Mössbauer effect spectroscopy.[58] Clearly the ferrocene and related intercalates in the FeOCl host lattice provide the opportunity to examine the lattice dynamical behavior of the guest and the host species, independently, and thus probe the behavior of these anisotropic solids at two very different points in the lattice. Such studies, combined with variable (low) temperature Fourier transform infrared studies and susceptibility measurements, should add very significantly to our detailed knowledge of these fascinating materials.

Our research has been generously supported by the Division of Materials Research of the National Science Foundation, as well as by grants from the Research Council and the Center for Computer and Information Services, Rutgers University. This support is herewith very gratefully acknowledged. The author is deeply indebted to his students and co-workers, many of whom have made major contributions to the results herein discussed. In particular the contributions of Dr. Y. Maeda, Dr. A. J. Rein, T. K. McGuire, and R. Cassell have been of central importance in the pursuit of this research.

(57) Schäfer-Stahl, H.; Abele, R. *Mater. Res. Bull.*, **1980**, *15*, 1157.
(58) Schäfer-Stahl, H.; Abele, R. *Angew. Chem., Int. Ed. Engl.*, **1980**, *19*, 477. Schäfer-Stahl, H. *Mater. Res. Bull.* **1980**, *15*, 1091.

Tropomyosin: A Model Protein for Studying Coiled-Coil and α-Helix Stabilization

JAMES A. TALBOT and ROBERT S. HODGES*

Medical Research Council Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7

Received July 23, 1981 (Revised Manuscript Received March 29, 1982)

Tropomyosin is a key control protein in muscle contraction[1-3] capable of transmitting subtle conformational changes. Information in the amino acid sequence directs alignment of chains in parallel and in-register, formation and stabilization of the coiled coil, and end-to-end aggregation. In addition, the sequence provides for binding sites for actin and troponin-T and for the possible transmission of a conformational change along its length. Tropomyosin has been previously reviewed,[4,5] but we wish to discuss the requirements for the stabilization of the coiled-coil structure at the molecular level. Our comprehension of the fine control of coiled-coil stability that permits it to perform its functions depends on our understanding of the factors that are important in an α helix and a coiled coil. Work in our and other laboratories has aimed at elucidating these design principles.

Examination of X-ray crystallographic bond lengths and angles for small peptides along with the assumptions that intrachain hydrogen bonds should be maximized and linear led Pauling et al.[6] to postulate the α helix. Since then many synthetic polypeptides have been demonstrated by wide-angle X-ray diffraction to form helices with the predicted pitch of 5.4 Å per turn and with a translation along the helix axis of 1.5 Å per residue.[7]

Circular dichroism and other optical techniques have shown that single chain polypeptides are often α helical in nonaqueous solvents or at a pH far from neutrality.[8-11] Polypeptides containing a large proportion of charged amino acids, even lysine and glutamic acid copolymers where strong charge repulsion is absent, have little α helix at neutral pH when the side chains are in an aqueous environment.[12,13] Many polypeptide chains do not form helices in aqueous solutions, although they do so as part of the sequence of a globular protein molecule.[14] As a first approximation, then, α helices will not form when the side chains of the polypeptide are in an aqueous environment, since hydrogen bonds in the presence of water are exceedingly unstable.[15]

Why then do α helices exist in globular proteins like myoglobin and the highly α-helical fibrous proteins of the k–m–e–f (keratin, myosin, epidermis, fibrinogen) group of which tropomyosin is a member? In myoglobin an interior of hydrophobic side chains excludes

Robert S. Hodges was born in Saskatoon, Saskatchewan, carried out his undergraduate studies in biochemistry at the University of Saskatchewan, and received his Ph.D. degree in Biochemistry at the University of Alberta. After a postdoctoral stay in the laboratory of Dr. Bruce Merrifield at The Rockefeller University, he joined the University of Alberta in 1974. He is presently Associate Professor of Biochemistry and member of the MRC Group in Protein Structure and Function at the University of Alberta.

James A. Talbot was born in Ottawa, Ontario, and received both his B.Sc. and Ph.D. degrees in Biochemistry from the University of Alberta. He is presently in Medical School at the University of Toronto. He will be doing part-time research there with Dr. D. H. MacLennan at the Banting and Best Department of Medical Research.

(1) S. Ebashi, K. Maruyama, and M. Endo, Eds., "Muscle Contraction, Its Regulatory Mechanisms", Springer Verlag, New York, 1980.
(2) W. F. Harrington in "The Proteins", Vol. IV, 3rd ed., H. Neurath, R. L. Hill, and C.-L. Boeder, Eds., Academic Press, New York, 1979.
(3) W. D. McCubbin and C. M. Kay, *Acc. Chem. Res.*, **13**, 185 (1980).
(4) L. B. Smillie, *PAABS Revista*, **5**, 183 (1976).
(5) L. B. Smillie, *Trends Biochem. Sci.*, **4**, 151 (1979).
(6) L. Pauling, R. B. Corey, and H. R. Branson, *Proc. Natl. Acad. Sci. U.S.A.*, **37**, 205 (1951).
(7) C. H. Bamford, A. Elliot, and W. E. Hanby, "Synthetic Polypeptides", Academic Press, New York, 1956, pp 215–261.
(8) J. Applequist and P. Doty, "Polyamino Acids, Polypeptides and Proteins", M. A. Stahman, Ed., University of Wisconsin Press, Madison, WI, 1962, pp 161–175.
(9) M. Idelson and E. R. Blout, *J. Am. Chem. Soc.*, **80**, 4631 (1958).
(10) G. D. Fasman, "Polyamino Acids, Polypeptides and Proteins", M. A. Stahman, Ed., University of Wisconsin Press, Madison, WI, 1962, pp 221–224.
(11) J. T. Yang and P. Doty, *J. Am. Chem. Soc.*, **79**, 761 (1957).
(12) C. Cohen and K. C. Holmes, *J. Mol. Biol.*, **6**, 423 (1963).
(13) E. R. Blout and M. Idelson, *J. Am. Chem. Soc.*, **80**, 4909 (1958).
(14) J. A. Schellman and C. Schellman, "The Proteins", Vol. II, 2nd ed., H. Neurath, Ed., Academic Press, New York, 1964, pp 1–137.
(15) I. M. Klotz and J. S. Franzen, *J. Am. Chem. Soc.*, **82**, 5241 (1960).

0001-4842/82/0115-0224$01.25/0

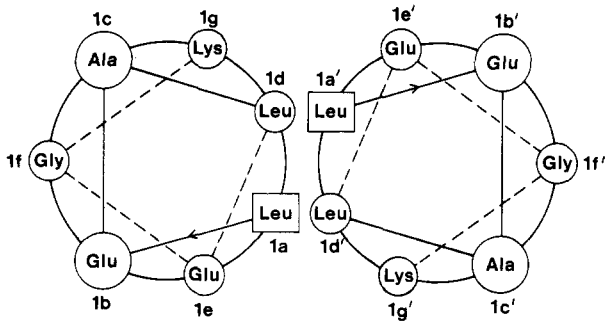

Figure 1. End-on view looking from the NH_2-terminal end of two helices in a coiled coil containing the heptapeptide sequence Leu-Glu-Ala-Leu-Glu-Gly-Lys used in the synthesis of a coiled-coil model.[51] The α helices descend into the page on proceeding from residue a to g. The chains are in-register and parallel. The nonpolar residues a and a' and d and d' interact and are responsible for the formation and stabilization of the coiled coil. The number preceding the letter designates the heptad starting from the N-terminal end of the sequence.

water and provides the additional stabilization required to form an α helix in this interior.[16] The situation with the fibrous proteins was at first glance different and more complicated. Tropomyosin is a rodlike protein, close to 100% α helix as estimated by its optical rotatory dispersion and wide-angle X-ray diffraction.[17,18] It has the largest charge density of any known protein, remains fully helical over a wide pH range (2–9), and is exceptionally stable in acid.[19,20] How can tropomyosin exist as an α helix in aqueous solution? The clue to the stability of tropomyosin was the finding that its pitch is 5.1 Å compared to 5.4 Å for synthetic peptides in the α form.[21] Crick[22,23] and Pauling[24] pointed out that an α helix wrapping around one or more α helices at an angle near 20° would result in a coiled coil. The small deformation required for this and the tilt results in 3.5 residues per turn and a pitch of 5.1 Å along the coiled-coil axis. Crick[23] showed that this kind of packing results in side chains that intercalate in a "knobs-into-holes" manner. If these are hydrophobic groups, the α helix would be stabilized, and both globular proteins and tropomyosin would be stabilized by nonpolar side-chain interactions.

The amino acid sequence of rabbit skeletal α-tropomyosin exhibits a number of repeating features.[25–27] The most striking is a repeating pattern of hydrophobic residues occurring in two series at seven-residue intervals. This repeat, first identified by Hodges et al.,[26] led to the proposal that tropomyosin and other coiled-coil fibrous proteins were stabilized by hydrophobic residues at positions a and d of a repeating heptad sequence $(a\text{–}b\text{–}c\text{–}d\text{–}e\text{–}f\text{–}g)_n$ (Figure 1). We refer to the sequence that results (N-X-X-N-X-X-X-N-X-X-N-X-X-X..., where N is a nonpolar residue) as having a 4–3 repeat. In tropomyosin, 71 nonpolar, 7 neutral, and 4 charged amino acids occupy the 82 a and d positions. The X-ray structure at 15-Å resolution implies that there are no major deviations from the coiled-coil structure except at the ends of the molecule.[28,29]

The 4–3 repeat leads to a hydrophobic face with a hydrophobe every 3.5 residues on the average. The α helices in hemoglobin and myoglobin (globular proteins) contain nonpolar residues that repeat every 3.6 residues.[30,31] X-ray crystallographic studies indicated that this repeat results from an almost total exclusion of polar residues from the face of the α helix oriented toward the interior of the globular molecule. We conclude that the constraints imposed on the position of hydrophobes in an α-helical sequence of a globular protein or a coiled coil are similar. The difference is that in tropomyosin, because of the deformation of the α helix and the consistent 4–3 repeat, a narrow hydrophobic face is maintained. This face is oriented toward the major helix axis indefinitely as the two α helices coil about one another while an undistorted α helix in a globular protein with a perfect 4–3 repeat has a broader hydrophobic face which will eventually shift across the face of the helix.

A structure clearly anticipated by Crick[23] and later used in the segmented rope model proposed for tropomyosin,[32–34] involves short α helices (<50 residues) that interact in a "knobs-into-holes" manner. The resultant 20° tilt (Figure 2a,b) would give a 5.1-Å reflection, but the lack of peptide backbone deformation would mean that the hydrophobes of the 4–3 repeat shift across the face of the helix until they are no longer capable of interacting with their opposite residues on the other helix (Figure 2c). These structures might be called "cruciforms", since they cross, to distinguish them from similar but different coiled-coil structures where continuous α-helix deformation occurs. The segmented rope model follows a "cruciform" of 20 to 30 Å with a bend to correct the deterioration in packing, and then another stretch of cruciform, and so on. This model concentrates all the necessary deformation in the bend, and in light of the constant 4–3 repeat and X-ray results,[28,29] the cruciform structure seems unlikely for tropomyosin.

Many reports have appeared in the literature of similarities in the sequence of their protein and tropomyosin.[35–47] Some of these have suggested, on this

(16) J. C. Kendrew, H. C. Watson, B. E. Strandberg, R. E. Dickerson, D. C. Phillips, and V. C. Shore, *Nature (London)*, **190**, 666 (1961).
(17) C. Cohen and A. G. Szent-Gyorgyi, *J. Am. Chem. Soc.*, **79**, 149 (1957).
(18) E. F. Woods, *Biochemistry*, **8**, 4336 (1969).
(19) S. Lowey, *J. Biol. Chem.*, **240**, 2421 (1965).
(20) M. Noelken and A. Holtzer, in "Biochemistry of Muscle Contraction", J. Gergely, Ed., Little, Brown and Co., Boston, 1964, p 374.
(21) W. T. Astbury, R. Reed, and L. C. Spark, *Biochem. J.*, **43**, 782 (1948).
(22) F. H. C. Crick, *Nature (London)*, **170**, 882 (1952).
(23) F. H. C. Crick, *Acta Crystallogr.*, **6**, 689 (1953).
(24) L. Pauling and R. B. Corey, *Nature (London)*, **171**, 59 (1953).
(25) D. Stone, J. Sodek, P. Johnson, and L. B. Smillie, in "Proceedings of the IX Federation of European Biochemical Societies Meeting, Proteins of Contractile Systems", E. N. A. Biro, Ed., North Holland Publishing Co., Amsterdam, 1975, pp 125–136.
(26) R. S. Hodges, J. Sodek, L. B. Smillie, and L. Jurasek, *Cold Spring Harbor Symp. Quant. Biol.*, **37**, 299 (1972).
(27) J. Sodek, R. S. Hodges, L. B. Smillie, and L. Jurasek, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 3800 (1972).
(28) G. N. Phillips, Jr., E. E. Lattman, P. Cummins, K. Y. Lee, and C. Cohen, *Nature (London)*, **278**, 413 (1979).
(29) G. N. Phillips, Jr., J. P. Fillers, and C. Cohen, *Biophys. J.*, **32**, 485 (1980).
(30) M. Schiffer and A. B. Edmundson, *Biophys. J.*, **7**, 121 (1967).
(31) M. F. Perutz, J. C. Kendrew, and H. C. Watson, *J. Mol. Biol.*, **13**, 669 (1965).
(32) D. A. D. Parry, *J. Theor. Biol.*, **26**, 429 (1970).
(33) R. D. B. Fraser and T. P. MacRae, *J. Theor. Biol.*, **3**, 640 (1961).
(34) R. D. B. Fraser and T. P. MacRae, *Nature (London)*, **189**, 572 (1961).
(35) W. G. Crewther, A. S. Inglis, and N. M. McKern, *J. Biochem.*, **173**, 365 (1978).
(36) K. H. Gough, A. S. Inglis, and W. G. Crewther, *J. Biochem.*, **173**, 373 (1978).
(37) A. D. McLachlan, *J. Mol. Biol.*, **122**, 493 (1978).

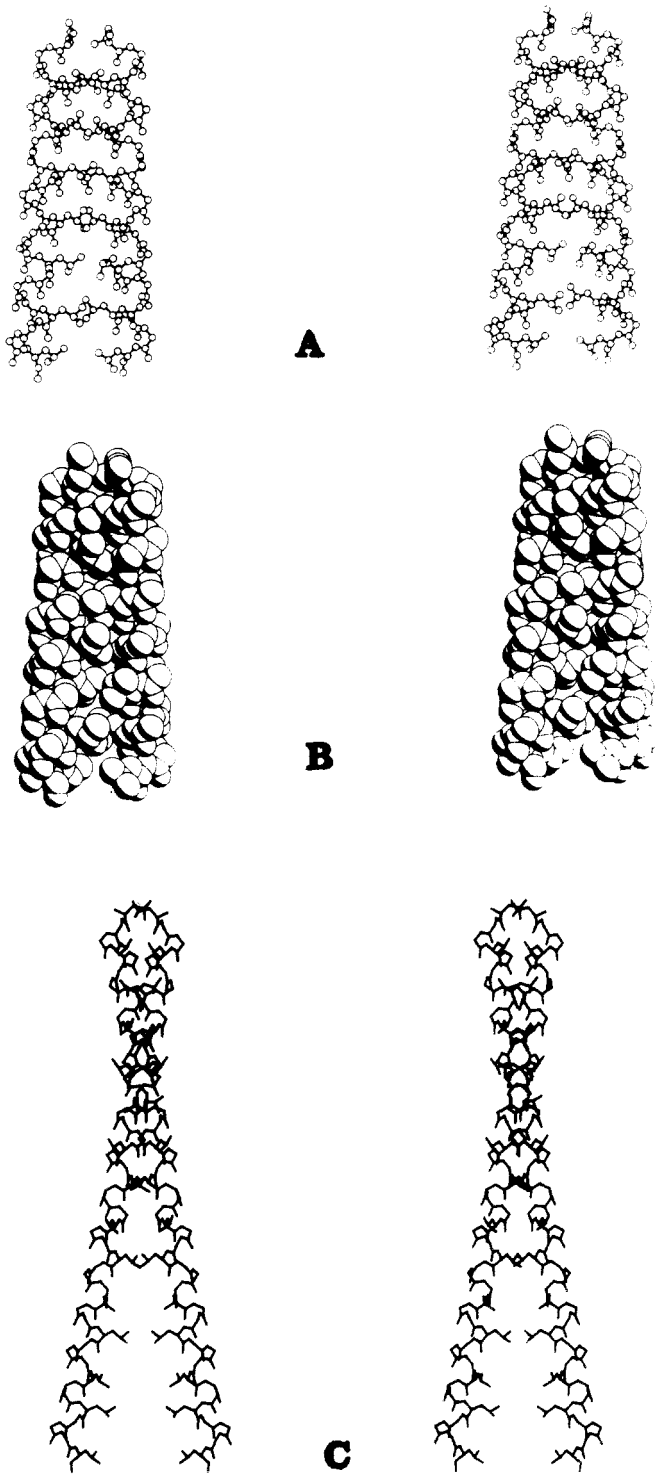

Figure 2. Stereo drawings of a "cruciform" structure containing the heptad repeat (Leu-Gly-Gly-Leu-Gly-Gly-Gly)$_n$. The two undeformed helices are parallel in-register and packed in a "knobs-into-holes" manner at an angle of 20° to one another. (a) Cruciform consisting of 2 α helices of 25 residues viewed from the side. (b) The same cruciform as in (a) but with the van der Waals radii of the atoms (except hydrogen) included to emphasize the packing of side chains. (c) Two 50 residues α-helices in a cruciform structure showing more clearly how the "knobs-into-holes" packing deteriorates. The packing is good at the top and clearly nonexistent at the bottom. The carbon–carbon distances for the β and γ carbons of the interacting leucine residues a–a′ and d–d′ calculate to be within 4–6 Å for the first 3 heptads and increase to 23 Å by residue 8a (position 50). These drawings can more easily be viewed in stereo with a stereoscope obtained from Hubbard Scientific Company, PO Box 105, Northbrook, IL.

basis, that the proteins may be coiled coils. Such an interpretation must be approached with caution since this similarity may reflect that the protein contains α helices with hydrophobic faces. If both the 4–3 repeat and 5.1-Å reflection are observed, then the question of which proteins contain coiled coils or "cruciforms" will most likely be resolved by the X-ray crystallographic structures.

Hydrophobic Requirements for Coiled-Coil Stabilization

We have examined many synthetic polyheptapeptides to investigate the requirements for hydrophobes at positions "a" and "d". The polypeptides were constructed by two methods. The earlier studies involved polymerizing heptapeptides in solution.[48–50] This was followed by a more elegant synthesis where a polyheptapeptide (M_r 4625) was constructed by fragment couplings of heptapeptides to the growing peptide chain on a solid support[51] to provide a coiled coil of defined molecular weight in high yield. This peptide had the same molar ellipticity as tropomyosin, a molecule seven times larger, both essentially 100% α helical.[51] From the results of these studies, we have been able to demonstrate that coiled-coil stability increases with the number of hydrophobic residues at positions a and d (chain length effect). The heptapeptide (sequence shown in Figure 1) demonstrates no α helicity in aqueous solution whereas the polyheptapeptide of the same sequence (M_r 4625, 8 M urea) shows high helicity which is, however, less stable to increasing temperature in benign medium ($t_{1/2}$ = 72.5 °C; $t_{1/2}$ is the temperature at which half the α-helical structure is lost compared to the fraction of helix at 10 °C) than a larger polyheptapeptide (M_r 9500, 8 M urea) with the same hydrophobes in positions a and d ($t_{1/2}$ = 90 °C).[51] These results also suggest that there is a minimum chain length for the coiled coil for polyheptapeptides of this sequence between 1 heptapeptide (maximum 2 leucine–leucine hydrophobic interactions) and six heptapeptides (maximum 11 leucine–leucine hydrophobic interactions).[51]

The minimum chain length for the formation of a coiled coil is sequence dependent. One reason for this is that coiled-coil stability is increased as the residues occupying the a and d hydrophobic positions increase in hydrophobicity. Thus, polyheptapeptides containing

(38) R. F. Doolittle, D. M. Goldbaum, and L. R. Doolittle, *J. Mol. Biol.*, **120**, 311 (1978).
(39) D. A. D. Parry, *J. Mol. Biol.*, **120**, 545 (1978).
(40) R. Henderson and P. N. T. Unwin, *Nature (London)*, **257**, 28 (1975).
(41) D. M. Engelman and G. Zaccai, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 5894 (1980).
(42) C. W. Ward and T. A. Dopheide, *Aust. J. Biol. Sci.*, **33**, 441 (1980).
(43) G. Stubbs, S. Warren, and K. Holmes, *Nature (London)*, **267**, 216 (1977).
(44) Y. Nakashima, R. L. Wiseman, W. Konigsberg, and D. A. Marvin, *Nature (London)*, **253**, 68 (1975).
(45) L. Makowski, D. L. D. Caspar, and D. A. Marvin, *J. Mol. Biol.*, **140**, 149 (1980).
(46) B. N. Manjula and V. A. Fischetti, *J. Exp. Med.*, **151**, 695 (1980).
(47) J.-P. Capony and M. Elzinga, *Biophys. J.*, **33**, 148a (1981).
(48) S. A. St. Pierre and R. S. Hodges, *Biochem. Biophys. Res. Commun.*, **72**, 581 (1976).
(49) S. A. St. Pierre and R. S. Hodges, *Can. J. Biochem.*, **55**, 636 (1977).
(50) R. S. Hodges, S. A. St. Pierre, and F.-S. Tjoeng, in "Peptides Proceedings of the Fifth American Peptide Symposium", M. Goodman and J. Meienhifer, Eds., Wiley, New York, 1977, p 411.
(51) R. S. Hodges, A. K. Saund, P. C. S. Chong, S. A. St. Pierre, and R. E. Reid, *J. Biol. Chem.*, **256**, 1214 (1981).

two alanines at these positions are less stable than ones containing two leucines.[51]

The studies outlined above have demonstrated that a large part of the stability of the coiled coil and consequently the α helicity is due to the presence of hydrophobes at positions a and d exactly as predicted. In fact the polyheptapeptide with only leucine in the hydrophobic positions has such good stability that it has provoked additional investigations. The peptide has greater stability than CM-tropomyosin ($t_{1/2}$ = 72.5 and 41 °C, respectively) in spite of the fact that the peptide has a maximum of 11 hydrophobic interactions, all of them leucine–leucine interactions, while CM-tropomyosin has a maximum of 71 (25 of which are Leu–Leu interactions, 23 involve Val, Met, Ile, and Tyr interactions, and 23 involve Ala–Ala interactions). To account for this discrepancy, it is necessary to have some kind of destabilization of the coiled coil in tropomyosin. The sequence studies indicate that many of the hydrophobic positions are occupied by alanine and a few by charged residues. These would destabilize the coiled coil relative to one composed entirely of leucine. In portions of the sequence where poorer hydrophobes predominate, we might expect these areas to be less stable.

α-Helical Potential Requirements for a Coiled Coil

Another intriguing possibility for destabilization of the coiled coil lies in the α-helical potential of the residues at positions other than a and d. We have shown that the replacement of alanine by serine at positions c and f in the sequence Leu-Glu-Ala-Leu-Glu-Ala-Lys results in a much lower α-helicity of the polyheptapeptide.[51] It is likely that the α helices in coiled coils are made possible by the hydrophobes at a and d but the α-helical potential of the other residues can modify the α-helical stability. The sum of these factors would then determine the helicity and stability of a particular region of the sequence.[51]

Previously[26] it was pointed out that there is an alternation of groups with bulky and less bulky side chains occupying the a and d positions as one proceeds through the tropomyosin amino acid sequence. Thus, regions rich in small hydrophobic residues, e.g., alanine, are separated by regions rich in bulky side chains such as leucine, valine, and isoleucine. A recent analysis in our laboratory[52] has shown that this periodicity of less bulky and bulky hydrophobes is clearly related to the periodicity of the averaged α-helical potential, as defined by Chou and Fasman.[53] Those regions rich in bulky hydrophobes in positions a and d of the repeating heptad correspond to regions of low α-helical potential, while those with low averaged hydrophobicity parameters in positions a and d (i.e., segments rich in alanine) correspond to maxima in the averaged α-helical potential. The exact contributions of these counteracting effects on the stabilities of particular regions of the coiled-coil structure are difficult to assess at present. However, the polyheptapeptide (Leu-Glu-Ser-Leu-Glu-Ser-Lys)$_n$ has an average α-helix parameter of 1.16, which is similar to or lower than tropomyosin (1.18) and the majority of tropomyosin fragments,[54] yet this peptide shows greater stability ($t_{1/2}$ is 42–58 °C higher).[51] This suggests that the nonpolar interactions in the a and d positions make a major contribution to the stabilization of the coiled coil.

Charged Interactions for Coiled-Coil Stabilization

In assessing the importance of possible ion pair interactions we come again to the meaning of the phrase, aqueous environment. Charged moieties may not interact significantly in an aqueous environment. It may be assumed that, since in an α helix the side chains are oriented outwardly toward the environment, the composition of the solvent solely determines the environment. Yet if a charged residue is partially surrounded by hydrophobic residues, its microenvironment is not entirely aqueous.[55] Thus, charged interactions may contribute substantially to stabilizing an α-helix. Even more interesting is the possibility that this may contribute to coiled-coil stability. If we visualize a small section of the coiled coil as a sandwich with the α helices as the bread, then the interacting hydrophobic residues are the filling, and inspection of a model reveals that the filling is only partially accessible to the solvent. Salt bridges that formed between the two chains and bridge the filling would undoubtedly be in a partially nonaqueous microenvironment and could increase the stability of the coiled coil.

Analysis of the sequence of tropomyosin revealed that positions e and g are generally occupied by charged residues. Position g is predominantly basic while e is generally acidic.[24] The nonrandom distribution of these residues suggests that solubility is not their only function. It has been suggested that ionic interactions between the chains such as those between the e′ and g or e and g′ positions (Figure 1) may bring the chains into register[25,56] and stabilize the coiled coil. It is of interest that the two histidines of tropomyosin, His-153 and His-276, occupy outer positions f and c (Figure 1) in the coiled coil, yet chemically induced dynamic nuclear polarization studies (CIDNP) have shown that these histidines are not available for reaction with the flavin dye until the protein is denatured.[57] One explanation would be that the histidines are prevented from interaction with the dye by their microenvironment, or ion-pair formation, or hydrogen bonding made possible by their microenvironment, even though they are on the outside of the molecule.

Although the tropomyosin molecule is stable over a surprisingly wide range of pH, it does denature under severely basic conditions.[19] This occurs because the net negative charge on the two chains increases as the basic residues are deprotonated. The electrostatic repulsion that results in the denaturation can be partially overcome by the addition of 1 M KCl. In an analogous manner the presence of positively and negatively charged side chains near one another in a polypeptide chain may eliminate the possibility of charge repulsion. Scheraga has suggested that this charge compensation may play a role in stabilization of α helices of highly

(52) R. S. Hodges, J. R. Pearlstone, P. C. S. Chong, and L. B. Smillie, unpublished results.
(53) P. Y. Chou and G. D. Fasman, *Trends Biochem. Sci.*, 2, 128 (1977).
(54) M. D. Pato, A. S. Mak, and L. B. Smillie, *J. Biol. Chem.*, **256**, 593 (1981).
(55) C. R. Cantor and P. R. Schimmel, in "Biophysical Chemistry, Part 1, The Conformation of Biological Macromolecules", W. H. Freeman, San Francisco, 1980, p 290.
(56) A. D. McLachlan and M. Stewart, *J. Mol. Biol.*, **98**, 293 (1975).
(57) B. F. P. Edwards and B. D. Sykes, personal communication.

charged polypeptides.[58-61] Thus, in the case of tropomyosin at neutral pH, besides the possible contribution of specific ion-pair formation to stabilization of the coiled coil, these more general electrostatic effects may ensure that the net charge on a chain is not so high as to preclude the association of two chains.

The increased stability of coiled coils at low pH cannot be explained by salt linkages, general electrostatic effects, or hydrophobic interactions (which should be independent of pH). Clearly some new contribution at low pH, which is yet not understood, must be involved.[19,20]

Experimental Investigations of the Factors Involved in α-Helical Coiled-Coil Stabilization

The areas that follow cover what we consider to be investigations most likely to aid our understanding of tropomyosin and by extension of other proteins. It is no coincidence that these appear tailor-made for the use of synthetic peptides.

Tyrosine as a Probe of the Hydrophobic Positions. Of the six tyrosines in tropomyosin, five are found in the hydrophobic a and d positions. Spectral and reactivity properties of tyrosine, in contrast to the usual occupants of the hydrophobic positions, allow it to serve as an excellent probe of these positions. Initial studies of these tyrosines indicated that they were fully accessible to solvent on the basis of pH titrations.[19] More recent investigations have indicated that the molecule denatures in the pH range studied and the original work may show only that the tyrosines are accessible because the structure is unfolded.[62] These same studies, however, demonstrated that the tyrosines were less accessible to nondenaturing perturbants than free tyrosine in solution or tyrosines in the denatured molecule. The main factor in determining accessibility was found to be the size of the perturbant, with those having a mean diameter greater than 4 Å showing decreased accessibility with increased size.[62] This is consistent with the tyrosines being "sandwiched" between the α helices and accessible by way of the crevice. In support of this are the following findings: firstly, iodination of tropomyosin by ^{125}I and lactoperoxidase showed that the five tyrosines in the hydrophobic a and d positions were iodinated to the same extent while tyrosine-261 in position e was labeled to a level about threefold over that of the others;[63] secondly, while the tyrosines are not exposed enough to react with flavin dye in laser CIDNP experiments until the protein is denatured,[57] they can be fully nitrated with tetranitromethane, a highly hydrophobic reagent.[64] NMR evidence has shown that at a pH of 7.95, the chemical shifts of the tyrosines correspond closely to those of standard amino acids broadened only by increase in molecular weight due to being part of tropomyosin. This suggests that the residues are relatively exposed to solvent and none so restricted in a unique environment as to produce a different chemical shift.[65] At a pH of 3.9, where the coiled coil is more stable, the tyrosines appear to be immobilized.[65]

In summary, then, it appears that hydrophobic positions occupied by tyrosines are in a partially hydrophobic environment but are partially exposed to solvent molecules, especially those of a mean diameter less than 4 Å. At physiological pH the tyrosines have considerable rotational freedom, but this can be removed under conditions that strengthen the coiled coil. Whether this is an accurate picture of all hydrophobic positions or only those occupied by tyrosines has yet to be established. Although tyrosine does contain a hydrophobic aromatic ring as part of its phenolic side chain, the hydroxyl moiety imparts considerable polar character to this side chain. It may be that tyrosine is not truly representative of what hydrophobes ordinarily experience at a and d positions because its presence disrupts the structure. If less bulky and/or polar "reporter" groups are investigated in tropomyosin or its analogues, it may be found that their accessibility to solvent and mobility is even more limited than is that of the tyrosines.

Differential Stabilities of Regions of Tropomyosin. From our previous studies, as discussed above, we feel that the stability of coiled coils is to a great extent determined by hydrophobic interactions. There appears to be some evidence including our peptide studies mentioned earlier that the α-helical potential of the residues in the sequence may aid in stabilizing the coiled coil.[54,66] Another factor which almost certainly modifies the stability of the coiled coil is electrostatic repulsion and attraction.[54,65,67] The sum of the effect of these factors at any point in the sequence is probably responsible for the stability of the structure at that point, and by studying the variations in stability along the molecule, we may learn something of the relative contributions of these factors. That regions of the tropomyosin molecule vary in stability is well documented,[28,65-77] but only recently have these studies begun to indicate which regions were more or less stable. Several studies are consistent, with the central region 126–183 being less stable than the rest of the molecule. This has been shown by susceptibility of this region to proteolytic degradation,[54] by direct study of fragments,[78] by the inability to cross-link at Cys-190 at concentrations of denaturant where the molecule is still intact,[68] and by differential scanning calorimetry of fragments containing this region.[67] It has been suggested that the region around Cys-190 (173–196, 11 negative, 3 positive

(58) F. R. Maxfield and H. A. Scheraga, *Macromolecules*, 8, 491 (1975).
(59) M. K. Dzgert, G. T. Taylor, F. Cardinaux, and H. A. Scheraga, *Macromolecules*, 9, 794 (1976).
(60) Y. Kobayashi, F. Cardinaux, B. D. Zweifel, and H. A. Scheraga, *Macromolecules*, 10, 1271 (1977).
(61) Y. Konishi, J. W. van Nispen, G. Davenport, and H. A. Scheraga, *Macromolecules*, 10, 1264 (1977).
(62) B. Nagy, *J. Biol. Chem.*, 252, 4557 (1977).
(63) A. S. Mak and L. B. Smillie, *J. Mol. Biol.*, 149, 541 (1981).
(64) B. Nagy and H. Bialkowska, *Biophys. J.*, 33, 238a (1981).
(65) B. F. P. Edwards and B. D. Sykes, in "NMR in Biology", R. A. Dwek, I. D. Campbell, R. E. Richards, and R. J. B. Williams, Eds., Academic Press, New York, 1977, p 157.
(66) L. B. Smillie, M. B. Pato, J. R. Pearlstone, and A. S. Mak, *J. Mol. Biol.*, 136, 199 (1980).
(67) D. L. Williams, Jr., and C. A. Swenson, *Biochemistry*, in press.
(68) S. S. Lehrer, *J. Mol. Biol.*, 118, 209 (1978).
(69) E. F. Woods, *Aust. J. Biol. Sci.*, 29, 405 (1976).
(70) R. W. Cowgill, *Biochim. Biophys. Acta*, 168, 417 (1969).
(71) M. Pato and L. B. Smillie, *FEBS Lett.*, 87, 95 (1978).
(72) M. Pato, A. S. Mak, and L. B. Smillie, *J. Biol. Chem.*, 256, 593 (1981).
(73) E. F. Woods, *Int. J. Pept. Prot. Res.*, 1, 29 (1969).
(74) A. Holtzer and Y.-Y. H. Chao, *Biochemistry*, 14, 2164 (1975).
(75) C. Cohen, D. L. D. Caspar, D. A. D. Parry, and R. M. Lucas, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 205 (1971).
(76) C. Cohen, D. L. D. Caspar, J. P. Johnson, K. Nauss, S. Margossiam, and D. Parry, *Cold Spring Harbor Symp. Quant. Biol.*, 36, 205 (1972).
(77) M. S. Pont and E. F. Woods, *Int. J. Protein Res.*, 3, 177 (1971).
(78) E. E. Eckard and R. W. Cowgill, *Biochim. Biophys. Acta*, 434, 406 (1976).

residues) has the largest excess of negative charges in the TM structure[67] and that electrostatic repulsion may destabilize this portion of the sequence.[54,67] This hypothesis gains some support from the finding that tropomyosin is stabilized by the addition of neutral salt, and so electrostatic repulsion would seem to play an important role in controlling the stability of the intact TM molecule[69] and some of its fragments.[67]

The specific cleavage points of tropomyosin by trypsin and chymotrypsin have also been explained by anomalies in the sequence.[54] The tryptic cleavage point occurs at Arg-133, which is very close to Asp-137, the only aspartic acid residue to occupy an a or d position. The chymotryptic cleavage point Leu-169 is close to the region of excess negative charge and is also found in a sequence of four consecutive bulky hydrophobes (169–172) that occurs nowhere else in the sequence.[54] Testing of the effect of any of these conditions could most easily be done with synthetic peptides.

Transmission of Conformational Changes. It would be difficult to visualize a conformational change occurring in a coiled coil resembling our most stable polyheptapeptides, which thermal and denaturant studies have characterized as rigid and inflexible.[51] Tropomyosin, on the other hand, has been characterized by NMR as being quite flexible[65] and less stable to heat and denaturants than our polyheptapeptides.[51] For this reason it is not surprising that transmission of conformational changes occurs along the length of tropomyosin.[79–81]

NMR studies show that disulfide bridge formation, or S-carboxymethylation of the cysteines at position 190 (Cys-190 is in a hydrophobic position a of the heptad repeat, Figure 1) in the coiled coil, causes broadening of the histidine resonances at position 153 some 55 Å away.[80] Cooperativity between histidines-153 and -276 has been suggested from NMR data of pH titrations of polymerized tropomyosin.[81,82] If perturbations at one site in tropomyosin can be transmitted along the molecule, the possibility exists that the careful control of tropomyosin's overall stability and variation in regional coiled-coil stability has provided a means for a conformational change to be transmitted along the coiled coil during muscle contraction. This change could account for the movement of the tropomyosin molecule in the grooves of the actin helix.

Generation of Parallel and In-Register Chains. One aspect of the difficulty encountered by maximizing the hydrophobic interactions in a coiled coil has revealed something about the sequence specificity necessary to align the helices. When we constructed polypeptides of M_r 9500 (8 M urea), they formed concatemers in benign media because of the "sticky ends" left when the coiled coils formed incorrectly in staggered fashion.[48] To overcome this inability of the polymers to form in-register, the previously described polyheptapeptide of MW 4625 in 8 M urea was constructed.[51] The cysteine near the amino terminus of the chain was oxidized, forming a disulfide bridge and bringing the chains in-register and parallel. This arrangement worked perfectly but, much to our surprise, we found that when the in-register coiled coil was reduced, completely denatured in 8 M urea, and then renatured without benefit of the disulfide bridge, the original helicity was regained.[51] We believe that at shorter chain lengths any staggering of the chains would reduce the hydrophobic interactions to an unacceptable level and as a result the in-register configuration is energetically favored. At longer chain lengths with only leucines in the hydrophobic positions, very stable coiled coils are formed even in a staggered conformation, making annealing into the in-register state difficult. Other possibilities have been advanced as explanations for the ability of tropomyosin to form in-register, and they have been discussed. However, it is possible that the specificity is due mainly to maximizing hydrophobic interactions in a protein where any loss of these interactions by staggering results in a substantial loss of stability.

Biologically Interesting Proteins That May Involve Coiled-Coil or "Cruciform" Structures. Many proteins have been mentioned in the literature as containing potential coiled-coil regions. The first group of these and probably still the best documented is the k–m–e–f group (keratin, myosin, epidermis, fibrinogen). Each of these has a rodlike structure, which exhibits both the 1.49- and 5.1-Å reflections as shown by wide-angle X-ray diffraction. It is most likely that the rod portion of myosin (~200 000 daltons) and paramyosin (~220 000 daltons) with helical contents greater than 90%, along with tropomyosin, are true examples of two-stranded α-helical coiled coils. The 4–3 repeat has been identified in the partial sequence of the myosin rod.[47] While the evidence is not unassailable, it appears that fibrinogen and keratin have three-stranded coiled coils. Fibrinogen consists of three nonidentical chains and has a molecular weight of 340 000. It has been proposed that fibrinogen contains two regions of three-stranded coiled coils involving 40–45 residues each.[38,39,83] It is also suggested that the primary driving force for assembly of the three nonidentical chains is the hydrophobic interactions of the three chains in the supercoiled regions. Although fibrinogen does exhibit the 5.1-Å diffraction pattern, the 4–3 repeat is not nearly as well developed as that seen in tropomyosin, and it may well turn out that the molecule contains three chains in a cruciform or segmented rope structure rather than a true coiled coil. On the other hand, keratin, because of the size of the helical segments (just over 100 residues) and the excellent hydrophobic 4–3 repeat, is probably a true coiled coil and may be three-stranded.[35–37,84]

Many other proteins, hemagglutinen membrane glycoprotein of influenza virus,[42,85] tobacco mosaic virus coat protein, PF1 and fd filamentous bacterial virus coat proteins,[43,45,86,87] streptococcal M protein,[46,88] murein lipoprotein from *Escherichia coli*,[37,89,90] and bac-

(79) M. D. Pato, A. S. Mak, and L. B. Smillie, *J. Biol. Chem.*, **256**, 602 (1981).
(80) B. F. P. Edwards and B. D. Sykes, *Biochemistry*, **19**, 2577 (1980).
(81) B. F. P. Edwards and B. D. Sykes, *Biochemistry*, in press.
(82) B. F. P. Edwards and B. D. Sykes, *Biochemistry*, **17**, 684 (1978).
(83) R. F. Doolittle, *Polym. Prepr., Am. Chem. Soc., Div. Polym. Chem.*, **20**, 47 (1979).
(84) P. M. Steinert, *J. Mol. Biol.*, **123**, 49 (1978).
(85) I. A. Wilson, J. J. Skehel, and D. C. Wiley, *Nature (London)*, **289**, 366 (1981).
(86) D. A. Marvin and B. Hohn, *Bact. Rev.*, **33**, 172 (1969).
(87) C. Nave, A. G. Fowler, S. Malsey, D. A. Marvin, H. Siegrist, and E. J. Wachtel, *Nature (London)*, **281**, 232 (1979).
(88) B. Hosein, M. McCarty, and V. A. Fischetti, *Biochemistry*, **76**, 3765 (1979).
(89) W. F. DeGrado, F. J. Kezdy, and E. T. Kaiser, *J. Am. Chem. Soc.*, **103**, 679 (1981).

teriorhodopsin from the purple membrane of *Halobacterium halobium*,[41] have been shown to exhibit at least one of the main features of coiled coils, that is, the 5.1-Å reflection or the 4–3 hydrophobic repeat. Whether these proteins are in fact examples of coiled coils, "cruciform" structures, or simply α helices with hydrophobic surfaces for helix–helix or helix–lipid interactions must await more experimental evidence.

In conclusion, we hope we have communicated in this Account some of our enthusiasm for the use of tropomyosin and its synthetic analogues as a vehicle to investigate the relationship of primary (amino acid sequence) to secondary (α helix) and quaternary structure (coiled coil), and to its biological functions. Tropomyosin occupies an almost unique position. It represents to us the next step in the investigations of amino acid polymer research that started with homopolymers and progressed to the more sophisticated aqueous soluble block copolymers and the random copolymers of Scheraga. Tropomyosin represents an aqueous soluble polymer consisting of a repeating heptapeptide in sequence that differs from the polymers in these earlier studies in that it has well-defined biological functions. In addition the coiled coil or its possible variants (segmented rope, cruciform) appear to have been repeated by a parsimonious Nature in a variety of biologically interesting proteins. Thus, the study of tropomyosin using synthetic analogues appears to be to us a field unmatched in simplicity of experimental design and has a good potential for increasing our understanding of protein conformation and function.

Our many colleagues in the University of Alberta MRC Group in Protein Structure and Function have been invaluable as resources to be drawn on in this review. In particular we thank Drs. L. Smillie and B. Sykes for access to papers in press. Dr. M. James was a particularly enlightening participant in several discussions. The expert assistance of Masao Fujinaga and Donna Clare in the design and execution of the stereo drawings and figures, respectively, is gratefully acknowledged. We are grateful to Dr. C. Swenson for a preprint of his publication, to Dr. D. Parry, Dr. W. McCubbin, and Pele Chong for helpful discussions, and to Dr. A. Holtzer for his critical evaluation of the final manuscript.

(90) V. Braun, H. Rotering, J. Ohms, and H. Hagenmaier, *Eur. J. Biochem.*, **70**, 601 (1976).