Residual Helical Structure in the C-Terminal Fragment of Cytochrome *c*

Yutaka Kuroda

Protein Engineering Research Institute, 6-2-3, Furuedai, Suita, Osaka 565, Japan

Received July 14, 1992; Revised Manuscript Received November 10, 1992

ABSTRACT: Several reports have pointed out the existence of both kinetic and equilibrium intermediate states in protein folding. In cytochrome *c*, it has been shown that the N- and C-terminal helices form in the early stages of folding and remain stable in the molten globule state (a compact equilibrium intermediate). These two facts prompted me to synthesize and examine the helical content, in aqueous solution, of the peptides corresponding to the three major helices of cytochrome *c*. These peptides are 15 residues long. This paper reports that little if any helix is present in the N-terminal and 61–75 peptides, regardless of the pH and salt concentration. However, the C-terminal peptide showed a far-UV CD spectrum characteristic of an α-helix (27% helicity). The helical content of the C-terminal peptide increased to 43% as salt (2 M Na_2SO_4) was added. The dimerization of the C-terminal peptide with the N-terminal peptide by an SS bridge stabilized the helical structures (14% to 63% helicity). These results strongly suggest that the C-terminal helix is essential for both the folding and the stability of cytochrome *c*. Furthermore, although the N-terminal segment does not form helices by itself, its interaction with the C-terminal helix would enhance the stability of the subdomain containing the two helices.

Recent refolding experiments on small globular proteins monitored by H–D exchange[1] (Roder et al., 1988; Udgaonkar & Baldwin, 1988, 1990; Miranker et al., 1991) have shown kinetic intermediates, which contain some of the native elements of regular secondary structures. These experiments support the framework model (Kim & Baldwin, 1982) and suggest that the interactions between secondary structures are of importance in the folding mechanism (Wetlaufer, 1973; Karplus & Weaver, 1976). On the other hand, equilibrium intermediates such as the molten globule state (Ohgushi & Wada, 1983; Kuroda et al., 1992) have been detected, and their stable substructures analyzed, again using H–D exchange (Baum et al., 1989; Jeng et al., 1990; Hughson et al., 1990). For cytochrome *c*, kinetic experiments have shown that the N- and C-terminal helices fold first (Roder et al., 1988) and also that they remain relatively stable in the equilibrium molten globule state (Jeng et al., 1990). Therefore, one may expect these secondary structures to be stable as isolated peptides. In addition, since the N- and C-terminal helices are close to each other in the native structure, the dimerization of these peptides may produce a structured fragment. Such structured, or at least partly structured, fragments have already been reported [see Wright et al. (1988) for a review] for myoglobin (Waltho et al., 1990), bovine pancreatic trypsin inhibitor (Oas & Kim, 1988), RNase A (Bierzynski et al., 1982; Brown & Klee, 1971), barnase (Sancho et al., 1992), and *trp* repressor (Tasayco & Carey, 1992). For cytochrome *c*, similar experiments have been reported, but only the addition of lipids in conjunction with changes in the peptide sequence enabled the observation of a helical structure in the C-terminal peptide (Collawn & Paterson, 1990).

This paper reports the measurement of the helical content of synthetic peptides corresponding to the N- and C-terminal helices and to the segment containing both the residues of the 60's and 70's helices of cytochrome *c*. The far-UV CD spectra of the peptides in aqueous solution showed that the C-terminal peptide was in a helical conformation at low temperature. No addition of lipid or organic solvent was required. Under the same conditions, the N-terminal peptide and 61–75 peptide exhibited little if any helical structure. The SS-bridged dimeric NC-terminal peptides also showed a large helical content when salt was added. The dimeric N-terminal peptides did not form helices, but surprisingly, the dimeric C-terminal peptides had almost 100% helicity. I discuss the possible implications of the present results on the folding and stability of cytochrome *c*.

MATERIALS AND METHODS

The peptides were synthesized by standard protocols (solid-phase method) with an Applied Biosystems peptide synthesizer (Model 430A) using the 9-fluorenylmethoxycarbonyl (Fmoc) chemistry. The N-terminus was acetylated before cleavage from the resin [4-[(2′,4′-dimethoxyphenyl)-Fmoc-aminomethyl]phenoxy resin] with trifluoroacetic acid. After cleavage, the crude peptides were purified by reversed-phase HPLC (Senshu Pak, C_{18}, 5 μm, 300 Å) with a water/acetonitrile gradient containing 0.1% trifluoroacetic acid and lyophilized. Amino acid analysis of the peptides was performed using a Beckman analyser (Model 6300E). Peptide hydrolysis was carried out in 6 N HCl for 1 h at 150 °C with a Pico-TAG station (Waters).

The dimers were obtained by overnight air oxidation of the cysteines at room temperature (pH 7). The dimeric peptides were then further purified with the reversed-phase HPLC column and lyophilized. The purity of the peptides and of the dimeric fragments was checked with reversed-phase HPLC and was greater than 95%. The amino acid composition of the dimeric peptides was also analyzed. We chose the positions of the cysteines such that the spatial relationship of the helices in the native structure would not be disturbed. This was carried

[1] Abbreviations: CD, circular dichroism; far-UV CD, far-ultraviolet circular dichroism, indicating the wavelength region between approximately 200 and 240 nm; H–D exchange, hydrogen–deuterium exchange; HPLC, high-performance liquid chromatography; C-terminal peptide, peptide containing the residues 89–103; N-terminal peptide, peptide containing residues 1–15; 61–75 peptide, peptide containing residues 61–75; NC1 and NC2, dimeric fragments containing the N- and C-terminal peptides bridged at positions 1 and 93 and positions 10 and 97, respectively; CC1 and CC2, dimeric fragments containing two C-terminal peptides bridged at positions 93 and 97, respectively; NN1 and NN2, dimeric fragments containing two N-terminal peptides bridged at positions 1 and 10, respectively.

0006-2960/93/0432-1219$04.00/0

Table I: Peptide Sequences[a]

N-terminal peptide

Gly[1]-Asp-Val-Glu-Lys[5]-Gly-Lys-Lys-Ile-Phe[10]-Val-Gln-Lys-Ala(Cys)-Ala[15]

C-terminal peptide

Thr[89]-Glu-Arg-Glu-Asp-Leu-Ile[95]-Ala-Tyr-Leu-Lys-Lys[100]-Ala-Thr-Asn

61-75 peptide

Glu[61]-Glu-Thr-Leu-Met[65]-Glu-Tyr-Leu-Glu-Asn[70]-Pro-Lys-Lys-Tyr-Ile[75]

[a] The sequences of the three peptides are shown with residue numbers corresponding to those of the native horse cytochrome *c*. The 61–75 peptide includes the two α-helices extending from residues 61 to 70 and from residues 71 to 75. In the native structure, the two helices are separated by a bend at Pro[71]. In the N-terminal peptides, Cys[14] was replaced by an alanine to avoid undesired SS bridging. The designed SS bridges were formed by replacing either Gly[1] and Asp[93] in the first design or Phe[10] and Tyr[97] in the second design by cysteines. These residues were chosen because their C_β are close to each other (Gly[1] C_α–Asp[93] C_β = 4.3 Å and Phe[10] C_β–Tyr[97] C_β = 5.6 Å). The two SS bridges are indicated by lines, and the residues replaced by cysteines in the dimeric fragments are marked by asterisks. The two fragments are called the NC1 (bridged by cystines at positions 1 and 93) and the NC2 (positions 10 and 97) fragments. In constructing these two fragments, byproducts in which the respective N- and C-terminal peptides are bridged to themselves were obtained. These fragments are called the CC1, NN1, CC2, and NN2 fragments. The sequence of the unbridged C-terminal peptide is identical to that of the native cytochrome *c*. In the sequence of the unbridged N-terminal peptide, Cys[14] is replaced by an alanine and Gly[1] by a cysteine. This peptide is the same as that used for constructing the NC1 fragment. The NC fragment means both the NC1 and the NC2 fragment. The same nomenclature is used for the NN and CC fragment.

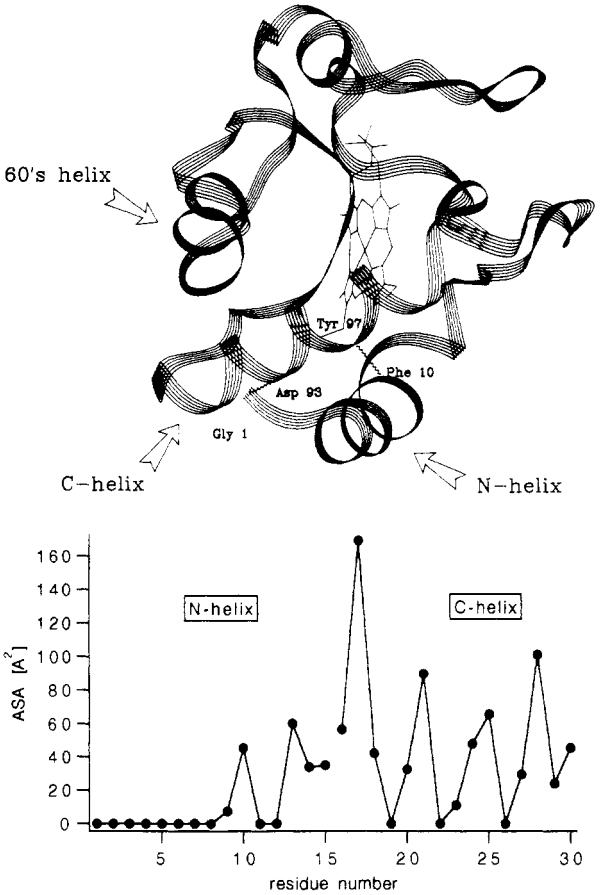

FIGURE 1: (a) Ribbon model of native horse cytochrome *c* (Bushnell et al., 1990). The three synthetic peptides are indicated by arrows, and the locations of the two SS bridges (one for each design) are shown by lines linking the C_α atoms. (b) The difference between the accessible surface area of the NC fragment (namely, the structure constructed by keeping only the N- and C-terminal helices of the native structure) and that of the corresponding residues in the complete native structure plotted versus the residue number. On the horizontal scale, numbers 1–15 correspond to residues Gly[1] to Ala[15] (the N-terminal peptide) and numbers 16–30 correspond to residues Thr[89] to Asn[104] (the C-terminal peptide).

out with the program Insight (Biosym, San Diego, CA) running on a Silicon Graphics Iris work station.

Stock solutions of the peptides (1 mg/mL) in unbuffered water were prepared and diluted (50–100 times) in the indicated buffers. The pH of the buffers was measured, as well as that of the samples after the far-UV CD measurements. The value of $[\theta]$ was computed using the peptide concentration determined by amino acid analysis.

CD spectra were recorded on a J-600 spectropolarimeter (Japan Spectroscopic). The temperature was controlled by circulating water around an optical cuvette and was measured using a thermistor inserted directly into the sample.

RESULTS

The primary structures of the synthesized peptides are shown in Table I and their relative spatial positions in the native structure are displayed in Figure 1a. The lack of contact between the 60's helix and the N-terminal helix is noteworthy. The contact area between the N- and C-terminal helices is large and residues 1–12 are in contact only with the C-terminal helix (Figure 1b). The only exception is Phe[10], which interacts with other parts of the protein and is therefore more exposed to the solvent in the (ideal) fragment's structure than in the native structure. On the other hand, the C-terminal helix interacts with both the N-terminal helix and the rest of the protein. Although the contact area for the heme moiety with the N- and C-terminal helices is limited (Figure 1a), some of the methyls interact importantly with the C-terminal helix.

The helicity of the C-terminal helix is ~27% in water and, it increases to ~43% when salt (2 M Na_2SO_4) is added (Figure 2a and Table II). The spectrum of the C-terminal peptide measured in 2 M Na_2SO_4 is characteristic of an α-helix, with negative $[\theta]$ values of about −17 000 and −20 000 deg cm^2 dmol^{-1} at 222 and 208 nm, respectively, and a positive value at 200 nm (Figure 2a). In comparison, the data suggest little if any helix in either the N-terminal peptide or the 61–75 peptide, under the same conditions (Figure 2b,c). The helix content of the 61–75 peptide increases to 15% when salt is added, but this value is still low. The second factor that plays an important role in the formation of helical structures in the C-terminal peptide is the interaction between helices (Figure 3). The helical content of the fragments containing the C- and N-terminal peptides is larger than that expected from the simple arithmetic sum of the monomeric peptides spectra (Figure 3a, Table II). The way of linking the two peptides is not essential although the helical content of the NC2 fragment, which is bridged by cystines at positions 10 and 97, is larger than that of NC1. At peptide concentrations lower than 50 μM, mixing the monomeric N- and C-terminal peptides did not enhance the helical content, and the spectrum was identical with the arithmetic sum of the N- and C-terminal peptide spectra (data not shown). The helical contents of both fragments increase when salt is added. Figure 3b shows that the CC1 and CC2 fragments have a very high helical content (~80%). It is worth noting that, the CC2 fragment has a large helical content even in the absence of salt. On the other hand, the N-terminal peptide is not stabilized by its

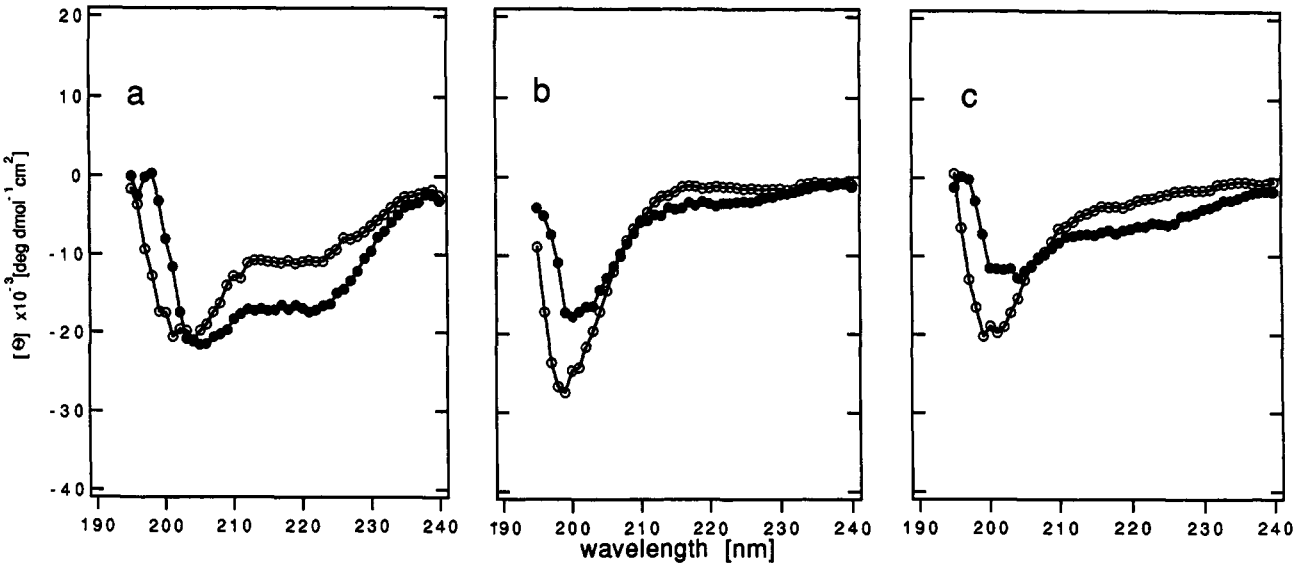

FIGURE 2: Far-UV CD spectra of the monomeric peptides in 25 mM phosphate buffer (pH 6.4) at 5 °C in the presence (●) or absence (○) of 2 M Na_2SO_4. The peptide concentration was ~5 μM, and the optical path length was 0.5 cm. (a) Spectra of the monomeric C-terminal peptide. The helical content computed from $[\theta]_{222}$ is 27% in water and 43% in the presence of salt. (b) Spectra of the N-terminal peptide. The helical content is 3% in the absence and 8% in the presence of salt. (c) Spectra of the 61–75 peptide. The helical content is 7% in the absence and 15% in the presence of salt.

Table II. Summary of the Mean Residue Ellipticity at 222 nm and of the Corresponding Helicity[a]

	no salt		2 M Na_2SO_4		4 M $NaClO_4$	
peptide	$[\Theta]_{222}$	% Hel	$[\Theta]_{222}$	% Hel	$[\Theta]_{222}$	% Hel
monomeric						
N-ter	−1.2	3	−3.3	8		
61–75	−2.7	7	−6.2	15		
C-ter	−10.9	27	−17.1	43	−12.1	31
dimeric						
NC1	−5.1	14	−20.7	53	−14.4	37
NC2	−9.8	26	−24.6	63	−17.9	46
CC1	−12.1	31	−26.6	68	−21.2	54
CC2	−23.2	59	−32.1	80	−27.9	71
NN1	−0.4	2	−2.6	7.5		

[a] The mean residue ellipticities at 222 nm (in 10^3 deg cm^2 dmol^{-1}) of the peptides at 5 °C and under different conditions are listed. This table summarizes the CD spectra shown in Figures 2, 3, and 5. The helicity is expressed in percent and was calculated using $[\theta]_{222} = -39\,500$ deg cm^2 dmol^{-1} for the complete helix and $[\theta]_{222} = +415$ deg cm^2 dmol^{-1} for the complete coil [Scholtz et al. (1991), with T = 5 °C]. Essentially the same results can be computed with the limits reported by Merutka et al. (1991).

dimerization (Figure 3c). As expected, the helical content decreases as the temperature increases (Figure 4). Both the monomeric C-terminal peptide (Figure 4a) and the dimeric NC1 fragment (Figure 4b) conserve some helical structures at 20 °C. However, the far-UV CD spectra measured at 20 °C are not as typical of α-helices as those measured at 5 °C. The fully denatured state is present at a rather high temperature, higher than 40 °C. Figure 5 and Table II show that the stabilization of the helices can also be obtained with $NaClO_4$, whose chaotropic effect differs from that of Na_2SO_4. Finally, neither the monomeric C-terminal peptide nor the dimeric CC1 peptides showed any dependence on the peptide concentration over the range 1–50 μM (Figure 6). Therefore, the helix formation is not due to the formation of large aggregates.

DISCUSSION

The main result concerning the stability of the protein is the difference in helical content between the three peptides as well as the fact that bridging the N- and C-terminal peptides enhances the stability of the fragment. However, the large helical content of the C-terminal peptide observed in aqueous solution is also noteworthy. This is because only a few short peptides are able to form stable secondary structures in aqueous solution. These are often redesigned from natural sequences to enhance their stability [for example, Lyu et al. (1989)]. For comparison, the fractional helicity of a 14-residue-long peptide, containing mainly alanine, is 40% at 0 °C (Scholtz et al., 1991). The helical content observed in the monomeric C-terminal peptide at 5 °C is ~27% in water and is ~43% in 2 M Na_2SO_4. The helicity of the C-terminal is observable by far-UV CD spectra. This differs from the nascent helix where NMR data indicate a set of turnlike structures but the far-UV CD spectra is not characteristic of α-helices (Dyson et al., 1988).

The stabilization of protein structures by salts can have different origins. The first cause is the chaotropic effect of the salt, which is important at high concentration of salt (Von Hippel & Schleich, 1969). However, in this study, the change in the hydrophobic properties of the solvent is likely to be minor. This is suggested because the same qualitative results were obtained for both $NaClO_4$ and Na_2SO_4 (Figure 5 and Table II), even though ClO_4^- ion is destabilizing and SO_4^{2-} ion is stabilizing. The second cause is the decrease of the repulsive electrostatic forces (Goto et al., 1990a,b; Goto & Nishikiori, 1991). The increase of stability of the residual structures when charges are neutralized may be explained as follows. In isolating the fragments, one can preserve, to a certain extent, the contact interactions between secondary structures (nonspecific between hydrophobic residues, specific interaction between side chains, etc.). However, the electrostatic contribution from the rest of the protein is likely to be largely disturbed. For most fragments, it will be necessary to neutralize the electrostatic interaction, but exceptions may occur, where the electrostatic interaction within the fragment is less destabilizing or may even be stabilizing. One noteworth· example is the C-peptide of RNase A, in which a salt brid stabilizes the α-helical conformation (Bierzynski et al., 19[illegible] This is not the way the C-terminal peptide is stabilized i

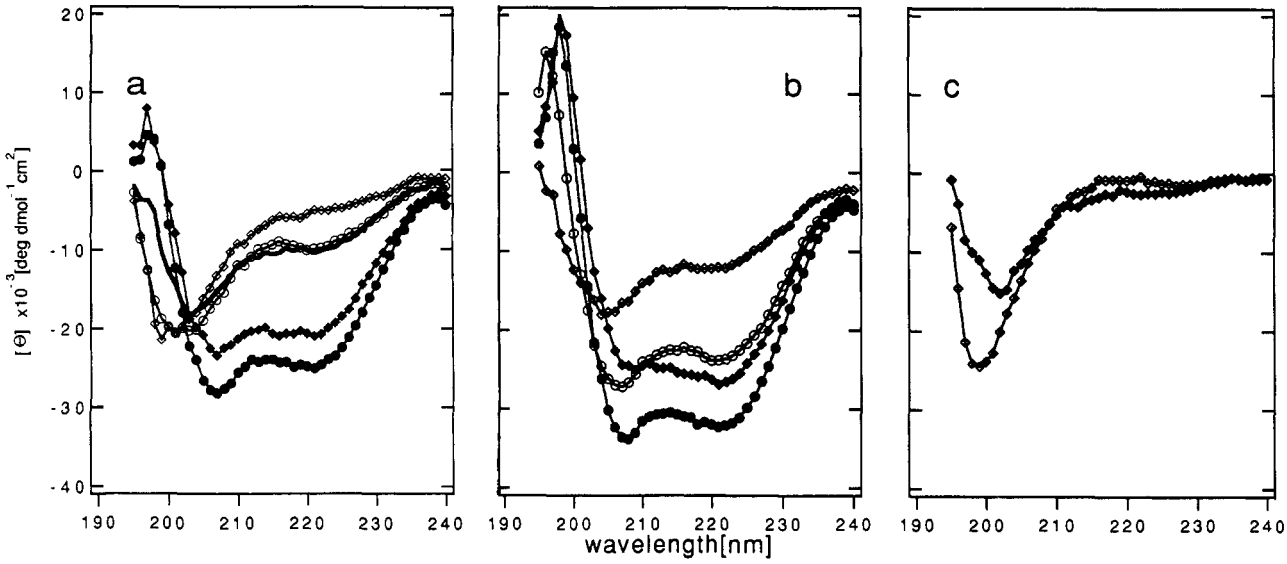

FIGURE 3: Far-UV CD spectra of the dimeric fragments in 25 mM phosphate buffer (pH 6.4) at 5 °C in the presence (filled symbol) or absence (open symbol) of 2 M Na_2SO_4. (a) Spectra of the dimeric CN1 (◇) and CN2 fragments (○). The bold continuous line represents the theoretical line obtained by the addition of the spectra of the monomeric N- and C-terminal peptides in 2 M Na_2SO_4 at 5 °C (Figure 2a,b, filled symbols). (b) Spectra of the dimeric C-terminal peptides CC1 (◇) and CC2 (○). (c) Spectra of the dimeric N-terminal peptide NN1.

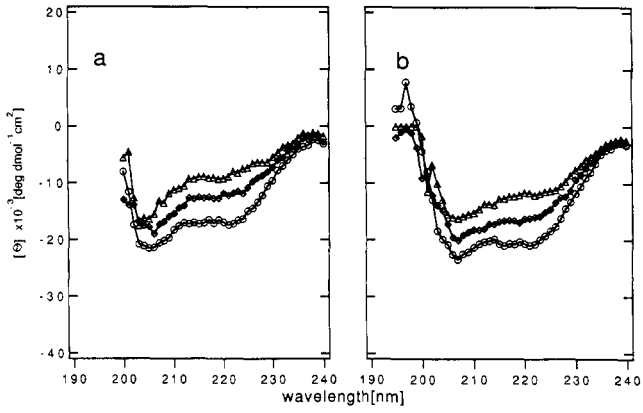

FIGURE 4: Temperature dependence of the far-UV CD spectra of (a) the monomeric C-peptide and (b) the dimeric CN1 fragment in 25 mM phosphate buffer (pH 6.4) containing 2 M Na_2SO_4. The temperatures are 5 (○), 20 (◇), and 40 °C (△).

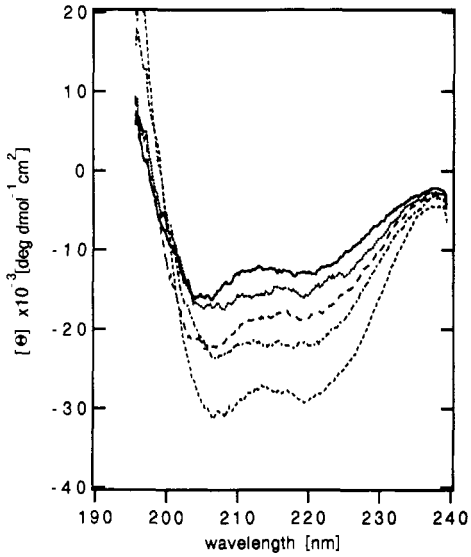

FIGURE 5: Far-UV CD spectra of the C-terminal peptide (—) and NC1 (···) NC2 (– –), CC1 (– · –), and CC2 (- - -) fragments in 25 mM phosphate buffer (pH 6.4) at 5 °C in the presence of 4 M $NaClO_4$. The concentration of $NaClO_4$ was set to 4 M in order to keep the charge concentrations (both positive and negative) constant.

helical conformation, because the addition of salt enhances the helical content.

The substantial difference between the helical content of the N-terminal and C-terminal peptides was not at all expected from previous reports. For example, H–D exchange studies shows that both the N- and the C-terminal helices are stable in a partly denatured globular state (Jeng et al., 1990). The accessibility of the two helices does not also explain the helicity difference between the two peptides. In the native structure (Figure 2a), the N-terminal helix is relatively exposed to the solvent molecules whereas the C-terminal helix is buried. Therefore, on the basis of accessibility, one may expect to observe some helical structure in the isolated N-terminal peptide but not necessarily in the C-terminal peptide. Although the N-terminal peptide ends at Ala^{14} (Table I), which is not a preferred residue at the C-terminal end of α-helices (Richardson & Richardson, 1988), the absence of helical structure in the N-terminal helix is probably not due to a poor design. Indeed, the helical content of the 61–75 peptide is also low (8%), even though the unfavorable Lys^{60} residue was omitted. In addition, an 18-residue peptide analogue where His^{14}-Ala^{15}-Gln^{16}-Ala^{17}-His^{18} was added to the N-terminal peptide was tested. This peptide contains two histidines near the C-terminus, and histidine is often found at the C-terminal end of α-helices (Richardson & Richardson, 1988). Furthermore, the N-terminal helix is possibly stabilized by the proximity of Cys^{14} and Cys^{17}, which are linked by thioether bonds to the heme moiety in the native cytochrome *c*. The heme moiety would constrain the conformation of the peptide and act as a helix initiator (in the native cytochrome *c*, the helix ends at residue 15). Cys^{17} and the Cys^{14} were changed respectively to Ala and His to avoid the formation of intermolecular SS bridges. These modifications and the addition of zinc or other metals (Ghadiri & Choi, 1990) should mimic the role of the heme and stabilize a hypothetical nascent helical structure, by bringing the two histidines close to each other. However, until now, I did not observe any formation of stable helices in the 18-residue N-terminal peptide with or without addition of metals. This would suggest that the helical structure of the C-terminal peptide is stabilized by local

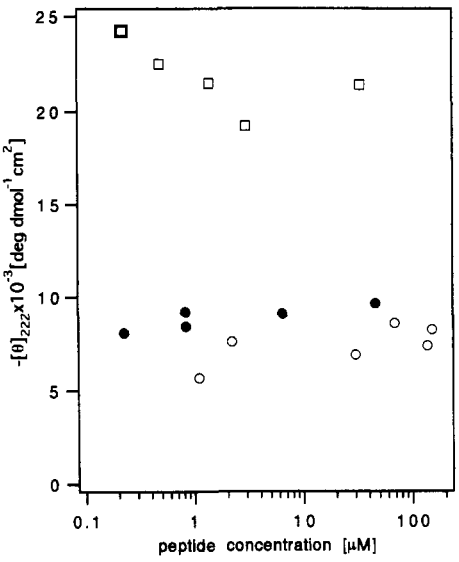

FIGURE 6: Peptide concentration dependence of the mean residue ellipticity at 222 nm for the monomeric C-terminal peptide in 25 mM phosphate buffer (pH 6.4) at 10 °C without salt (○) or containing 2 M Na_2SO_4 (●) and for the CC2 fragment (□) in the same condition but without salt. The optical path of the cell ranged from 0.1 to 2 cm.

interactions within the helix, whereas this is not sufficient to stabilize the helical structure in the N-terminal peptide.

The dimerization of the peptides by SS bridges was used to analyze the nonspecific interactions between the two helices. The cysteines are placed in order not to disturb the spatial arrangement of the N- and C-terminal helices in the native structure. The present interaction differs from that occurring in the native cytochrome *c* in two essential points. First, the rigid tertiary structure and highly specific interactions characteristic of the native structure are probably absent, and only fluctuating interactions between helices are likely to be present. Second, the interaction of the helices, in particular of the C-terminal helix, with the rest of the protein is absent. Nonetheless, the helical content increases when the peptides are dimerized, regardless of how the C-terminal peptides are bridged. This result was anticipated from both the relative position of the helices and the calculation of the accessible surface area, which shows that, in the native protein structure, the N-terminal helix interacts only with the C-terminal helix. Further reasons to expect this result were the H–D exchange studies (Jeng et al., 1990; Roder et al., 1988), which reported that the helical structures are stabilized by the docking of the N- and C-terminal helices to each other. The unexpected high helical contents of the CC fragments, where the C-terminal is dimerized with itself, suggest that nonspecific interactions stabilize the helices in the C-terminal peptide. On the other hand, bridging the N-terminal peptide with itself does not increase the helicity (Figure 3c), suggesting that some specific interactions which are not reproduced in the NN fragments stabilize the helix in the native cytochrome *c*.

The results of equilibrium studies of fragments cannot be directly related to the folding and stability properties of cytochrome *c*. However, the present results agree with the proposition of Roder et al. (1988) that the docking of the N- and C-terminal helices stabilizes the subdomain and also with the observation that the subdomain containing the N- and C-terminal helices remains stable in the molten globule state (Jeng et al., 1990). Yet, as mentioned above, a new and important observation is the large difference in stability between the two helices. Therefore, the folding model of cytochrome *c* should emphasize the intrinsic property of the C-terminal segment to form helices. The ability of the C-terminal peptide to fold independently suggests that the C-terminal segment folds first and that it is an essential structural piece. In contrast, the N-terminal and residues 60's segment, could not fold in helices without further interaction. The structural role of the N-terminal helix would be to stabilize a potential helix in the C-terminal region. The specific interactions between side chains in the native cytochrome *c* would further enhance the stability of the protein and especially of the subdomain and give the final tight packing.

I have mainly discussed the relationship between the fragment's structures and the structure of the native protein. However, it seems unrealistic that the N- and C-terminal helices have a single, well-defined, spatial relationship as in the native state. The fluctuating interactions between helices resemble that occurring in the molten globule state where most of the specific interactions between side chains are absent. On the other hand, the difference is that no globule, which probably provides a hydrophobic environment to the helices, is present in the dimeric fragment. At present, it seems that a necessary condition to form helices in isolated peptides is that the corresponding segments have a large stability not only in the native but also in partly denatured globular states (Waltho et al., 1990; Hughson et al., 1990). This may be a general rule since in the isolated peptides or fragments no specific interactions are present.

In summary, the isolated C-terminal peptide of cytochrome *c* remains largely helical in aqueous solution. The differences in helical content observed among the three peptides are probably observable in peptides corresponding to α-helices of other proteins. This difference shows clearly that the formation of some helices is determined by local sequence whereas others are stabilized by the overall folding and therefore by long-range interactions.

ADDED IN PROOF

While this paper was in press, a theoretical paper predicting a possible difference between the stability of the N- and C-terminal helices appeared (Rooman & Wodak, 1992).

ACKNOWLEDGMENT

I thank Dr. H. Nakamura (PERI) for providing me with the opportunity to complete this study, and for discussion. I thank Prof. Y. Goto (Osaka University) for discussion, proofreading, and advice. I thank Dr. T. Tanaka and Mr. T. Nakai for advice on peptide synthesis, and Dr. M. Oobatake for calculation of the accessible surface area. I thank Prof. G. D. Brayer (University of British Columbia, Canada) for the coordinates of horse cytochrome *c*.

REFERENCES

Baum, J., Dobson, C. M., Evans, P. A., & Hanley, C. (1989) Characterization of a partly folded protein by NMR methods: Studies on the molten globule state of guinea pig α-lactalbumin, *Biochemistry 28*, 7–13.

Bierzynski, A., Kim, P. S., & Baldwin, R. L. (1982) A salt bridge stabilizes the helix formed by isolated C-peptide of RNase A, *Proc. Natl. Acad. Sci. U.S.A. 79*, 2470–2474.

Brown, J. E., & Klee, W. A. (1971) Helix–coil transition of the isolated amino terminus of ribonuclease, *Biochemistry 10*, 470–476.

Bushnell, G. W., Louie, G. V., & Brayer, G. D. (1990) High-resolution three-dimensional structure of horse heart cytochrome *c*, *J. Mol. Biol. 214*, 585–595.

Collawn, J. F., & Paterson, Y. (1990) Stabilization of helical structure in two 17-residue amphipathic analogues of the C-terminal peptide of cytochrome *c*, *Biopolymers 29*, 1289–1296.

Dyson, H. J., Rance, M., Houghten, R. A., Wright, P. E., & Lerner, R. A. (1988) Folding of immunogenic peptide fragments of proteins in water solution II. The nascent helix, *J. Mol. Biol. 201*, 201–217.

Ghadiri, M. R., & Choi, C. (1990) Secondary structure nucleation in peptides. Transition metal ion stabilized α-helices, *J. Am. Chem. Soc. 112*, 1630–1632.

Goto, Y., & Nishikiori, S. (1991) Role of electrostatic repulsion in the acidic molten globule of cytochrome *c*, *J. Mol. Biol. 222*, 679–686.

Goto, Y., Calciano, L. J., & Fink, A. L., (1990a) Acid-induced folding of proteins, *Proc. Natl. Acad. Sci. U.S.A. 87*, 573–577.

Goto, Y., Takahashi, N., & Fink, A. L. (1990b) Mechanism of acid-induced folding of proteins, *Biochemistry 29*, 3480–3488.

Hughson, F. M., Wright, P. E., & Baldwin, R. L. (1990) Structural characterization of a partly folded apomyoglobin intermediate, *Science 249*, 1544–1548.

Jeng, M., Englander, S. W., Elöve, G. A., Wand, A. J., & Roder, H. (1990) Structural description of acid-denatured cytochrome *c* by hydrogen exchange and 2D NMR, *Biochemistry 29*, 10433–10437.

Karplus, M., & Weaver, D. L. (1976) Protein-folding dynamics, *Nature 260*, 404–406.

Kim, P. S., & Baldwin, R. L. (1982) Specific intermediates in the folding reactions of small proteins and the mechanism of protein folding, *Annu. Rev. Biochem. 51*, 459–489.

Kuroda, Y., Kidokoro, S., & Wada, A. (1992) Thermodynamic characterization of cytochrome *c* at low pH: Observation of the molten globule state and of the cold denaturation process, *J. Mol. Biol. 223*, 1139–1153.

Lyu, P. C., Marky, L. A., & Kallenbach, N. R. (1989) The role of ion pairs in α-helix stability: Two new designed helical peptides, *J. Am. Chem. Soc. 111*, 2733–2734.

Merutka, G., Shalongo, W., & Stellwagen, E. (1991) A model peptide with enhanced helicity, *Biochemistry 30*, 4245–4248.

Miranker, A., Radford, S. E., Karplus, M., & Dobson, C. M. (1991) Demonstration by NMR of folding domains in lysozyme, *Nature 349*, 633–636.

Oas, T. G., & Kim, P. S. (1988) A peptide model of a protein folding intermediate, *Nature 336*, 42–48.

Ohgushi, M., & Wada, A. (1983) "Molten-globule state": A compact form of globular proteins with mobile side-chains, *FEBS Lett. 164*, 21–24.

Richardson, J. S., & Richardson, D. C. (1988) Amino acid preferences for specific locations at the ends of α-helices, *Science 240*, 1648–1652.

Roder, H., Elöve, G. A., & Englander, S. W. (1988) Structural characterization of folding intermediates in cytochrome *c* by H-exchange labelling and proton NMR, *Nature 335*, 700–704.

Rooman, M. J., & Wodak, S. J. (1992) Extracting information on folding from the amino acid sequence: Consensus regions with preferred conformation in homologous proteins, *Biochemistry 31*, 10239–10249.

Sancho, J., Neira, J. L., & Fersht, A. R. (1992) An N-terminal fragment of barnase has residual helical structure similar to that in a refolding intermediate, *J. Mol. Biol. 224*, 749–758.

Scholtz, J. M., Qian, H., York, E. J., Stewart, J. M., & Baldwin, R. L. (1991) Parameters of helix–coil transition theory for alanine-based peptides of varying chain lengths in water, *Biopolymers 31*, 1463–1470.

Tasayco, M. L., & Carey, J. (1992) Ordered self-assembly of polypeptide fragments to form nativelike dimeric *trp* repressor, *Science 255*, 594–597.

Udgaonkar, J. B., & Baldwin, R. L. (1988) NMR evidence for an early framework intermediate on the folding pathway of ribonuclease A, *Nature 335*, 694–699.

Udgaonkar, J. B., & Baldwin, R. L. (1990) Early folding intermediate of ribonuclease A, *Proc. Natl. Acad. Sci. U.S.A. 87*, 8197–8201.

Von Hippel, P. H., & Schleich, T. (1969) Effects of neutral salts on the structure and conformational stability of macromolecules in solution, in *Structural Stability of Biological Macromolecules* (Timasheff, S. N., & Fasman, G. D., Eds.) Vol. 2, pp 417–574, Marcel Dekker, Inc., New York.

Waltho, J. P., Feher, V. A., & Wright, P. E. (1990) The H-helix of myoglobin as a potential independent protein folding domain, in *Current Research in Protein Chemistry* (Villafranca, J. J., Ed.) pp 283–293, Academic Press Inc., San Diego, CA.

Wetlaufer, D. B. (1973) Nucleation, rapid folding, and globular intrachain regions in proteins, *Proc. Natl. Acad. Sci. U.S.A. 70*, 697–701.

Wright, P. E., Dyson, H. J., & Lerner, R. A. (1988) Conformation of peptide fragments of proteins in aqueous solution: implications for initiation of protein folding, *Biochemistry 27*, 7167–7175.