matsueda, G. R., & Stewart, J. M. (1981) in *Peptides 2*, 45–50.

Merutka, G., Lipton, W., Shalongo, W., Park, S. H., & Stellewagen, E. (1990) *Biochemistry 29*, 7511–7515.

Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M., & Baldwin, R. L. (1990) *Nature (London) 344*, 268–270.

Phillips, D. R., Fitzgerald, L. A., Charo, I. F., & Parise, L. V. (1988) *Ann. N.Y. Acad. Sci. 509*, 177–187.

Rudolph, R., Fuchs, I., & Jaenicke, R. (1986) *Biochemistry 25*, 1662–1669.

Ruoslahti, E., & Pierschbacher, M. D. (1986) *Cell 44*, 517–518.

Ruoslahti, E., & Pierschbacher, M. D. (1987) *Science 238*, 491–497.

Ruoslahti, E., Hayman, E. G., & Pierschbacher, M. D. (1985) *Arteriosclerosis 5*, 581–594.

Shiba, E., Fossel, E., Lindon, J., McEver, R., & Salzman, E. W. (1988) *Arteriosclerosis 8*, 668a.

Talbot, J. A., & Hodges, R. S. (1982) *Acc. Chem. Res. 15*, 224-230.
Tam, J. P., Heath, W. F., & Merrifield, R. B. (1983) *Int. J. Pept. Protein Res. 21*, 57-65.
Williams, R. W. (1983) *J. Mol. Biol. 166*, 581-603.
Williams, R. W. (1986) *Methods Enzymol. 130*, 311-331.
Williams, R. W., Dunker, A. K., & Peticolas, W. L. (1984) *Biochim. Biophys. Acta 791*, 131-144.

Stereochemistry of the Concerted Enolization Catalyzed by Δ^5-3-Ketosteroid Isomerase†

A. Kuliopulos,‡ G. P. Mullen, L. Xue,§ and A. S. Mildvan*
Department of Biological Chemistry, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, Maryland 21205

Received August 24, 1990; Revised Manuscript Received October 31, 1990

ABSTRACT: The reaction catalyzed by Δ^5-3-ketosteroid isomerase has been shown to occur via the concerted enolization of the Δ^5-3-ketosteroid substrate to form a dienolic intermediate, brought about by Tyr-14, which hydrogen bonds to and protonates the 3-keto group, and Asp-38, which removes and axial (β) proton from C-4 of the substrate, in the same rate-limiting step [Xue, L., Talalay, P., & Mildvan, A. S. (1990) *Biochemistry 29*, 7491-7500; Kuliopulos, A., Mildvan, A. S., Shortle, D., & Talalay, P. (1989) *Biochemistry 26*, 3927-3937]. Since the axial C-4 proton is removed by Asp-38 from above the substrate, a determination of the complete stereochemistry of this rapid, concerted enolization requires information on the direction of approach of Tyr-14 to the enzyme-bound steroid. The double mutant enzyme, Y55F + Y88F, which retains Tyr-14 as the sole Tyr residue, was prepared and showed only a 4.5-fold decrease in k_{cat} (12 000 s^{-1}) and a 3.6-fold decrease in K_M (94 μM) for Δ^5-androstene-3,17-dione, in comparison with the wild-type enzyme. Deuteration of the aromatic rings of the 10 Phe residues further facilitated the assignment of the aromatic proton resonances of Tyr-14 in the 600-MHz TOCSY spectrum at 6.66 ± 0.01 ppm (3,5H) and at 6.82 ± 0.01 ppm (2,6H). Variation of the pH from 4.9 to 10.9 did not alter these shifts, indicating that the pK_a of Tyr-14 exceeds 10.9. Resonances assigned to the three His residues titrated with pK_a values very similar to those found with the wild-type enzyme. The binding of 19-nortestosterone, a product analogue and substrate of the reverse isomerase reaction, induced downfield shifts of -0.12 and -0.06 ppm of the 3,5- and 2,6-proton resonances of Tyr-14, respectively, possibly due to deshielding by the 3-keto group of the steroid, but also induced +0.29 to -0.41 ppm changes in the chemical shifts of 8 of the 10 Phe residues and smaller changes in 10 of the 12 ring-shifted methyl resonances, indicating a steroid-induced conformation change in the enzyme. NOESY spectra in H_2O revealed strong negative Overhauser effects from the 3,5-proton resonance of Tyr-14 to the overlapping 2α-, 2β-, or 6β-proton resonances of the bound steroid but no NOE's to the 4- or 6α-protons of the steroid. These observations indicate the NOE's to be from Tyr-14 to the 2α- and 2β-protons rather than to the 6β-proton of the steroid. Prolonged incubation of the enzyme–steroid complex in ^2H_2O resulted in deuteration of the 6β-, 4-, and 2α-positions of the steroid. The remaining 2β-proton retained 13% of the original NOE from Tyr-14, indicating that 87% of the NOE was to the 2α-proton. These observations are consistent with a specific and asymmetric orientation of Tyr-14 with respect to the bound steroid that places to phenolic ring of Tyr-14 beside C-2 and far from C-4 and C-6 of the steroid. This orientation permits Tyr-14 to form a hydrogen bond to the lone electron pair of the carbonyl oxygen of the steroid product that is trans to the C-4–C-5 double bond. On the basis of the geometry of this product complex and the fact that the axial C-4 β-proton of the substrate is removed by Asp-38 from above the steroid, we conclude that an orthogonal (rather than suprafacial or antarafacial) arrangement of the proton donor and proton acceptor, with respect to the bound substrate, is stereoelectronically appropriate for a rapid, concerted enolization.

The enzyme Δ^5-3-ketosteroid isomerase of *Pseudomonas testosteroni* catalyzes the isomerization of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids by a stereospecific and conservative transfer of the 4β-proton to the 6β-position by way of an enolic intermediate (Batzold et al., 1976; Bantia & Pollack, 1986; Eames et al., 1990) (Figure 1). Primary, secondary, and combined deuterium kinetic isotope effects have established the enzyme-catalyzed enolization of the bound substrate to be concerted and rate limiting (Xue et al., 1990). The high velocity of this concerted enolization of 54 000 s^{-1} is brought about by the combined actions of Tyr-14, which protonates the 3-keto group, and Asp-38, which removes the axial (β) proton from C-4 of the substrate. Indeed, as revealed by the additive effects on catalysis of mutating Tyr-14 and Asp-38, the total catalytic power of the enzyme can be accounted for by general acid–base catalysis by these two residues in the concerted and rate-limiting enolization step of the isomerase reaction (Kuliopulos et al., 1989, 1990a).

† This work was supported by NIH Grants DK28616 (to A.S.M.) and DK07422 to Paul Talalay, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine. The 600-MHz NMR spectrometer was funded by NIH Grant RR03518, NSF Grant DMB8612318, and The Johns Hopkins University School of Medicine.

* To whom correspondence should be addressed.
‡ Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02114.
§ Present address: Department of Medicinal Chemistry, University of Illinois at Chicago, Chicago, IL 60680.

0006-2960/91/0430-3169$02.50/0