Participation of the Protein Ligands in the Folding of Cytochrome *c*†

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ABSTRACT: Absorption spectral, circular dichroic spectral, and viscosity measurements indicate that the compact low-spin conformation characteristic of native cytochrome *c* is quantitatively recovered from its extended high-spin conformation at pH 2 by titration to pH 4.0. This conformational transition has a midpoint of 2.5 and is very cooperative. Comparison of the pH transitions of native and various carboxymethylated derivatives of cytochrome *c* indicates that recovery of the compact conformation of the protein is coincident with coordination of histidyl-18 and does not require coordination of a second protein ligand. Extensive carboxymethylation of cytochrome *c* including histidyl-18 stabilizes an unfolded high-spin conformation of the protein throughout the pH range 2–7.

Horse heart ferricytochrome *c* is a relatively small globular protein containing 104 residues arranged in a known sequence along a single polypeptide chain (Margoliash *et al.*, 1961). The protein contains no free sulfhydryl groups or disulfide bonds. A single heme moiety is covalently attached to the polypeptide by two thioether bridges. In the crystalline state, the polypeptide chain is folded about the heme moiety forming a nearly spherical molecule with a hydrophobic interior and a hydrophilic exterior (Dickerson *et al.*, 1971). The heme iron is coordinated with two strong-field protein ligands, histidyl-18 and methionyl-80, and the heme moiety forms numerous noncovalent interactions with the polypeptide chain. The native conformation in solution as deduced by a variety of physical and chemical measurements is generally the same as that described for the crystalline state.

The small size of cytochrome *c*, the absence of disulfide bonds, and the availability of a variety of measurements to characterize the conformation of the protein in solution makes cytochrome *c* an attractive model for studying the mechanism of folding of a polypeptide chain into its native conformation. The polypeptide chain of cytochrome *c* is extensively unfolded in the presence of concentrated urea solutions at neutral pH and is quantitatively refolded into the native conformation upon removal of the urea (Stellwagen, 1968b). However, spectral studies indicate that two strong-field protein ligands are coordinated with the heme iron in concentrated urea solution (Babul and Stellwagen, 1971). In order to study the entire folding process it is necessary to dissociate these protein ligands by acidification of the protein solution. This report examines the folding of the polypeptide chain of cytochrome *c* and various carboxymethylated derivatives upon neutralization of acidified protein solutions in the absence of urea and the roles of the two protein ligands for the heme iron in the refolding process.

Materials and Methods

Materials. Horse heart cytochrome *c*, type III and VI, was obtained from the Sigma Chemical Co. The protein preparations were quantitatively oxidized to ferricytochrome *c* which was used exclusively throughout this work. Bromoacetic acid was purchased from the Eastman Kodak Co. and recrystallized from petroleum ether (bp 30–60°) prior to use.

Methods. Amino acid compositions were determined using a Spinco 120C analyzer after hydrolysis of carboxymethylated derivatives of cytochrome *c* in 6 N HCl for 24 hr *in vacuo*. The content of each amino acid was calculated relative to the sum of arginine, proline, leucine, and valine, assuming that each molecule of protein contains 13 of these residues. No corrections were made for destruction of amino acids during acid hydrolysis. The content of half-cystine and methionine was determined as cysteic acid and methionine sulfone, respectively, after oxidation with performic acid as described by Hirs (1967) prior to acid hydrolysis. Carboxymethylmethionine is not oxidized under these conditions (Neuman *et al.*, 1962).

All absorption spectra were obtained using a Cary Model 14 recording spectrophotometer. Extinction coefficients used to calculate the concentration of native ferricytochrome *c* at pH 7 and 410 nm, (CmMet)(CmHis) *c*[1] at pH 7 and 408 nm, $(CmMet)_2$ *c* at pH 8 and 406 nm, $(CmMet)_2(CmHis)_3$ *c* at pH 7 and 403 nm in 0.1 M imidazole, hemopeptide 14–21 at pH 7 and 406 nm in 0.1 M imidazole, and the carboxymethylated hemopeptide at pH 7 and 403 nm in 0.1 M imidazole were 1.06×10^5, 1.06×10^5, 1.27×10^5, 1.06×10^5, 1.06×10^5, and 1.05×10^5 M^{-1} cm^{-1}, respectively. Circular dichroic spectra were obtained with a Cary Model 60 ORD–CD apparatus. Viscosity measurements, solvent perturbation difference spectra, and spectrophotometric titrations of the phenolic ionization of tyrosyl residues at 243 nm were obtained as described previously (Stellwagen and Van Rooyan, 1967).

Results

Cytochrome c. At neutral pH, the visible absorption spec-

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[1] Abbreviations used are: (CmMet)(CmHis) *c*, cytochrome *c* having one methionyl and one histidyl residue carboxymethylated; $(CmMet)_2$ *c*, cytochrome *c* having both methionyl residues carboxymethylated; $(CmMet)_2(CmHis)_3$ *c*, cytochrome *c* having two methionyl, three histidyl, and one-half of one lysyl residue carboxymethylated.

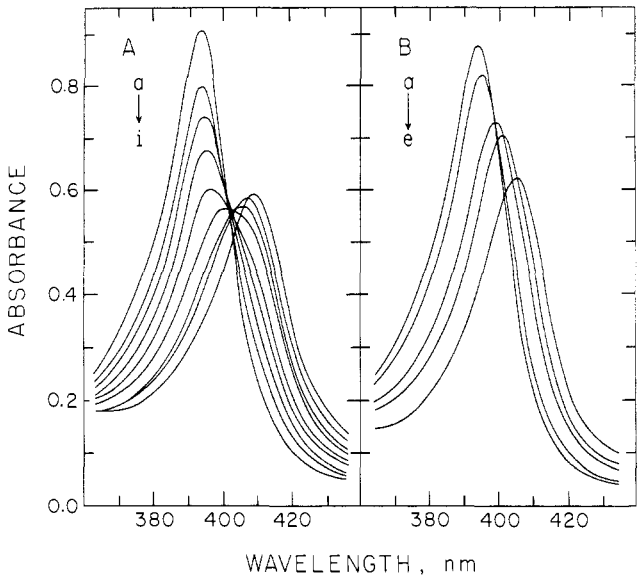

FIGURE 1: Soret absorbance spectra of native and $(CmMet)_2$ cytochrome *c*. (A) Native protein, 5.5×10^{-6} M. (a) pH 2.16, (b) pH 2.30, (c) pH 2.37, (d) pH 2.41, (e) pH 2.46, (f) pH 2.53, (g) pH 2.65, (h) pH 3.69, and (i) pH 7.10. (B) $(CmMet)_2$ *c*, 5.0×10^{-6} M. (a) pH 2.19, (b) 2.61, (c) pH 3.05, pH 4.69, and (e) pH 7.25. The ionic strength was 0.01, the temperature 25°, and the light path 10 mm.

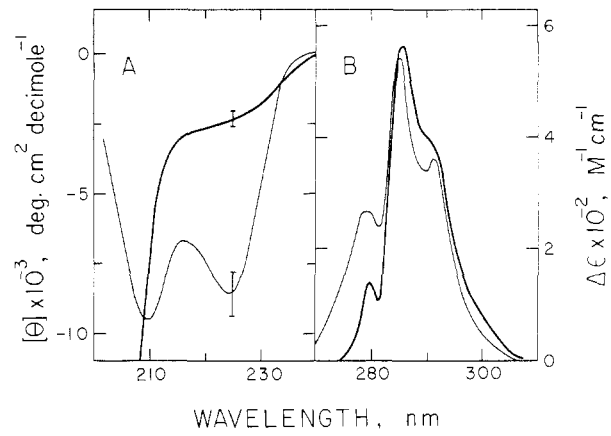

FIGURE 3: Spectral properties of cytochrome *c* and derivatives. (A) Circular dichroic spectra. The heavy line represents the spectrum of cytochrome *c*, 1.58×10^{-6} M, in water acidified to pH 2 with HCl and the spectrum of $(CmMet)_2(CmHis)_3$ *c*, 1.04×10^{-6} M, in 10 mM phosphate buffer (pH 7.0). The light line represents the spectrum of cytochrome *c*, 1.58×10^{-6} M, in H_2O, pH 7, and the spectrum of this solution acidified with HCl to pH 2 and subsequently adjusted to pH 7 with NaOH. These spectra were obtained at 25° using a cell having a path length of either 10 or 50 mm. (B) Solvent perturbation difference spectra in 20% ethylene glycol. The heavy line represents the spectrum of the $(CmMet)_2(CmHis)_3$, *c*, 8.75×10^{-5} M, in 10 mM imidazole (pH 7). The heme perturbation spectrum was subtracted as before (Stellwagen and Van Rooyan, 1967). The light line represents the spectrum calculated (Herskovits and Sorensen, 1968) for four exposed tyrosyl and one exposed tryptophanyl residue.

trum of ferricytochrome *c* has maxima at 410, 530, and 695 nm. At pH 2.0, the maxima in the visible spectrum occur at 395 and 620 nm. After neutralization of the acidified protein solution, the visible absorption spectrum is identical with that of the native protein at pH 7.0. The changes in the Soret maximum after addition of increasing concentrations of base to an acidified cytochrome *c* solution are shown in Figure 1A. The change in absorbance at 395 nm with increasing pH describes a single transition having a midpoint at pH 2.5, as shown in Figure 2A. A $\Delta\epsilon_{395}$ of 11.4×10^4 M^{-1} cm^{-1} was calculated between pH 2 and 7. The change in absorbance at 620 nm with increasing pH describes a transition identical with that shown for the unmodified protein in Figure 2A at 395 nm.

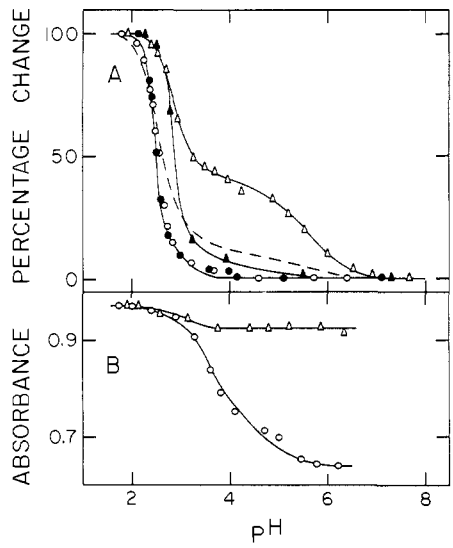

FIGURE 2: Changes in the properties of cytochrome *c* and derivatives upon neutralization of acidified solutions. (A) Proteins. The circles refer to cytochrome *c* and the triangles to $(CmMet)_2$ *c*. (○, △) Absorbance at 395 nm; (●, ▲) reduced viscosity. The dashed line represents the decrease in the absorbance of (CmMet)(CmHis) *c* at 395 with increasing pH. (B) Peptide. (○) Hemopeptide 14–21, (△) carboxymethylated hemopeptide. All measurements were done at 25° in an ionic strength of 0.01–0.02.

A solution of cytochrome *c* containing 4 mg of protein/ml at pH 7 and an ionic strength of 0.01 has a reduced viscosity of 2.6 ml/g, characteristic of a nearly spherical molecule. At pH 2 the reduced viscosity is increased to 22.5 ml/g. This value is considerably larger than the intrinsic viscosity of 15.1 ml/g calculated (Tanford *et al.*, 1966) for a randomly coiled polypeptide containing 104 amino acid residues. This calculation indicates that cytochrome *c* at pH 2 has the conformation of an extended chain due to the repulsive forces generated by the high net positive charge on the molecule at low pH. After neutralization the reduced viscosity is again 2.6 ml/g. The change in reduced viscosity with increasing pH describes a transition in common with that observed by the spectral measurements, as shown in Figure 2A.

The circular dichroic spectra of cytochrome *c* at pH 7, after acidification to pH 2, and after acidification to pH 2 and subsequent titration to pH 7 are shown in Figure 3A. The spectra at pH 7 and pH 2 are similar to those previously reported by Myer (1968).

Carboxymethylated Cytochrome c. As shown in Table I, reaction of cytochrome *c* for 3 days at 25° with 0.2 M bromoacetate in 0.1 M phosphate buffer (pH 7) results in the carboxymethylation of 0.8 histidyl residue and 0.8 methionyl residue. The principal histidyl derivative is 1-carboxymethylhistidine as observed previously (Stellwagen, 1966). The loss of 0.4 of a tyrosyl residue is attributed to halogenation during acid hydrolysis by trace bromide ion (Gurd, 1967) since 4.0 tyrosyl residues are titrated spectrophotometrically at 243 nm prior to acid hydrolysis. At neutral pH, the visible absorption spectrum of the carboxymethylated derivative, (CmMet)(CmHis)

TABLE I: Amino Acid Compositions.[a]

Amino Acid	Cytochrome *c*	(CmMet)(CmHis) *c*	$(CmMet)_2$ *c*	$(CmMet)_2(CmHis)_3$ *c*
Alanine	6	5.9 ± 0.0	5.8 ± 0.0	5.9 ± 0.1
Arginine	2	2.0 ± 0.1	1.9 ± 0.0	1.9 ± 0.1
Aspartic acid	8	8.1 ± 0.1	8.0 ± 0.2	7.9 ± 0.1
$^1/_2$-Cystine[b]	2	1.6 ± 0.1	1.8 ± 0.2	2.0 ± 0.2
Glumatic acid	12	12.9 ± 0.2	12.7 ± 0.3	12.0 ± 0.1
Glycine	12	12.5 ± 0.2	12.0 ± 0.3	11.7 ± 0.1
Histidine	3	2.1 ± 0.2	3.0 ± 0.0	0.1 ± 0.0
1-Carboxymethyl-		0.7 ± 0.1	nd	Trace
3-Carboxymethyl-		nd	nd	0.4 ± 0.1
1,3-Dicarboxymethyl-		0.1 ± 0.1	nd	2.6 ± 0.0
Isoleucine	6	5.7 ± 0.5	5.2 ± 0.1	5.7 ± 0.0
Leucine	6	6.0 ± 0.1	6.0 ± 0.0	6.1 ± 0.2
Lysine	19	19.3 ± 0.3	18.9 ± 0.4	18.9 ± 0.4
ε-Monocarboxymethyl-		nd	nd	nd
ε-Dicarboxymethyl-		Trace	nd	0.5 ± 0.2
Methionine[b]	2	1.2 ± 0.1	0.2 ± 0.0	Trace
Phenylalanine	4	3.7 ± 0.2	3.7 ± 0.1	3.8 ± 0.0
Proline	4	4.0 ± 0.1	4.0 ± 0.1	3.8 ± 0.0
Serine	0	0.4 ± 0.2	nd	0.4 ± 0.2
Threonine	10	9.4 ± 0.1	9.2 ± 0.3	9.1 ± 0.1
Tryptophan	1	na	na	na
Tyrosine	4	3.4 ± 0.2	3.1 ± 0.2	2.9 ± 0.0
Valine	3	3.0 ± 0.1	3.0 ± 0.0	3.1 ± 0.1

[a] The values reported represent the average of two analyses. [b] Determined as cysteic acid and methionine sulfone after performic acid oxidation. na, not analyzed, nd, not detected.

c, has a Soret maximum at 408 nm having an ϵ of 1.06×10^5 M^{-1} cm^{-1} and a small maximum at 695 nm having a ϵ of 685 M^{-1} cm^{-1}. These values indicate that neither histidyl-18 or methionyl-80 has been carboxymethylated (Stellwagen, 1966, 1968a). Carboxymethylation of cytochrome *c* under these conditions is known to selectively carboxymethylate histidyl-33 and methionyl-65 (Harbury, 1966).

At pH 1.8, the visible absorption spectrum of (CmMet)-(CmHis) *c* is identical with that of the unmodified protein at the same pH. The change in absorbance at 395 nm with increasing pH has a midpoint at pH 2.6 but is less cooperative than that of the unmodified protein, as shown by the dashed line in Figure 2A. A $\Delta\epsilon_{395}$ of 10.9×10^4 M^{-1} cm^{-1} was calculated between pH 2 and 7. The change in absorbance at 620 nm with increasing pH describes a transition similar to that shown in Figure 2A for this derivative at 395 nm.

Reaction of cytochrome *c* for 28 hr at 25° with 0.2 M bromoacetate in 0.2 M citrate buffer (pH 3) as described by Ando *et al.* (1965) and Tsai and Williams (1965) results in the selective carboxymethylation of 1.8 methionyl residues as shown in Table I. The reduction in the tyrosine content is attributed to halogenation during acid hydrolysis since 4.1 tyrosyl residues are titrated spectrophotometrically prior to acid hydrolysis. No significant reduction in the histidyl, lysyl, or cysteinyl content is observed. Since cytochrome *c* contains only two methionyl residues, both methionyl-65 and -80 are virtually completely carboxymethylated by this procedure. At neutral pH, this derivative $(CmMet)_2$ *c*, has a reduced viscosity of 3.2 ml/g and a visible absorption spectrum having maxima at 406 and 530 nm. At pH 2.2, maxima in the visible absorbance spectrum appear at 394 and 620 nm and the reduced viscosity is increased to 24.4 ml/g. After neutralization of the acidified solution of $(CmMet)_2$ *c*, the visible spectrum and reduced viscosity are characteristic of this derivative at pH 7. The changes in the Soret absorption after addition of increasing concentrations of base to an acidified solution of $(CmMet)_2$ *c* are shown in Figure 1B. The change in absorbance at 395 nm with increasing pH describes a two-step transition having midpoints at about pH 2.9 and 5.5. As shown in Figure 2A, a $\Delta\epsilon_{395}$ of 10.0×10^4 M^{-1} cm^{-1} was calculated for $(CmMet)_2$ *c* between pH 2 and 7. The change in absorbance at 620 nm with increasing pH also exhibits a two-step transition with similar midpoint values. However, the first step in the transition at 620 nm accounts for about 30% of the total change as contrasted with about 60% at 395 nm. The dependence of the reduced viscosity on increasing pH describes a single-step transition having a midpoint at pH 2.9, as shown in Figure 2A.

As shown in Table I, reaction of cytochrome *c* for 5 days at 25° with 0.2 M bromoacetate in 0.1 M phosphate buffer and 9 M urea (pH 7.0) results in the carboxymethylation of all three histidyl residues and both methionyl residues. About 80% of the histidyl residues are carboxymethylated to the 1,3-dicarboxymethyl derivative. In addition, 0.5 of an ε-dicarboxymethyllysine residue is detected indicating that about 3% of the lysyl residues are carboxymethylated by these reaction conditions. Again the loss of 1.1 tyrosyl residues is attributed to halogenation during acid hydrolysis since 4.0 tyrosyl residues are detected by spectrophotometric titration of this carboxymethylated protein prior to acid hydrolysis.

The visible absorption spectrum of this derivative, $(CmMet)_2(CmHis)_3$ *c*, exhibits maxima at 390 and 620 nm through-

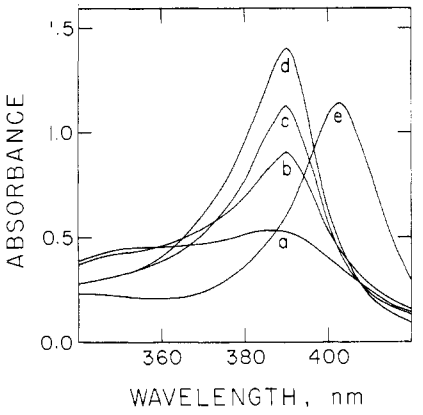

FIGURE 4: Soret absorbance of $(CmMet)_2(CmHis)_3$ *c*. (a–c) Protein in 10 mM phosphate buffer (pH 7): (a) 1.08×10^{-4} M solution in 1 mm light path; (b) 10-fold dilution in 10-mm light path; (c) 100-fold dilution in 100-mm light path; (d) 10-fold dilution, adjusted to pH 2.0; (e) 10-fold dilution with 0.1 M imidazole buffer (pH 7.0). All spectra were obtained at 25°.

out the pH range from pH 2 to 9. At pH 7 the Soret absorption decreases with increasing protein concentration as shown in Figure 4. Similar changes in the Soret absorbance of hemopeptide 14–21 with increasing peptide concentration are proposed to result from aggregation of the hemopeptide involving a stacking of heme moieties (Urry and Pettegrew, 1967). By contrast, the Soret absorption of $(CmMet)_2(CmHis)_3$ *c* is concentration independent at pH 2, indicating that the intermolecular charge repulsions at low pH disperse the aggregated species. The Soret maximum of $(CmMet)_2(CmHis)_3$ *c* at neutral pH appears at 403 nm in the presence of 0.1 M imidazole as shown in Figure 4. The Soret absorption in the presence of imidazole at pH 7 is independent of protein concentrations indicating that coordination of the extrinsic ligand depolymerizes the aggregated species. The reduced viscosity of $(CmMet)_2(CmHis)_3$ *c* in the presence of 0.1 M imidazole is 17.6 ml/g, a value somewhat larger than that calculated for a random coil containing 104 residues, 15.1 ml/g. This discrepancy suggests that a small amount of aggregated species may persist in the presence of imidazole. The near-ultraviolet solvent perturbation difference spectrum of $(CmMet)_2(CmHis)_3$ *c* in 10 mM imidazole compares favorably with the spectrum calculated for four exposed tyrosyl and one exposed tryptophanyl residues as shown in Figure 3B. Since all extrinsic ligands absorb strongly in the far-ultraviolet region at concentrations necessary for coordination (Nanzyo and Sano, 1968), it is not possible to determine the circular dichroic spectrum of the predominately monomeric form of $(CmMet)_2(CmHis)_3$ *c*. The ultraviolet circular dichroic spectrum of the polymerized $(CmMet)_2(CmHis)_3$ *c* in the absence of added ligand at neutral pH is identical with that of the unfolded protein at acid pH as shown in Figure 3A. These results indicate that $(CmMet)_2(CmHis)_3$ *c* either in the presence or absence of added ligands retains little if any of the native conformation and that $(CmMet)_2(CmHis)_3$ *c* has a great propensity to aggregate.

Hemopeptide 14–21. The hemopeptide containing residues 14–21 in the protein sequence was prepared by proteolytic digestion of the protein by pepsin and trypsin as described by Harbury and Loach (1960). At neutral pH, the Soret maximum of this peptide occurs at 397 nm. Upon acidification of the peptide solution to pH 2.0, the Soret maximum is shifted to 394 nm. As shown in Figure 2B, the transition from pH 2 to 7 has a midpoint at pH 3.8 and a $\Delta\epsilon_{395}$ of 5.4×10^4 M^{-1} cm^{-1} using a concentration of hemopeptide at which the monomeric form predominates. Addition of an extrinsic ligand, either imidazole or *N*-acetylmethionine, to a concentration of 0.1 M in a solution of hemopeptide 14–21 at neutral pH shifts the Soret maximum from 397 to 406 nm, giving a $\Delta\epsilon_{395}$ of 3.8×10^4 M^{-1} cm^{-1}. Carboxymethylation of histidyl-18 in the hemopeptide 14–21 causes the Soret maximum to remain at 390 nm throughout the pH range 2–7. At neutral pH, the extinction of the Soret maximum at 390 nm decreases with increasing concentration of the carboxymethylated hemopeptide 14–21 in a manner similar to that shown in Figure 4 for $(CmMet)_2(CmHis)_3$ *c*. This polymerization is responsible for the small decrease in absorbance at 395 nm of the carboxymethylated hemopeptide 14–21 observed between pH 2 and 4, as shown in Figure 2B. Addition of 0.1 M imidazole to the carboxymethylated hemopeptide at neutral pH shifts the Soret maximum from 390 to 403 nm.

Discussion

Coordination of the heme iron of ferricytochrome *c* with two strong-field protein ligands, such as nitrogen or sulfur atoms, produces a low-spin complex having a Soret maximum above 400 nm. Coordination with two weak-field ligands, such as oxygen or halogen atoms, produces a high-spin complex having a Soret maximum between 390 and 395 nm, while coordination of one strong-field and one weak-field ligand produces an equilibrium mixture of high- and low-spin configurations having a Soret maximum between 396 and 400 nm (Margoliash and Schjecter, 1966). The high-spin complex and the equilibrium mixture of spin configurations are further characterized by a maximum at 620 nm. Protonation or dicarboxymethylation of the imidazole nitrogens of histidyl-18 or monocarboxymethylation of the sulfur methionyl-80 would be expected to prevent coordination of these residues with the heme iron. If the modified residue is replaced by a weak-field ligand the Soret maximum should shift from above 400 to below 400 nm.

Acidification of cytochrome *c* to pH 2.0 shifts the Soret maximum from 410 to 395 nm and produces a maximum at 620 nm indicating the replacement of the two strong-field polypeptide ligands in the native conformation by two weak-field ligands at low pH presumably supplied by the solvent. Concurrently, the reduced viscosity increases from 2.6 to 22.4 ml per g indicating the compact native conformation has assumed the conformation of an extended coil. The visible circular dichroic spectrum of cytochrome *c* at pH 2 is that typical of an unstructured polypeptide with an exposed heme moiety. Neutralization of acidified cytochrome *c* rapidly and quantitatively produces a conformation having the same reduced viscosity, absorption spectrum and circular dichroic spectrum as the native protein. It was concluded therefore that the native conformation is unfolded at pH 2 in minimal ionic strength and is quantitatively recovered upon neutralization of the acidified solution. Preliminary stopped-flow spectrophotometric measurements indicate that the Soret absorption typical of native cytochrome *c* is recovered with a half-time of about 3 msec after neutralization of acidified cytochrome *c* at 25°.

Since a protonated imidazole side chain cannot function as a ligand, the midpoint of the spin-state transition of the unmodified protein, 2.5, must also be the apparent p*K* of the imidazole of histidyl-18 as suggested earlier by Theorell (1941). This low apparent p*K* value is presumably due to a

coupling of the dissociation of protonated imidazole with the preferential coordination of the dissociated form. The effect of coupling can be evaluated using hemopeptide 14–21. This octapeptide would be expected to have little if any structure. The monomeric form of the hemopeptide has a Soret maximum at 397 nm at neutral pH, indicating an equilibrium mixture of high- and low-spin configurations and a Soret maximum at 394 nm at pH 2, characteristic of a high-spin complex. A CPK model of the hemopeptide indicates that histidyl-18 is the only residue which can contribute a strong-field ligand. As shown in Figure 2B, the spin-state transition for hemopeptide 14–21 has an apparent pK of 3.8 and a $\Delta\epsilon_{395}$ of 5.4×10^4 M^{-1} cm^{-1}. Dicarboxymethylation of histidyl-18 in hemopeptide 14–21 causes a high-spin complex having a Soret maximum at 390 nm to persist throughout the pH range from pH 2 to 7, indicating that the spin-state transition is associated with coordination of histidyl-18. The coupling of the dissociation of the protonated imidazole of histidyl-18 with coordination of the dissociated form with the heme iron lowers the apparent pK of dissociation from pH 7.0 (Datta and Grzybowski, 1966) to 3.8. The further reduction of the apparent pK of histidyl-18 to pH 2.5 in cytochrome *c* must be due to coupling of the dissociation and subsequent coordination with the multiplicity of noncovalent interactions in the protein which stabilize the low-spin conformation.

The $\Delta\epsilon_{395}$ of 4.8×10^4 M^{-1} cm^{-1} observed for the coordination of histidyl-18 in the hemopeptide 14–21 with the heme iron indicates that only a portion of the $\Delta\epsilon_{395}$ of 11.4×10^4 M^{-1} cm^{-1} accompanying the spin-state transition of the protein is associated with coordination of histidyl-18. Addition of either 0.1 M imidazole or *N*-acetylmethionine to the equilibrium mixture of high- and low-spin configurations of hemopeptide 14–21 at neutral pH shifts the Soret maximum from 397 to 406 nm indicating coordination of the intrinsic ligand to form a low-spin complex. The attendant $\Delta\epsilon_{395}$ is 3.8×10^4 M^{-1} cm^{-1}. Therefore a sizeable portion of the $\Delta\epsilon_{395}$ of 11.4×10^4 M^{-1} cm^{-1} observed for the spin-state transition of the protein is also associated with coordination of the second protein ligand.

Carboxymethylation of a single methionyl and a single histidyl residue, methionyl-65 and histidyl-33 (Harbury, 1966) both surface residues (Dickerson *et al.*, 1971), at neutral pH to form (CmMet)(CmHis) *c* does not alter the features of the native conformation (Stellwagen, 1968a). Carboxymethylation of both methionyl residues at pH 3 to form $(CmMet)_2$ *c* similarly does not alter the gross features of the native conformation but does increase the exposure of the heme moiety (Stellwagen, 1968a). Both derivatives exist in compact low-spin complex conformations at neutral pH. As shown in Figure 2A, the spin state transition accompanying neutralization of acidified $(CmMet)_2$ *c* occurs in two steps having apparent midpoint values at about pH 2.9 and 5.5. By contrast, the spin-state transition accompanying neutralization of (CmMet)(CmHis) *c* in which methionyl-65 but not the natural ligand methionyl-80 is carboxymethylated exhibits a single step transition with a midpoint at pH 2.6. It is reasonable to assign the first step in the spin-state transition of $(CmMet)_2$ *c* having a midpoint at pH 2.9 and a $\Delta\epsilon_{395}$ of about 6.0×10^4 M^{-1} cm^{-1} to coordination of the unmodified ligand, histidyl-18, and the second step having a midpoint at about pH 5.5 and a $\Delta\epsilon_{395}$ of about 4.0×10^4 M^{-1} cm^{-1} to coordination of a second protein ligand. The structural transition of $(CmMet)_2$ as detected by viscosity measurements describes a single transition having a midpoint at pH 2.9 as shown in Figure 2A. It is concluded therefore that the majority of the folding of the extended conformation of cytochrome *c* in its compact native conformation is coincident with coordination of histidyl-18 and does not require coordination of methionyl-80 or any other protein ligand.

If coordination of histidyl-18 is necessary for, rather than only coincident with, formation of a compact structure, carboxymethylation of histidyl-18 should prevent formation of such a structure. Unfortunately, carboxymethylation of histidyl-18 can only be achieved in denaturing solvents with the simultaneous carboxymethylation of the other two histidyl residues, both methionyl residues, and one-half of one of the nineteen lysyl residues. The visible absorption spectrum of this derivative, $(CmMet)_2(CmHis)_3$ *c*, is typical of a high-spin complex throughout the pH range from pH 2 to 7 as shown in Figure 3. The far-ultraviolet circular dichroic spectrum (Figure 3A) and the reduced viscosity of the monomeric form at pH 7 both indicate that the polypeptide chain is unstructured. By contrast, carboxymethylation of methionyl-65, methionyl-80, or histidyl-33 does not alter the compact low-spin conformation of the protein at pH 7. The high-spin random conformation of $(CmMet)_2(CmHis)_3$ *c* must then be the result of carboxymethylation of histidyl-18 or -26, the carboxymethylation of one-half of a lysyl residues, or some combination thereof. Histidyl-26 does not appear to have a critical role in maintaining the native conformation serving only to hydrogen bond with prolyl-44 (Dickerson *et al.*, 1971). In *Neurospora crassa* cytochrome *c*, histidyl-26 is replaced by a glutamine residue (Heller and Smith, 1966) without adversely effecting the function of the protein. All nineteen lysyl residues in horse heart cytochrome *c* are located on the surface of the protein (Dickerson *et al.*, 1971) and can be guanidinated without altering the properties of the protein (Hettinger and Harbury, 1964). A decrease of 6 units in the net positive charge of unfolded $(CmMet)_2(CmHis)_3$ *c* as compared to folded (CmMet)(CmHis) *c* at neutral pH would not be expected to destabilize the compact structure so extensively. By difference then, carboxymethylation of histidyl-18 appears primarily responsible for the loss of the native conformation following extensive carboxymethylation of the protein. It is most likely that the loss of the ligand function of dicarboxymethylhistidyl-18 rather than the increase in the size of the residue or the presence of a negative charge is primarily responsible for the conformational changes accompanying carboxymethylation of this residue.

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Some Physical Properties of the Cholinergic Receptor Protein from *Electrophorus electricus* Revealed by a Tritiated α-Toxin from *Naja nigricollis* Venom†

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ABSTRACT: The cholinergic receptor protein present in excitable membrane fragments is labeled by an α-toxin purified from the venom of *Naja nigricollis*. After tritiation *in vitro*, the α-toxin possesses a specific activity of 14 Ci/mmole and exhibits properties identical with those of the native toxin. Reversible cholinergic effectors, like carbamylcholine, decamethonium, *d*-tubocurarine, or gallamine, and two affinity labeling reagents, protect against [^{3}H]α-toxin binding. The number of [^{3}H]α-toxin binding sites in membrane fragments is found to be 10 to 20 nmoles per g of membrane protein. The receptor–[^{3}H]α-toxin complex can be separated easily from the free toxin in solution by ammonium sulfate precipitation in the presence of 1% Triton X-100; this property is used to develop an assay for the free receptor protein in solution. Extraction of labeled membrane fragments by 1% sodium deoxycholate or 1% Triton X-100 preserves the association of the toxin to a macromolecule which precipitates in the absence of detergent but migrates as a single band on gel electrophoresis in the presence of 1% sodium deoxycholate. The [^{3}H]α-toxin–receptor complex as well as the free receptor protein in the presence of either a charged detergent (deoxycholate) or a neutral one (Triton X-100) sediments in sucrose gradients with a standard sedimentation coefficient of 9.5 S and is eluted on a Sepharose 6B column with *Escherichia coli* β-galactosidase (mol wt 540,000). Sedimentation and gel filtration data cannot be reconciled assuming that the receptor protein is a classical globular protein with a normal density. Treatment of a deoxycholate extract by sodium dodecyl sulfate gives a unit of apparent mol wt 55,000 by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. This unit is the smallest one seen before dissociation of the receptor–toxin complex.

In previous papers (Changeux *et al.*, 1970a,b; Changeux *et al.*, 1971) we described and critically discussed a binding assay which led to the first unambiguous characterization *in vitro* of the physiological receptor site to which acetylcholine binds in the course of its electrogenic action (Nachmansohn, 1959, 1971). This assay was based on the joint capacity for the receptor site to bind typical cholinergic effectors (such as decamethonium or *d*-tubocurarine) or α-toxins from snake venoms (like α-bungarotoxin (Lee and Chang, 1966) or the α-toxin from *Naja nigricollis* (Boquet *et al.*, 1966)). It was further shown that the macromolecule which carries this site can be extracted by deoxycholate in a soluble form and in appreciable amounts from the electric organ of the fish *Electrophorus electricus*. The macromolecule, a protein distinct from the enzyme acetylcholinesterase, retains *in vitro*, and *in solution*, most specific binding properties of the physiological receptor (Changeux *et al.*, 1970a, 1971; Meunier *et al.*, 1971a).

More recently Miledi *et al.* (1971) published findings on *Torpedo* electric tissue labeled with radioactive [^{131}I]α-bungarotoxin. They described the irreversible binding of the radioactive toxin to membrane fragments, the protection by

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