Circular Dichroism Studies of the Conformation of Synthetic Peptides in the Carboxyl-Terminal Region of Cytochrome *c*†

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ABSTRACT: Circular dichroism studies (CD) on synthetic peptides in the carboxyl-terminal sequence of cytochrome *c* were carried out. It was observed that peptides required a critical chain length before assuming a secondary structure, that is, synthetic peptides in water showed α-helical specifics first in the octapeptide and then in the undecapeptide where it was more apparent. The helix content in the undecapeptide was still less than that observed in native cytochrome *c*. Methanol enhanced α-helix formation in the undecapeptide. CD studies in sodium dodecyl sulfate with deca- to dodecapeptides revealed evidence of a β to α transition with a change of sodium dodecyl sulfate concentration. These model experiments can be extended to the hypothesis that peptides having a specific amino acid sequence will show a predisposition for forming α helix or β structure, thereby providing a more stable secondary structure by long-range interactions with appropriate hydrophobic environment in other parts of the protein.

It is well established that the primary structure of a protein determines its conformation (Anfinsen, 1964). Whether the protein chain begins to fold into its native conformation as it is synthesized on the polyribosomal system or whether a stable conformation after synthesis is completed remains to be clarified. Conformational analysis has been carried out on a series of synthetic poly(amino acids) or natural peptides isolated from native protein. Goodman *et al.* (1969, 1971) indicated in their circular dichroism (CD) studies that L-isoleucine and γ-ethyl-L-glutamate oligomers form β and α conformations beginning at the heptamer. Singhal and Atassi (1970) and Epand and Sheraga (1968) prepared various peptides by chemical cleavage of myoglobin and studied same by optical rotary dispersion and CD. These studies revealed that conformation of each of these peptides was less helical in the free form than was expected from the conformation within the intact protein. Helical content increased with the addition of methanol, but still was much lower than the values possessed by the peptides within native protein. This contradicts Phillips' (1967) suggestion that the native conformation forms during biosynthesis. Studying the conformation of synthetic peptides which are identical with known protein structures may lead to a greater comprehension of the manner in which folding takes place in proteins.

Dickerson *et al.* (1971) reported that residues 92–102 form an α-helical structure in horse heart cytochrome *c* as observed by X-ray diffraction. This observation prompted us to investigate the CD of synthetic peptides in the carboxyl-terminal region of cytochrome *c*. Demonstrated herein is the significance of the specific amino acid sequence and the solvent.

Materials and Methods

Synthesis of Peptides. Synthetic peptides were prepared by a simplified technique of the solid-phase procedure of Merrifield (1964). Boc[1]-amino acids utilized for the study were purchased from Kyoto Daiichi Kagaku Co. Ltd. Shaking the vessel containing the resin was eliminated without untoward effects.

The Boc-amino acid (or peptide) resin was washed with CH_2Cl_2, treated with trifluoroacetic acid–CH_2Cl_2 (1:1, v/v) for 30 min, then washed with CH_2Cl_2, next neutralized twice with 15% triethylamine–CH_2Cl_2, followed by washing with CH_2Cl_2, with special care being taken to remove acid or base from the resin. The desired Boc-amino acid was added in a threefold excess in CH_2Cl_2 and after 10 min an identical excess of *N,N'*-dicyclohexylcarbodiimide in CH_2Cl_2 was added. The concentrated solution was left still to promote coupling; in the case of dicyclohexylcarbodiimide 2 hr was usually required. Complete removal of the Boc group was checked by the Dorman method (1969) and coupling rates of each were determined by amino acid analysis. In the case of an incomplete step, the reaction was repeated raising the level of deprotection or coupling up to 100%. At the end of one cycle, alcohol was used to remove the dicyclohexylcarbodiimide–urea after which washing with CH_2Cl_2 was done. The HF treatment in the presence of anisole was used in the procedure for deblocking and isolation of the crude peptides and was followed by extensive purification on a column (0.9 × 150.0 cm) of Sephadex G-25 Superfine, eluted with 0.2 M acetic acid. Amino acid composition was determined using a Hitachi automatic amino acid analyzer (KLA-3B) on samples which had been hydrolyzed with 6 N HCl in the presence of 20 μl of 5% phenol at 110° for 24 hr. Purity of the peptides was checked by paper electrophoresis with pyridine–acetic acid–H_2O buffer (10:100:890, v/v) at pH 3.6, followed by descending paper chromatography utilizing butanol–pyridine–acetic acid–H_2O (75:50:15:60, v/v).

CD Measurement. The CD curves were obtained with a Jasco ORD/UV-5 (J-2) apparatus. Stock solutions were prepared by weighing the vacuum-dried peptide into a volumetric flask, adding water, and diluting to the desired concentration. Amino acid analysis determined the exact content of the peptide in the solution. Methanol or sodium dodecyl

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[1] Abbreviation used is: Boc, *tert*-butyloxycarbonyl.

TABLE I: Amino Acid Compositions of Synthetic Peptides in the Carboxyl-Terminal Region of Cytochrome *c*.

91	92	93	94	95	96	97	98	99	100	101	102
						Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
						0.82	1.07	1.38	1.38	1.11	1.0
					Ala-	Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
					1.06	0.95	1.05	1.14	1.14	1.06	1.0
				Ile-	Ala-	Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
				0.97	1.02	0.95	1.01	0.96	0.96	1.02	1.0
			Leu-	Ile-	Ala-	Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
			1.01	1.02	1.03	0.90	1.01	1.06	1.06	1.03	1.0
		Asp-	Leu-	Ile-	Ala-	Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
		1.00	0.98	0.99	1.03	0.83	0.98	0.97	0.97	1.03	1.0
	Glu-	Asp-	Leu-	Ile-	Ala-	Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
	1.05	1.03	1.01	1.01	1.05	0.82	1.01	1.05	1.05	1.05	1.0
Arg-	Glu-	Asp-	Leu-	Ile-	Ala-	Tyr-	Leu-	Lys-	Lys-	Ala-	Thr
0.99	1.01	1.0	0.97	0.98	1.01	0.84	0.97	0.95	0.95	1.01	0.96

sulfate was added at various concentrations and the solution was then maintained for 10 min to ensure equilibrium. All spectra were scanned repetitively at 2.3 nm/min at room temperature. The solvent base line was recorded before and after all scanning. $[\theta]_R$ represents the molecular ellipticity based on the mean residue weight; its units are (deg cm^2)/dmol.

Results

Amino Acid Compositions of Synthetic Peptides. Synthetic peptides were prepared including residues 97–102, 96–102, 95–102, 94–102, 93–102, 92–102, and 91–102 of cytochrome *c*. Amino acid compositions for the purified peptides (Table I) were in good agreement with the expected values based on the structure of cytochrome *c* (91–102) as seen in Figure 1. Single homogeneous spots were obtained with ninhydrin reagents after electrophoresis and descending paper chromatography.

CD Spectra of Peptides in Water. CD spectra of solutions of the synthetic peptides in water at pH 6.0–6.5 were measured from 200 to 300 nm. All peptides studied herein were water soluble. The hexapeptide Tyr-Leu-Lys-Lys-Ala-Thr (cytochrome *c*, 97–102) in water showed a broad band at approximately 227 nm, as expected for the tyrosine residue, and a comparatively strong negative band below 200 nm (Figure 2). The low-wavelength band is attributed to the π–π^* transition of the amide chromatophore. Difficulties arise in the interpretation of the CD spectrum of the hexapeptide because of overlap with the positive Cotton effect of the tyrosine residue at 227 nm. The spectrum suggests, however, that the hexapeptide lacks a secondary structure.

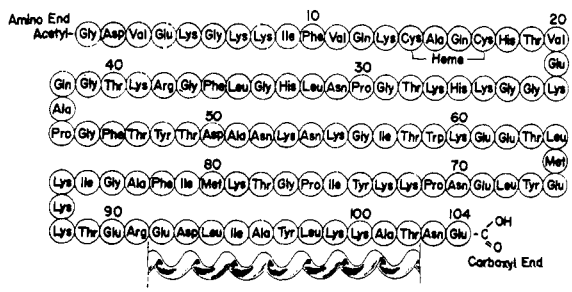

FIGURE 1: Sequence of residues 92–102 showing α helix of horse heart cytochrome *c*.

The positive band at 227 nm was replaced by a negative band at 220–222 nm when two residues (Ile-Ala) were added to the amino terminus to yield the octapeptide (Ile-Ala-Tyr-Leu-Lys-Lys-Ala-Thr), as shown in Figure 2. The negative band at 220–222 nm suggests that conformation changes appear in the octapeptide, with the formation of helical structure. As the chain length increased from 8 to 11 (Glu-Asp-Leu-Ile-Ala-Tyr-Leu-Lys-Lys-Ala-Thr cytochrome *c*, 92–102), the negative band increased in intensity, lending further support to the suggestion of helicity.

CD Spectra of Peptides in Methanol–Water. The effect of methanol on the CD behavior of the synthetic peptides was investigated. Figure 3 shows plots of the residue ellipticities as functions of the methanol concentration. For the hexa- and heptapeptide, $[\theta]_R$ = 222 nm was not changed by the addition of methanol, indicating the absence of conformational change, but a 3- to 4-nm red shift in the positive band was observed. This slight shift may be due to a hydrogen bond between hydroxyl groups of tyrosine and methanol. The negative ellipticity in the 200- to 240-nm region for octa-, nona-, deca-, and undecapeptide solutions showed an increased intensity when methanol was added, as expected for CD contributions from helical structure. Typical CD spectra of the undecapeptide in water and in 60% methanol are illustrated in Figure 4. The CD minima at 222 and 207–208 nm in the latter solvent are evidence of an α-helical structure. A plot of the residue ellipticity at 222 nm in 60% meth-

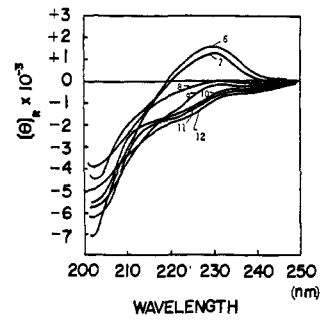

FIGURE 2: CD spectra of various synthetic peptides of cytochrome *c* in water; numerals denote: 6, hexapeptide (Tyr^{97} → Thr^{102}); 7, heptapeptide (Ala^{96} → Thr^{102}); 8, octapeptide (Ile^{95} → Thr^{102}); 9, nonapeptide (Leu^{94} → Thr^{102}); 10, decapeptide (Asp^{93} → Thr^{102}); 11, undecapeptide (Glu^{92} → Thr^{102}); 12, dodecapeptide (Arg^{91} → Thr^{102}).

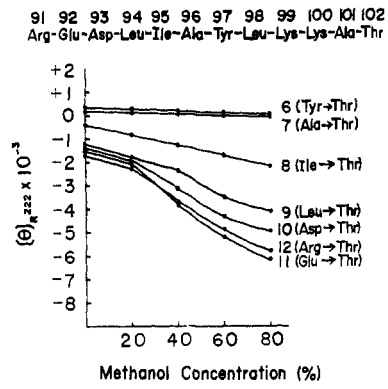

FIGURE 3: Mean residue ellipticity of various peptides at 222 nm as a function of methanol concentration.

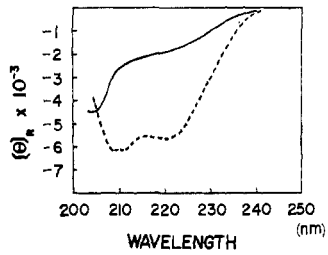

FIGURE 4: CD spectra of the undecapeptide, (Glu^{92} → Thr^{102}), in water (——) and in 60% methanol (- - -).

anol *vs.* the number of residues in the peptide chain is shown in Figure 5. The value of $[\theta]_R$ = 222 (negative) increases up to the undecapeptide level, but decreases again in the dodecapeptide. These data suggest that the total helix is less in the dodecapeptide than in the undecapeptide.

CD Spectra of Peptides in Sodium Dodecyl Sulfate Solution. All peptide solutions were completely soluble at high concentrations of sodium dodecyl sulfate. With the dodecapeptide, precipitation was observed only when sodium dodecyl sulfate was added to the peptide solution in a 5–10 molar ratio of sodium dodecyl sulfate:peptide. With the addition of sodium dodecyl sulfate to solutions of hexa- and heptapeptides, no change was observed in the CD behavior (Figure 6), suggesting that these peptides do not undergo significant conformational changes. This agrees with the results for both water and methanol–water systems. Typical β conformations, characterized by a single minimum at 218 nm, were observed in octa-, nona-, and decapeptides at sodium dodecyl sulfate concentrations of 1.25 mM (Figure 6). The molecular ellipticity at 218 nm in the nonapeptide was independent of the peptide concentration in either the presence or the absence of sodium dodecyl sulfate. It is highly unlikely, therefore, that intermolecular aggregation occurs in these

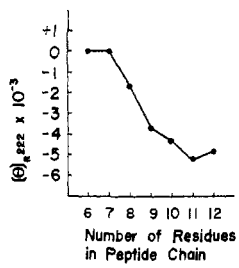

FIGURE 5: Mean residue ellipticity at 222 nm of the various peptides in 60% methanol.

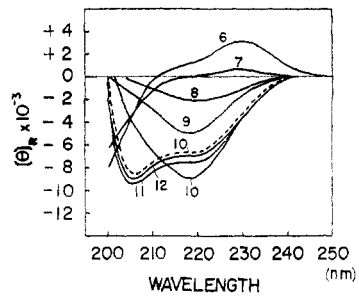

FIGURE 6: CD spectra of various peptides in sodium dodecyl sulfate solution acidified with H_2SO_4 to pH 2.0. Peptide concentration, 25 μM; sodium dodecyl sulfate concentration, 1.25 mM (——) and 3.75 mM (- - -). Numerals represent the number of peptide residues.

systems. At concentrations of sodium dodecyl sulfate over 3.75 mM, a β- to α-transition stage was quite evident in the case of decapeptide (Figure 6). In the undecapeptide, α-helix appeared to form even at the low concentration of 0.625 mM. β conformation was maximal when the molar ratio of sodium dodecyl sulfate to the undecapeptide was 8 (Figure 7). A greater hydrophobic environment can be expected with higher concentrations of sodium dodecyl sulfate. This environment stabilizes hydrogen bonds, resulting in the transition of β structure to α helix. Data related to the secondary structure of different peptides at various sodium dodecyl sulfate concentrations can be seen in Table II.

Discussion

The experimental results herein demonstrate the existence of a critical size for helix formation in the carboxyl region of cytochrome *c* which began at the undecapeptide level. Secondary conformation was, however, observed even in smaller peptides such as octa- and nonapeptides, which showed a negative ellipticity band at 220–230 nm. This band is influenced by the positive Cotton effect of the tyrosine residue in the peptides. The negative mean residue ellipticity would be greater than the actual recorded values if the positive band contributed by tyrosine were subtracted. These findings are in good agreement with the observations of Goodman *et al.* (1969, 1971) that L-isoleucine and γ-ethyl-L-glutamate homopolymers form β and α conformation, respectively, beginning at the heptamer stage. The present undecapeptide formed α-helical structure in water, although the helical content was still less than that observed in the native protein. Methanol enhanced the formation of α-helical structure and the largest negative $[\theta]_R$ value was obtained with the undecapeptide, the helical content being approximately 30% that of the native protein level. The use of methanol to increase and stabilize α helix has been demonstrated by many workers

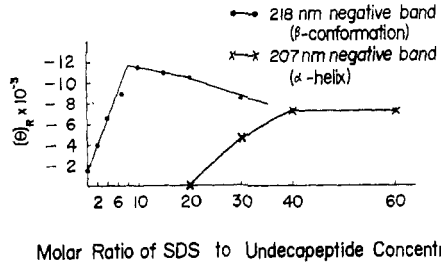

FIGURE 7: Mean residue ellipticity at 218 and 207 nm of the undecapeptide (Glu^{92} → Thr^{102}) as a function of sodium dodecyl sulfate (SDS) concentration at pH 3.

TABLE II: Effect of Sodium Dodecyl Sulfate on the Conformation of the Various Peptides.

Peptides 25 μM	Added Sodium Dodecyl Sulfate 3.75 mM (150)[b]	1.25 mM (50)	1.0 mM (40)	0.625 mM (25)	0.5 mM (20)	0.25 mM (10)	0.125 mM (5)
Decapeptide (Asp → Thr)	α[a]	β[a]	β	β	β	β	β
Undecapeptide (Glu → Thr)	α	α	α	α	β	β	β
Dodecapeptide (Arg → Thr)	α	α	α + β	β	β	ND[c]	ND

[a] α and β represent α helix and β conformation, respectively. [b] Parentheses indicate the molar ratio of sodium dodecyl sulfate: peptide. [c] ND = not determined by precipitation.

(Epand and Sheraga, 1968; also see references listed by Iio, 1971).

CD measurements in the presence of sodium dodecyl sulfate appear to provide a more progressive transition of conformation in the synthetic peptides. The transition was found to be largely dependent on the concentration of sodium dodecyl sulfate. The undecapeptide revealed α-helical conformation at 1 mM of sodium dodecyl sulfate and 25 mM of undecapeptide, whereas smaller peptides all showed β conformation at the same concentrations. This evidence strongly supports the hypothesis that the undecapeptide has a greater helix potential as compared to the smaller peptides. The effect of sodium dodecyl sulfate may be explained as follows. Sodium dodecyl sulfate anions combine with cationic sites of the two residues of lysine in the undecapeptide. It can be calculated that 6 mol of sodium dodecyl sulfate interact nonionically with the rest of the chain (nine amino acid residues), that is, 1.0 mol of sodium dodecyl sulfate appears to combine nonionically with a 1.5 equiv of amino acid residue in the peptide. The resulting hydrophobic environment appears to play an important role in stabilization of the α-helical conformation. Reynold and Tanford (1970) reported that sodium dodecyl sulfate combines with protein chains in a ratio of 1.4:1.0 by weight. Our observations are in good agreement with their findings.

The distribution of amino acid residues located at positions from 92 to 102 is of particular significance. Glutamic acid, assigned as a so-called helix former, is located in the amino terminus and other helix formers such as Leu-94 and -98, Ile-95, and Ala-96 and -101 are localized in the middle of this sequence. Other residues, Tyr-97, Thr-102, Lys-99 and -100, and Arg-91, are helix indifferent (Lewis *et al.*, 1970; Lewis and Scheraga, 1971). One residue, Asp-93, a helix breaker, is located at the amino terminus. It can be postulated that should a helix former be located at the amino terminus of the undecapeptide, then the remaining sequence will tend toward a helix formation.

It is not expected that the conformation of a synthetic peptide will correspond exactly to that of native protein. Nevertheless, it appears worthwhile to envisage such a small peptide as serving as an appropriate initiating site for a secondary structure. It was definitely found that synthetic octapeptide, based on the sequence of portions of the carboxyl terminus of cytochrome *c*, gave CD spectra compatible with organized structure. Additional specific amino acid residues forming the undecapeptide may provide a more stable conformation in such a small fragment, under favorable conditions. This strongly supports Dickerson's observation (Dickerson *et al.*, 1971) from X-ray studies.

Studies of other helical structures at residues 1–11, 14–18, 49–54, 62–70, and 71–75 in cytochrome *c* are in progress.

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