Uncooperative Block in the Tropomyosin Coiled Coil

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We have synthesized a 35-residue, capped peptide in order to test the idea that long, two-chain coiled-coil proteins are made up of segments ("cooperative blocks") that quasi-independently undergo two-state unfolding, as many globular proteins do.[1,2] Although protein folding is highly cooperative, small, excised subsequences of a protein chain sometimes can retain structural integrity. Coiled-coil proteins, whose structure comprises two parallel, registered α-helical chains in supertwisted association, are striking examples. Large segments of the tropomyosin molecule[3-5] and leucine zippers[6] remain coiled coils when detached from the parent proteins.

Tropomyosin is a protein molecule in which two 284-residue chains associate to form an unbroken coiled coil.[7] An investigation by differential scanning calorimetry (DSC) led to a detailed assignment of nine cooperative blocks, each supposed to unfold in a two-state manner, and a melting temperature was assigned to each block.[1,2]

We report here a direct test of this model using a synthetic peptide replica of one of these alleged blocks. The peptide synthesized is[8] Ac-CGGYENALDRAEQAEADKKAAEDRSKQLEDELVSL-NH_2. We call it CGGY($_{16}$Tm$_{46}$). The N-terminus is cysteine, for cross-linking; next, a two-glycine swivel provides flexibility for cross-linking;[6] next, a tyrosine facilitates concentration determination. Caps avoid unfavorable terminal charge–helix dipole interactions. The rest of the chain comprises residues 16–46 of the α-tropomyosin sequence, which is, when dimerized, virtually all of cooperative block 6.[9] This block is supposed to melt at 58 °C at a formal concentration of $\sim 10^{-4}$ F.[1,2] Concentration is relevant because the reduced coiled coils unfold to monomers.[10,11] Tropomyosin residue 16 is b in the pseudorepeating heptad (a–g) that characterizes coiled coils.[12] Such heptads demonstrate strong statistical preference for hydrophobes in heptad positions a and d, negative charges at e, and positive at g.[7,12]

CD experiments[13] on CGGY($_{16}$Tm$_{46}$) were conducted on both reduced and cysteine-cross-linked forms[14] in the usual medium, $NaCl_{500}NaPi_{50}(7.4)$,[15] with added dithiothreitol (5 mM) in the former case. The results are startling. At 2.5 °C, the CD spectrum (not shown) shows characteristic α-helix minima at 208 and 222 nm. However, the magnitudes and the CD_{222}/CD_{208} ratio for the reduced form are smaller than for cross-linked material and all are smaller than for native tropomyosin. These indicate less than full helicity for what was expected to be one of the most stable regions of the parent molecule.

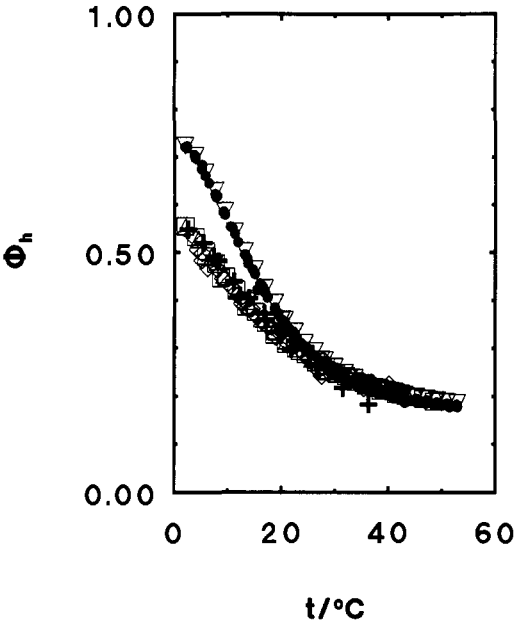

Figure 1. Fraction helix (from CD at 222 nm) vs temperature for CGGY($_{16}$Tm$_{46}$). Chain formality of reduced peptide: open diamonds, 2×10^{-6} F; plusses (some rereduced), $1-2 \times 10^{-5}$ F; open squares (rereduced), 2×10^{-4} F; open triangles, 2×10^{-3} F. Chain formality of cross-linked peptide: open dels, 1×10^{-5} F; solid circles, 2×10^{-4} F.

The equilibrium (i.e., reversible) thermal unfolding profiles are shown in Figure 1, which reveals further disagreement. The cross-linked protein is maximally only ~70% helical and unfolds relatively noncooperatively. The melting temperature is only 13 °C. Reduced CGGY($_{16}$Tm$_{46}$) is maximally 55% helical and melts at 5 °C, over 50 deg lower than the ~58 °C expected. Furthermore, the latter's unfolding is independent of concentration over a 1000-fold range ($\sim 10^{-6}$–10^{-3} F), indicating that the chains are not associated.[10] The small additional stability of the cross-linked peptide must be caused by weak secondary association of the tethered chains. It is noteworthy, too, that CGGY($_{16}$Tm$_{46}$) has no CD at 280 nm, where parent tropomyosin has a band ascribable to tyrosine–tyrosine interchain interactions.[16-18]

These unexpected properties cannot stem from the presence of the nonnative tetrapeptide at the N-terminal. The CGG trio is well tolerated at coiled-coil N-termini.[6] The tyrosine residue is also nondisruptive, since it appears in the a heptad position, where two of the six tyrosines of the native 284-residue chain are located.

Finally, small differences between CGGY($_{16}$Tm$_{46}$) and the full cooperative block 6, residues 12–48,[1,2] cannot account for the discrepancies, because there are no significant differences in fraction of hydrophobes or of positively or negatively charged groups, numbers of helix-favoring interactions, or fraction of occurrence of a given residue in the various heptad positions. We are forced to conclude that CGGY($_{16}$Tm$_{46}$) behaves quite contrary to expectations based upon the cooperative block model for tropomyosin. Suspicions aroused by the presence of noncanonical residues in critical heptad positions (S in a, L in e, D and E in g) apparently provide a better basis for judgment than the calorimetric assignments.

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(8) The protein product was sequenced on the resin and found satisfactory. After removal and capping, it was purified by C-18 reversed-phase HPLC to >95% purity, judged by HPLC, strong cation exchange chromatography, and amino acid composition. Fast-atom-bombardment mass spectroscopy gave 3895 Da for the primary ion, in exact agreement with the expected singly protonated peptide. Electrospray mass spectroscopy gave the expected 3893.9 Da, with other ions less than 5% of it.
(9) This is the most recent numbering (ref 2). An earlier version (ref 1) calls it block 1.
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(13) Ample detail on the CD determinations may be found in Holtzer et al. (Holtzer, M. E.; Bracken, W. C.; Holtzer, A. *Biopolymers* **1990**, *29*, 1045-1056) and in ref 10.
(14) Reduction was effected by using 5-10 mM dithiothreitol and stirring for 15 min at room temperature. Oxidation was effected by stirring overnight in air at room temperature. This oxidation is not complete, but cross-linked and non-cross-linked species are readily separated by C-4 reversed-phase HPLC. The rereduced cross-linked fraction was indistinguishable (Figure 1) from the product of direct reduction.
(15) We designate complex aqueous solvent media by giving the millimolarity as subscript to the solute species, followed by the pH in parentheses.
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While far smaller than expected from the idea of independent blocks, the helix content of CGGY($_{16}$Tm$_{46}$) is far *larger* than predicted from the Zimm-Bragg single-chain theory using host-guest parameters.[5] This may be caused by the substantial number of *intra*chain salt bridges possible in subsequence 16-46, although these are not, overall, significant in the parent chain.[19] Studies are in progress on other tropomyosin subsequences designed to shed light on intrinsic local stabilities.

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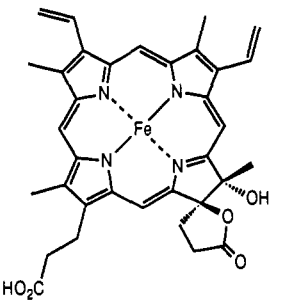

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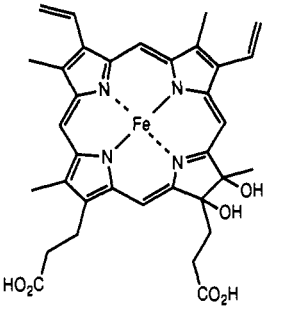

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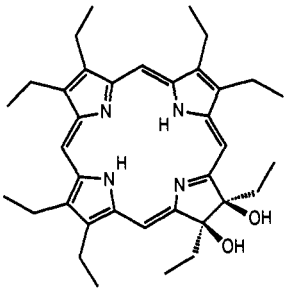

3

Molecular Structure of a Dihydroxychlorin. A Model of the Green Heme *d* and of a Photodynamic Therapy Sensitizer

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Heme *d* is the iron chlorin prosthetic group of bacterial terminal oxidases.[2,3] The molecular structures proposed for the green heme are alternatively a chlorin core with trans γ-spirolactone and hydroxyl groups at the saturated pyrrole ring[2] (**1**) or a dihydroxyprotochlorin IX[3] (**2**). **2** was shown[2] to readily cyclize to **1**. A similar spirolactone structure, but with the oxygens cis or on the same side of the pyrroline plane, has been suggested[4] for the green catalase HPII of *Escherichia coli*. In a different context, the burgeoning interest in the use of porphyrin derivatives for photodynamic therapy has focused attention on chlorins because they absorb at longer wavelengths than porphyrins and thereby allow deeper tissue penetration of incident light. Intriguingly, recent results have shown hydroxychlorins (and hydroxyporphyrins) to be effective photosensitizers for tumor eradication in vivo.[5,6] In particular, *cis*-dihydroxyoctaethylchlorin **3** (2,3-dihydroxy-2′,3′,7,8,12,13,17,18-octaethylporphyrin) showed better photonecrotic activity than the standard hematoporphyrin derivative used in clinical tumor phototherapy.[5] Chlorin **3** thus serves as a structural model both of one formulation of heme *d* and of the photodynamic sensitizer.

We present here a crystallographic determination of **3**. Not surprisingly, the molecule exhibits the structural characteristics of chlorins, i.e., elongated Cα-Cβ and Cβ-Cβ bonds in the pyrroline ring and significant distortion of the macrocycle, a feature also often found in hydroporphyrins.[7-12] Unexpectedly, the hydroxy groups of **3** form intra- and intermolecular hydrogen bonds, as evidenced by the short distances of 2.78 Å between the oxygens on the same molecule and 2.80 Å between the oxygens of adjacent molecules. The latter results suggest that if heme *d* is indeed a dihydroxychlorin, the macrocycle is likely to be anchored to its apoprotein via hydrogen bonds. As well, the propensity of **3** to form hydrogen bonds may contribute to its effectiveness,[5] and that of other hydroxyporphyrin derivatives,[5,6] as sensitizers in photodynamic therapy.

The molecular structure, atom names, and bond distances for **3**[13] are shown in Figure 1. The cis configuration of the hydroxyl

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(13) **3** was prepared by OsO_4 oxidation of octaethylporphyrin.[14] The compound crystallizes from chloroform/ethyl acetate with a half molecule of ethyl acetate of solvation $[C_{36}N_4O_2H_{48}]^{+}{}_2(C_4O_2H_8)]$, in the monoclinic space group $C2/c$, with a = 24.477 (8) Å, b = 14.696 (6) Å, c = 22.502 (16) Å, β = 114.08 (5)°, V = 7389.9 Å^3, Z = 8. Data collection at 200 K: Enraf-Nonius CAD4 diffractometer with graphite-monochromated Cu Kα radiation; scan range $4° \le 2\theta \le 100°$; 5018 reflections of the form $h, k, \pm l$ (excluding C-centering) measured, 4507 unique. The structure was solved using MULTAN 78 and refined with full-matrix least squares with anisotropic thermal parameters for the chlorin and isotropic thermal parameters for the molecule of solvation against the 2664 data with $F_o > 4\sigma(F_o)$ to final values of R_F = 0.068 and R_{wF} = 0.072. Hydrogens were included in calculated positions, except for those of the hydroxyl groups and the disordered solvation molecule. Additional refinement details are given in the supplementary material.

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