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CIRCULAR DICHROISM ANALYSIS OF A SYNTHETIC PEPTIDE CORRESPONDING TO THE α,α-CORNER MOTIF OF HEMOGLOBIN

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The α,α-corner is a helix-turn-helix super-secondary structural protein motif where the two α-helices cross at approximately right angles. This motif has been observed in a wide variety of proteins and thus, has been proposed to be a protein folding initiator. We sought to test this hypothesis by synthesizing a peptide corresponding to the α,α-corner of the α-chain of horse methemoglobin (residues 80-108) and examining its structure by circular dichroism. We found that the α,α-corner peptide is moderately helical in water and fully helical in trifluoroethanol, a solvent that approximates the hydrophobic surroundings of the excised portion of the protein. The helicity of our synthetic peptide suggests that the α,α-corner may in fact have some stability on its own and thus, may be capable of initiating protein folding.

Protein folding is a seminal problem in biochemistry. For some proteins, it has been suggested that first there is a collapse of the random coil such that the hydrophobic residues are removed from water and buried in a hydrophobic cluster. Next, secondary structure forms, yielding a loosely packed array of secondary structural units called a molten globule. Finally, the secondary structural units align properly, forming the protein's tertiary structure (1-7). Efforts are being made to provide clues to the precipitation of any of these steps. Efimov observed that a super-secondary structural motif, which he named an α,α-corner, occurs in a wide variety of proteins whose only common function is folding (8,9). Thus, Efimov proposed that the α,α-corner is a protein folding initiator. The α,α-corner is a helix-turn-helix motif where the two α-helices cross at approximately right angles. A detailed discussion of the structure and occurrence of α,α-corners is provided by Efimov (8, 9). We sought to test Efimov's proposal that the α,α-corner is a folding initiator. We expect that if the α,α-corner motif is a protein folding initiator, a synthetic α,α-corner peptide would exhibit helicity independent of the remainder of the protein. Thus, we sought to determine the helicity of the α,α-corner sequence from the α-chain of horse methemoglobin (residues 80-108) which is structurally a tetrameric analogue of myoglobin. Since myoglobin has been proposed to fold by the mechanism described above (1, 2, 5), the discovery of a protein folding initiator in

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hemoglobin would jibe well with the folding pathway that is currently proposed for myoglobin. We report here the circular dichroism analysis of a synthetic α,α-corner peptide and discuss its ramifications to protein folding.

Materials and Methods

All reagents were used without further purification. Peptide aac-heme was synthesized by solid phase methodology using Fmoc chemistry on an ABI 431A automated peptide synthesizer. Amino acid derivatives, solvents and other reagents were purchased from Advanced ChemTech (Louisville, Kentucky). The peptide was purified by reverse phase HPLC on a Phenomenex C_{18} column (25 x 1 cm or 25 x 2.25 cm) using a water/acetonitrile gradient with 0.1% trifluoroacetic acid. The peptide was determined to be greater than 98% pure by analytical reverse phase HPLC and characterized by liquid secondary ion mass spectrometry (m/e = 3312, $(M+H)^+$).

Circular dichroism spectra were recorded on a Jasco J-710 spectropolarimeter equipped with a Neslab RTE-210 circulating water bath. All spectra were obtained using a 1 mm path length cuvette. Concentrations were determined from amino acid analysis using norleucine as an internal reference. Other conditions are specified in the individual figures. Error bars represent one standard deviations.

Results

The sequence, Ac-LSDLSNLHAHKLRVDPVNFKLLSHCLLST-NH_2, was taken from Efimov's analysis of the α,α-corner from the α-chain of horse methemoglobin (8-10). We synthesized this peptide, aac-heme, with the N-terminus acetylated and the C-terminus amidated to avoid the introduction of charges that are not present in the native protein. A circular dichroism (CD) spectrum of aac-heme in aqueous solution is shown in **Fig. 1**. We

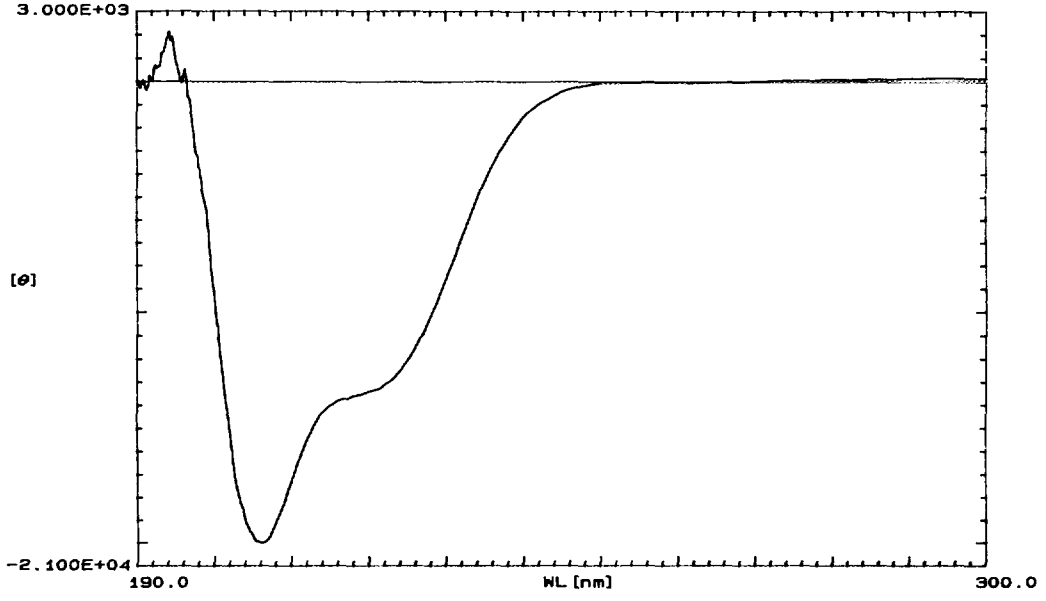

Fig. 1. Circular dichroism spectrum of aac-heme (93 μM) in 50 mM sodium borate, 0.1 M NaCl, pH 7, 4°C. θ is in deg•cm^2•dmol^{-1}.

determined the helicity of aac-heme by analysis of its CD spectra. Helicity is often measured using

$$(\theta_{obs} - \theta_0)/(\theta_{max} - \theta_0), \quad 1$$

where θ_{obs} is the observed ellipticity at 222 nm, θ_0 is the ellipticity at 222 nm of the fully denatured peptide and θ_{max} is the ellipticity at 222 nm for the peptide when it is fully helical (11). We determined θ_0 (0 ± 500 deg•cm^2•dmol^{-1}) by measuring θ_{222} in the presence of 6 M guanidinium hydrochloride. We determined θ_{max} by measuring θ_{222} in the presence of 60% trifluoroethanol (TFE). A CD spectrum of aac-heme in 60% TFE is shown in **Fig. 2**. The maximum at 195 nm and minima at 208 and 222 nm are characteristic of α-helices. The observed ratio of these ellipticities is typical of a fully helical peptide (12, 13). Furthermore, a plot of θ_{222} versus TFE concentration (**Fig. 3**) shows that ellipticity levels off at about 50% TFE. Thus, we take θ_{max} to be $-36{,}000 \pm 3000$ deg•cm^2•dmol^{-1}. This is a typical value for θ_{222} for a fully helical peptide of length 29 residues in TFE (13). **Fig. 4** shows a plot of θ_{222} versus concentration of aac-heme in aqueous solution. From the data in **Fig. 4**, equation 1 shows that the helicity of aac-heme in aqueous solution increases from 19% to 37% over the concentration range of 13-93 μM.

Discussion

Our synthetic peptide, aac-heme, represents a protein fragment that corresponds to the α,α-corner of the α-chain of horse methemoglobin. This peptide exhibits moderate helicity in aqueous solution and this helicity is concentration dependent. Concentration dependence of

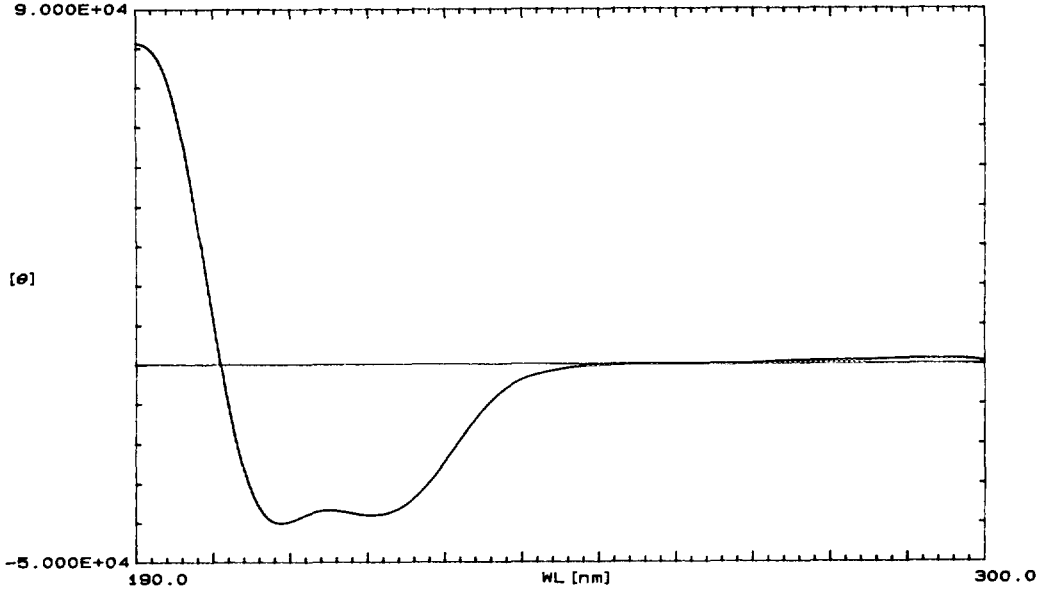

Fig. 2. Circular dichroism spectrum of aac-heme (12.7 μM) in 60% TFE, 4°C. θ is in deg•cm^2•dmol^{-1}.

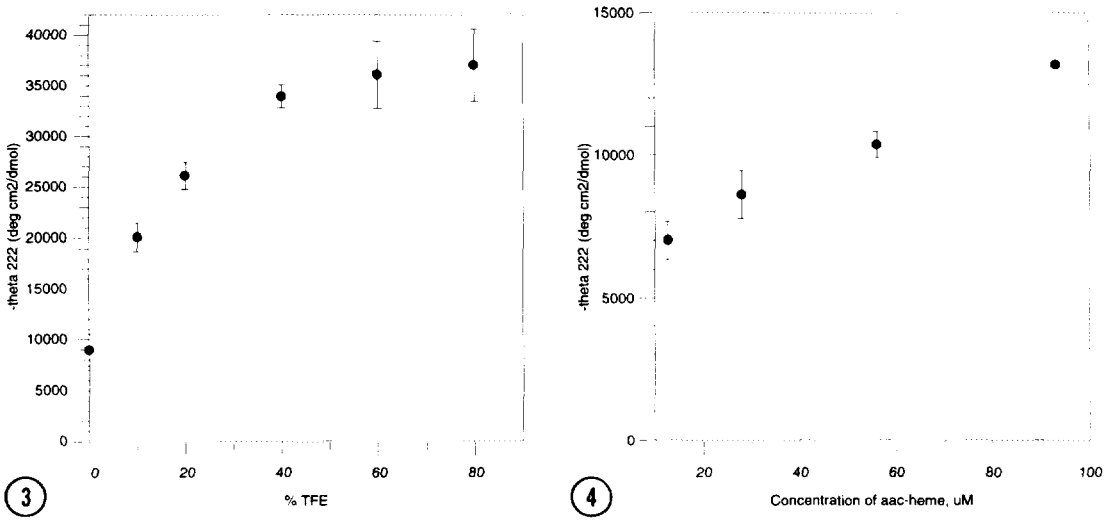

Fig. 3. Helicity of aac-heme as a function of TFE percentage. Error bars represent one standard deviation from the average of four determinations. (12.7 μM aac-heme, 4°C).

Fig. 4. Helcity of aac-heme as a function of concentration. Error bars represent one standard deviation from the average of two determinations. (50 mM sodium borate, pH 7, 0.1 M NaCl, 4°C).

helicity is often an indication of aggregation. This is particularly common in amphiphilic peptides (14) or in protein fragments (1, 15), which are devoid of the tertiary packing interactions that they encounter in the intact protein. Thus, the aggregation of aac-heme in aqueous solution is not surprising since aac-heme represents a portion of hemoglobin that normally holds many tertiary packing interactions with the remainder of the protein. The peptide aggregates to compensate for the absence of tertiary interactions. In the hydrophobic TFE solutions (16), aac-heme is fully helical suggesting that a hydrophobic environment enhances the helicity of this peptide. The moderate helicity of this α,α-corner peptide in water alone and the high helicity in the hydrophobic TFE solutions suggest that this α,α-corner sequence is capable of helical stability independent of the remainder of the protein and that the helicity is enhanced by a hydrophobic environment. These observations are consistent with a folding mechanism for the native hemoglobin whereby a hydrophobic collapse occurs first, followed by the formation of secondary structure. Our results suggest that the α,α-corner could well initiate the secondary structural formation in hemoglobin and that this initiation would be even more pronounced if it was preceded by a hydrophobic collapse of the unfolded hemoglobin. Thus, the α,α-corner may in fact be integral to the implementation of folding as Efimov proposed and may be what Kim called an "autonomous folding unit" (15). We are currently looking at mutants that eliminate aggregation so that we can examine the structure of the α,α-corner by NMR. We will also investigate the effect of mutation on the stability of an α,α-corner peptide and see how it relates to the stability and folding of the analogously mutated protein.

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