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The Design, Synthesis, and Crystallization of an Alpha-Helical Peptide

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ABSTRACT **Twelve- and sixteen-residue peptides have been designed to form tetrameric alpha-helical bundles. Both peptides are capable of folding into amphiphilic alpha-helices, with leucyl residues along one face and glutamyl and lysyl residues along the opposite face. Four such amphiphilic alpha-helices are capable of forming a noncovalently bonded tetramer. Neighboring helices run in antiparallel directions in the design, so that the complex has 222 symmetry. In the designed tetramer, the leucyl side chains interdigitate in the center in a hydrophobic interaction, and charged side chains are exposed to the solvent. The designed 12-mer (ALPHA-1) has been synthesized, and it forms helical aggregates in aqueous solution as judged by circular dichroic spectroscopy. It has also been crystallized and characterized by x-ray diffraction. The crystal symmetry is compatible with (but does not prove) the design. The design can be extended to a four-alpha-helical bundle formed from a single polypeptide by adding three peptide linkers.**

Key words: protein design, alpha-helical bundle, x-ray crystallography

INTRODUCTION

Recently the design of oligopeptides with predetermined structures and properties has met with some success. [1–6]. Several empirical methods are available for protein design, including homology with known structures, [2–7], empirical secondary structure prediction, [8–10] consideration of hydrophobic periodicity, [11–14] and solvation energies.[15] With these methods, it may be possible to design peptide segments having predictable secondary structures. By combining different secondary structural units within a single peptide chain it may be possible to design supersecondary structures [3,16,17] and eventually proteins with new properties. The trial-and-error process of design, synthesis, and structural characterization, such as that described here, may lead to improved empirical rules of protein folding.

We have chosen to construct a four-alpha-helical bundle protein (Fig. 1c). These proteins in nature consist of four sequential alpha-helices, packed antiparallel to one another, often with an angle of 18° between helices. [18] With this packing arrangement, the helices can diverge from a common point of closest approach, creating a binding pocket at one end of the bundle in which a prosthetic group can be accommodated. Natural proteins with this structural motif (including myohemerythrin, apoferritin, tobacco mosaic virus coat protein and cytochrome c′) exhibit diverse functions and little sequence homology; yet their x-ray structures reveal a common fold. [18] What enables differing sequences to fold into similar structures must be similar intermolecular forces.

Protein folding is driven by forces that seek to minimize exposure of apolar side chains to water.[15,19] In helical bundle proteins this is accomplished by packing the helices so that the apolar side chains of adjacent helices shield each other from water. The pattern of packing of the helices is often interdigitation. Residues protruding from positions i−4, i, and i+4 form a ridge (referred to here as a type 4 ridge) which packs against residues k−3, k, and k+3 (a type 3 ridge) of a neighboring helix. [22,23] This pattern of interaction is repeated between each pair of the helices, giving rise to a four-helical bundle such as that of Figure 1c. Also important for the stability of the bundle geometry may be helix-dipole-helix-dipole interactions. [24,25] The connecting loops between helices may be of variable length and sequence. Their contribution to stabilizing the four-helical bundle is uncertain, although they diminish the decrease in entropy that would otherwise be required for bringing the individual helices together.

DESIGN OF THE FOUR-HELICAL BUNDLE

Our design is based on the assumption that hydrophobic interactions among four amphiphilic helices plays the dominant role in determining the strucure. Thus, the helices were designed first. We assume that linking sequences can be added later.

Both the design and synthesis of the bundle are simplified by using the identical amino acid sequence for each of the four helices. Clearly, the use of a repeated sequence can simplify the synthesis of the

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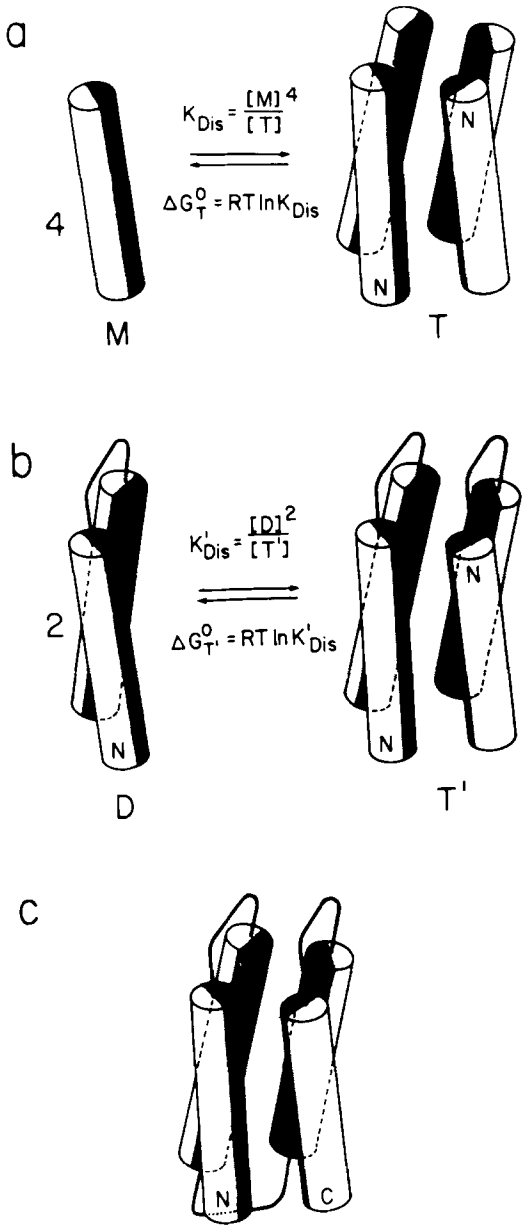

Fig. 1. A schematic illustration of an approach to the design of a four-helical bundle. In stage a, a monomeric amphiphilic alpha helix is designed which associates into a helical tetramer. The hydrophobic helical face is shaded. In stage b, two identical optimized helical sequences are joined by a peptide loop. The loop sequence can be optimized by measuring the dissociation constant for dimerization of the dimer. In stage c, the covalent tetramer can be constructed from optimized helical and loop sequences. Stages b and c will be described elsewhere. Notice in stage b that other aggregates can occur, such as a tetramer of "linear dimers."

protein, or of a gene encoding the protein. Moreover, for four identical but separate sequences, various designs can be evaluated experimentally by using the scheme depicted in Figure 1. Also, the design itself becomes a conceptually simpler problem with four identical helices: a sequence is sought which, upon application of a 222 symmetry operator, gives a structure with the characteristics of natural four-helical bundles. These include uniform and close packing of apolar side chains in the interior of the protein, helical crossing angles of approximately 18°, and polar residues exposed on the surface of the bundle.

In choosing the sequence of the helix, several criteria were used. First, residues were selected with intrinsic conformational preferences that most strongly favor helix formation.[8–10] Leucine was chosen as an apolar aliphatic residue, and glutamic acid and lysine were chosen as charged residues. The helix must also be amphiphilic (i.e., it must have an apolar and a polar face). The reason is that dehydration of the side chains on the apolar face can provide the driving force for bringing the helices together, while the polar face can provide water solubility to the ensuing aggregate. However, amphiphilicity alone is not enough to assure that the helices will associate into a four-helical bundle (e.g., references 3,29,30,31). A four-helical bundle requires that the hydrophobic residues are positioned so that they can interdigitate most effectively when they form a four-helical bundle, thus stabilizing this structure over other possible aggregates.

An initial model of a four-helical bundle was constructed by using Kendrew models and was refined with computer graphics and CPK models. The first bundle was constructed from four identical 16-residue helices with 222 symmetry, an interhelical spacing of approximately 10 Å, and an 18° tilt between neighboring helices. Once the spacing and the angle between neighboring helices are fixed, there remain just two degrees of freedom which may be varied while still maintaining 222 symmetry. These are the displacement of the four centers of mass with respect to the molecular center and the rotation of the helices about their helical axes. These were varied to achieve interdigitation of Leu residues and to shield most apolar groups from water and exposes all charged groups. In the final model, all but one Leu side chain in each helix is completely shielded from water. Residues of opposite charge were placed at positions i and i+4, with the goal of stabilizing the helix by electrostatic interactions. [32,33] The sequence of the helix designed is

Gly-Lys-Leu-Glu-Glu-Leu-Leu-Lys-Lys-Leu-Leu-Glu-Glu-Leu-Lys-Gly.

The glycyl residues at the N- and C-termini were included as coupling residues for linker sequence to be added at a later stage. Figure 2 illustrates a helical net projection of the sequence.

During the purification of a sample of this 16-residue peptide prepared by the solid phase method, a side product lacking the N-terminal four residues was isolated. It also contained an N-terminal acetyl group, which may stabilize the helix. This peptide (ALPHA-1)

ALPHA-1 Ac-Glu-Leu-Leu-Lys-Lys-Leu-Leu-Glu-Glu-Leu-Lys-Gly

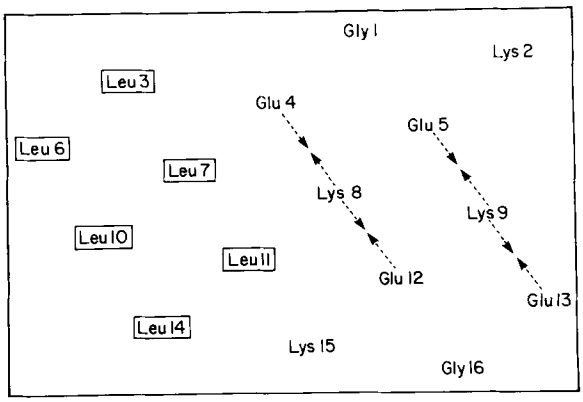

Fig. 2. A helical net diagram of the 16 residue analog of the ALPHA-1 sequence. The folded alpha-helix is amphiphilic, with Leu residues (in boxes) along one face and charged Glu and Lys residues along the opposite face. Electrostatic interactions between Glu and Lys residues on positions i and i + 4 are indicated by dashed arrows.

formed helical aggregates in aqueous solution and formed x-ray-grade crystals. It is the topic of the current paper, while the full-length 16-residue helical sequence will be discussed in a future publication.

MATERIALS AND METHODS

Peptide Synthesis and Spectroscopic Characterization

The peptide ALPHA-1 was synthesized by the solid phase technique starting with 1% crosslinked BocGly Merrifield resin (0.45 mmol/g; Pierce Chemical Company) by using a 990B Beckman synthesizer programmed as described previously. [26,27] The peptide was cleaved from the resin by reaction with HF/anisol (9:1) at 0° for 45 minutes. The crude peptide was purified in a single step by reverse phase high performance liquid chromatography (HPLC) by using a PRP-1 column (250 × 0.41 cm, Hamilton) and a linear gradient of 18–45% aqueous acetonitrile containing 0.1% trifluoroacetic acid over 30 minutes. The desired peptide eluted in a single major peak and was obtained in approximately 30% yield based on the loading of the first amino acid on the resin. The purified peptide appeared to be homogeneous by criteria of amino acid analysis and reverse phase HPLC.

Circular dichroic spectra were recorded at room temperature by using a 0.1–1.0-mm sample cell and a Jasco-J-500C spectropolarimeter which was calibrated with a dioxane solution of recrystallized androsterone. Peptide concentrations were calculated from the dry weight of the peptide with a molecular weight which was adjusted to include bound water and trifluoroacetate as determined by amino acid analysis. The concentration dependence of the circular dichroic spectra was analyzed as described previously.[11]

Crystal Growth

The HPLC-purified, synthetic peptide was used in the hanging drop vapor diffusion crystallization technique.[28] Twenty-microliter drops of a 5 mg/ml ALPHA-1 solution in 2.9 M $(NH_4)_2SO_4$, 50 mM Tris-HCl, pH 7.5, were hung from siliconized coverslips inverted over 1-ml reservoirs of 80–90%-saturated $(NH_4)_2SO_4$ in 0.1 M Tris-HCl, pH 7.5. Reservoirs were placed in the wells of Limbro Tissue Culture 24-well plastic plates (Flow Labs, Inc.) with vacuum grease applied to the rims to seal the coverslips. A granular precipitate formed in the drops at 11 days, followed by visible crystal growth from the precipitate after 2 months.

Chemical Composition of Crystals

A single 0.15-mm-diameter crystal was removed from a hanging drop and thoroughly rinsed. This was achieved by four glass capillary transfers through consecutive 20-μl drop washes in reservoir buffer containing 90%-saturated $(NH_4)_2SO_4$. After the last transfer, the crystal was dissolved in 70 μl of 0.1% TFA in H_2O. Fifty microliters of this solution was injected on a Pharmacia FPLC system equipped with a reverse phase C_2/C_{18} PepRPC HR 5/5 column. The peptide was eluted with a linear gradient of 0–100% aqueous acetonitrile containing 0.1% TFA over 30 minutes, operating with a 0.7 ml/minute flow rate. The absorption of the efluent at 214 nm and 280 nm was monitored by using a 1-cm optical path. As a control for the effectiveness of the crystal rinse procedure, an equivalent volume of transfer from the last rinse drop was subjected to the same reverse phase isolation procedure. As a positive control for ALPHA-1, a sample of the peptide used in the crystal growth mixtures was also used in the reverse phase isolation. A single peak eluting at 49% acetonitrile-TFA was collected from the chromatographed crystal sample. The solvent was evaporated by using a Savant Speed Vac Concentrator (RH 40-11), and samples were subjected both to analysis of amino acid composition and amino acid sequence.

Crystal Optical Photography

Optical microscopy of a crystal was carried out on a Wild M3 microscope at 40× with 20× eyepieces, fitted with a Nikon Microflex model PMFB attachment loaded with Plus X ASA 125 film.

Crystal X-Ray Photography

A single 0.15-mm-diameter crystal was mounted in a 0.5-mm-diameter glass capillary (Charles Supper Co.). X-ray precession photographs were recorded with Ni-filtered CuK radiation from an Elliot GX-6 rotating anode operating at 40 kV and 40 mA. Kodak DEF-5 film was used with a crystal-to-film distance of 75 mm for zero-level zones.

X-Ray Data Collection

X-ray data were collected for 0.2–0.35-mm crystals on the UCLA multiwire area detector with an Elliot GX-21 rotating anode source.

RESULTS

Synthesis and Solution Characterization of ALPHA-1

Initially, ALPHA-1 was isolated as a side product by reverse phase chromatography of a synthetic mixture of peptides obtained from an attempt to synthesize the above-mentioned 16-residue peptide by the solid phase technique. During the synthesis of the 16-mer the coupling of Glu_{13} onto Glu_{12} failed to go to completion.[34] The remaining free amino groups were therefore "capped" by reaction with 0.5 M acetic anhydride in methylene chloride to avoid the formation of deletion peptides during the subsequent synthetic steps. The 12-residue peptide (ALPHA-1) thusly formed could be isolated by reverse phase HPLC and was fortuitously found to have favorable properties. Its covalent structure was determined by amino acid analysis, fast atom bombardment mass spectroscopy, Edman sequence analysis (which confirmed that the N-terminus was blocked), and ultimately by an independent solid phase synthesis in which the acetylated 12-mer was the target molecule. The product from the latter synthesis was obtained in approximately 30% yield by reverse phase HPLC.

The synthetic peptide is in a random conformation in dilute aqueous solution, but forms alpha-helical aggregates as its concentration is increased. In dilute aqueous solution (<100 μM) ALPHA-1 exhibited a UV circular dichroic spectrum typical of peptides in random coil configurations, whereas at increasingly higher concentrations its spectrum became more typical of an alpha-helix (Fig. 3).[35] The concentration dependence of the ellipticity at 222 nm was evaluated to determine whether it conformed to a cooperative monomer to n-mer equilibrium by using the computer method described previously.[11] The data were found to be optimally described by a monomer to tetramer equilibrium with $\epsilon_{mon} = -5.5 \times 10^3$ deg cm^2/dmol, $\epsilon_{tet} = -21.7 \times 10^3$ deg cm^2/dmol, and $K_{diss} = 1.86 \times 10^{-9}$ M^3 (Fig. 4). From the dissociation constant, the free energy associated with folding four random coil monomers into a tetramer of alpha-helices was calculated to be −11.4 kcal/mol—a value which is comparable to values for small natural proteins.[36] The value of K_{diss} was, within experimental error, invariant between pH 8.5 and 7.0 and an ionic strength of 0.15 to 0.30 M, in MOPS [3-[N-morpholino]propanesulfonic acid] buffer and NaCl.

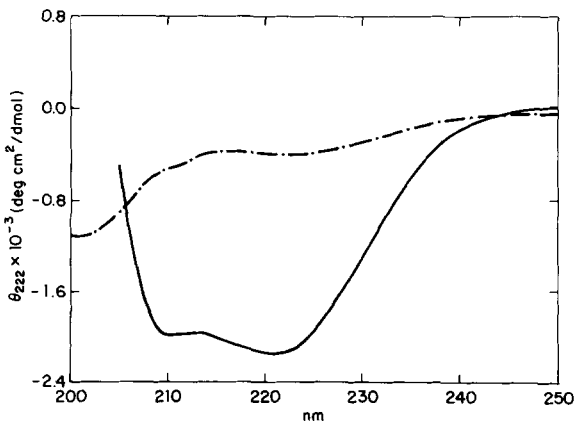

Fig. 3. The circular dichroic spectrum of ALPHA-1 dissolved in 0.15 M NaCl and 0.01 M MOPS [3-[N-morpholino]propanesulfonic acid], pH 7.0, at concentrations of 0.1 (dashed) and 10.0 mM (solid).

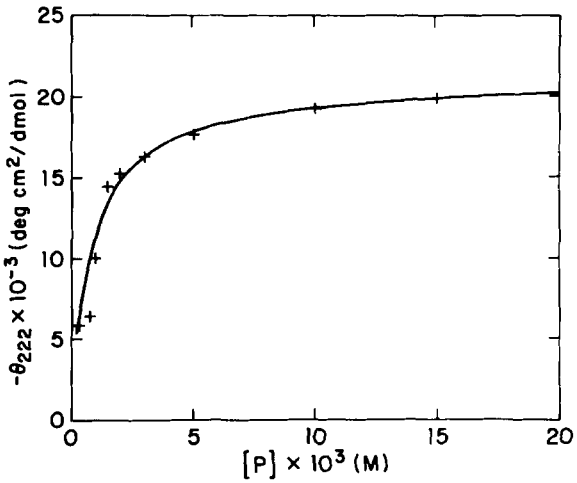

Fig. 4. The concentration dependence of the mean residue molar ellipticity as a function of ALPHA-1 concentration. The solid line is a theoretical curve for a monomer-tetramer equilibrium, with the parameters indicated in the text.

Crystal Morphology and Composition

Crystals grown from high salt mixtures containing ALPHA-1 are virtually perfect rhombic dodecahedrons (Fig. 5), exhibiting three mutually orthogonal four-folds, four three-folds, and six two-fold axes of symmetry. The largest crystals measure 0.35 mm in diameter.

The crystals are composed of the ALPHA-1 peptide as judged by reverse phase chromatography, amino acid composition, and N-terminal sequence analysis. A single crystal was extensively washed, then dissolved and chromatographed on a reverse phase system, yielding a single peak eluting with a retention

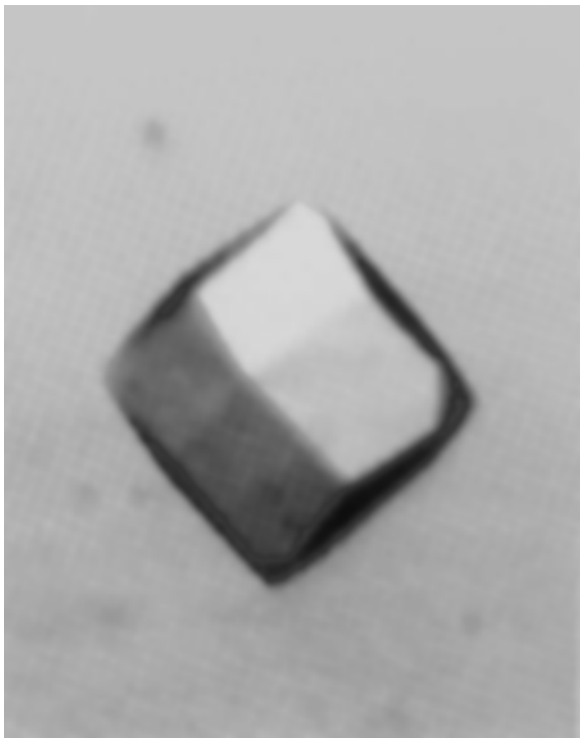

Fig. 5. An ALPHA-1 crystal (about 0.15 mm long) viewed approximately down a four-fold axis.

time which was indistinguishable from the authentic ALPHA-1 mixture. Amino acid analysis gave $Glu_{3.00}$, $Leu_{4.66}$, $Lys_{2.92}$, and $Gly_{1.41}$. Amino acid sequencing by the N-terminal Edman method failed, supporting the presence of the N-acetyl blocking group. No aromatic material was detected in the crystal or peptide samples by 280-nm absorbance.

Crystallographic Parameters

Precession photographs taken through the three mutually orthogonal four-fold axes of the crystal reveal the same principal zone with 4 mm symmetry shown in Figure 6. The lattice is centered, with the hk0 zone displaying reflections only of the class $h+k+1 = 2n$ and the hk1 zone displaying reflections only of the class h, k, and $h + k = 2n + 1$. Thus the space group is either I432 or $I4_132$ (or its enantiomorph $I4_132$), all with 48 asymmetric units per unit cell.[37] Distinguishing between I432 and $I4_132$ depends on the h00 reflections, all of which are weak or absent up to the 24th order.

The number of ALPHA-1 molecules per unit cell was determined by trial calculation of the Matthews parameter, V_M, the volume of the crystal occupied per dalton of protein. The unit cell dimension is $a = b = c = 62.3 \pm 0.1$ Å, with a calculated cell volume, V_c, of $2.42 (\pm 0.01) \times 10^5$ Å^3. The calculated molecular weight of ALPHA-1, $M_r = 1.455$, was used to compute $V_M = V_c/nM_r$ and assign n, the number of monomers per unit cell. This yielded values of 3.5 Å^3/dalton, 1.7 Å^3/dalton, and 1.1 Å^3/dalton for n = 1, 2, and 3, respectively. The n = 2 value is the most reasonable one, based on the range listed by Matthews.[38] This was confirmed by the observation of peaks 180° away from each other in the self rotation function (Dr. Duilio Cascio, unpublished results). Therefore, the asymmetric unit is a dimer and the unit cell contains 96 molecules of ALPHA-1.

Based on the helical conformation indicated by the circular dichroism data, the high concentration of ALPHA-1 in the crystals suggests that the molecules are packed together as alpha-helices. The ideal four-alpha-helical bundle structure composed of ALPHA-1 molecules would exhibit 222 symmetry in the tetramer. Both of the possible ALPHA-1 crystal space groups permit this symmetry.

In spite of the large, high-symmetry unit cell, the ALPHA-1 crystals appear suitable for data collection and x-ray structure analysis. The roughly spherical morphology of the crystal does not create asymmetric absorption effects in the x-ray beam. The well-defined facets of the crystal facilitate optical prealignment. The ruggedness of the crystal permits manipulations; the resistance to damage in the x-ray beam allows for long exposures (a precession photograph was recorded after 120 hours of beam exposure). The cubic 432 Laue symmetry permits the collection of a complete

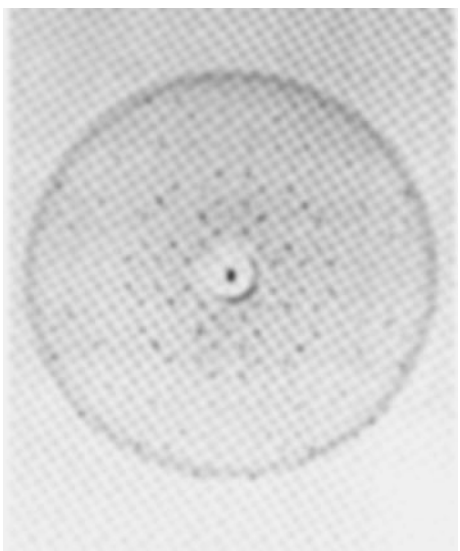

Fig. 6. A 10° hk0 x-ray precession photograph of an ALPHA-1 crystal, with the principal axes horizontal and vertical. CuK_α radiation was used; a = 62.3 Å.

data set with a small rotation angle. Reflections have been recorded out at 2.7-Å resolution with an F over sigma value greater than 5, and the reflection width subtends an angle of approximately 0.05°, indicating a small mosaic spread.

CONCLUSIONS

This paper describes the first step of an iterative procress aimed at the design of a four-helical-bundle protein. The designed peptide (ALPHA-1) is apparently long enough to form the structural core of such a protein. The evidence that this peptide forms helical tetramers is, at present, indirect. The concentration dependence of its circular dichroic spectrum is consistent with a highly cooperative monomer-tetramer equilibrium, and the extrapolated value of the mean residue ellipticity for the tetramer is consistent with the tetramer being predominantly alpha-helical. Ideally, it would be desirable to determine the molecular weight of the ALPHA-1 under conditions where it is aggregated. However, these experiments have not been successful, because of the very high concentrations of peptide (> 10 mg/ml) required for reasonably complete aggregation. This should present less of a problem in subsequent studies in which the stability of the tetramer has been improved. Indeed the 16-residue peptide described here elutes from size exclusion columns at a position consistent with it forming a tetramer (Ho and DeGrado, unpublished).

The ALPHA-1 crystals are suitable for x-ray structure analysis as judged by their high diffraction limit, low degree of mosaic spread, resistance to x-ray beam damage, well-defined morphology, and ease of data collection. In addition, the possible space groups are compatible with the designed 222 symmetry of the four-alpha-helical bundle.

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