Tyrosine and Tryptophan in Cytochrome *c**

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ABSTRACT: The optical rotatory dispersion of oxidized cytochrome *c* reveals distinct Cotton effect maxima at both 287 and 278 mμ; the peak at 278 mμ is absent in reduced cytochrome *c* [Ulmer, D. D. (1965), *Biochemistry 4*, 902]. The nature of the chromophore giving rise to the oxidation–reduction-dependent Cotton effect at 278 mμ was investigated by spectropolarimetric studies and chemical modifications of the oxidized and reduced proteins. Acetylimidazole modifies two tyrosyl residues in ferricytochrome *c* while less than one-half tyrosine is modified in the reduced cytochrome; however, acylation does not alter the optical rotatory dispersion, indicating that the oxidation–reduction-dependent Cotton effect does not arise from "free" tyrosyl groups. In contrast, titration with *N*-bromosuccinimide modifies one tryptophan, and, concomitantly, obliterates the peak at 278 mμ; this suggests that the oxidation–reduction-dependent Cotton effect arises from the single tryptophyl residue of the horse heart protein.

Such a conclusion is supported by studies of the optical rotatory dispersion of cytochromes isolated from different species, which vary in aromatic amino acid composition. Thus, tuna ferricytochrome, the only species having *two* tryptophans, exhibits a Cotton effect with a peak at 278 mμ nearly double in magnitude that observed in any other cytochrome. Neither apocytochrome, from which the heme has been removed, nor a peptic heme peptide from cytochrome *c*, which lacks aromatic amino acids, exhibit Cotton effects in the spectral range 270–300 mμ. Moreover, the Cotton effects generated by aromatic residues of the native cytochrome are markedly sensitive both to alkaline pH and to iron-binding ligands known to alter the relationship of the heme to the protein. These data suggest that the asymmetric environment of aromatic chromophores in cytochrome *c* is dependent upon an *interaction* of the heme with regions of the primary structure containing aromatic amino acids; oxidation–reduction appears to affect the nature of this interaction.

The reversible change in valence of the heme iron atom of cytochrome *c* induces remarkable alterations in the physicochemical properties of this protein. The oxidized and reduced cytochromes differ physically in thermal stability (Butt and Keilin, 1962), crystalline form (Hagihara *et al.*, 1958a–c), and surface charge or shape (Paléus and Neilands, 1950; Margoliash, 1954), and chemically in the reactivity both of the heme group, for ligands such as cyanide and azide (Theorell and Åkesson, 1941; Horecker and Kornberg, 1946; Horecker and Stannard, 1948), and of the protein moiety, in its susceptibility to proteolysis (Nozaki *et al.*, 1957, 1958). Such observations have been interpreted to indicate that the ferri- and ferrocytochromes differ in conformation and recent spectropolarimetric measurements lend additional support to this hypothesis (Ulmer, 1965; Urry and Doty, 1965).

The optical rotatory dispersion of cytochrome *c* also suggests that oxidation–reduction of the heme iron atom may alter the asymmetry of aromatic chromophores (Ulmer, 1965). Chemical modification of the oxidized and reduced proteins by group-specific reagents offers a means to examine this problem further. Differences between the two oxidation states in the reactivity of aromatic amino acids toward site-specific reagents could provide added insight as to the mechanism and, perhaps, the regions of primary structure involved in the oxidation-linked conformational change. Such a difference in reactivity has been shown recently for methionine (Matsubara *et al.*, 1965).

The present communication reports evidence indicating that the environment of both tyrosyl and tryptophyl residues differs in oxidized and reduced cytochrome *c*. In addition to the results of chemical modification of the horse heart protein, information pertinent to the role of these aromatic residues has been derived from spectropolarimetric studies of cytochromes from various species, which differ in their amino acid composition, and from examinations of a heme-peptide, obtained by peptic digestion of cytochrome *c*, and of the heme-free apocytochrome.

Methods and Materials

Horse heart cytochrome *c*, type III, was obtained commercially (Sigma Chemical Co.). Human and tuna cytochrome *c* and a crystallized preparation from horse heart were gifts of E. Margoliash. Oxidized cytochromes

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were prepared by exposing samples of the protein to 0.001 M ferricyanide followed by extensive dialysis *vs.* several changes of 0.1 M sodium phosphate buffer, pH 6.8. The cytochromes were reduced with neutralized ascorbate or dithionite followed by dialysis or by passing the protein over a column of Sephadex G-25 to remove the reducing agent. Cytochrome concentrations were based upon the weight of salt-free samples of protein dried at 104°, upon iron analyses (Cameron, 1965), or were determined spectrophotometrically at 550 mμ, on samples of protein reduced with dithionite, employing a millimolar extinction coefficient of 27.7 (Margoliash and Frohwirt, 1959). The apoprotein and heme group of cytochrome *c* were isolated according to Paul (1950, 1951), while a heme-peptide was obtained by means of pepsin digestion (Margoliash *et al.*, 1959). The composition of the peptide, containing the heme moiety and amino acid residues 11–21, was confirmed by amino acid analysis (Spackman *et al.*, 1958).

Acylation with acetylimidazole was performed according to Simpson *et al.* (1963) employing protein concentrations of 4–6 mg/ml. The reaction was carried out at 23°, in 0.02 M Veronal, pH 7.5, followed by dialysis for 18 hr at 4° *vs.* several changes of 0.1 M sodium phosphate, pH 6.8. Acetylation of the reduced cytochrome was carried out in the presence of either ascorbate or platinum black and hydrogen gas. *N*-Acetylimidazole was purchased from Aceto Chemical Corp. and stored over phosphorus pentoxide *in vacuo* after recrystallization from dry benzene or isopropenyl acetate. The extent of modification of tyrosyl residues was determined by measurements of spectra based on the value, ΔA_{278} = 1160/mole of tyrosyl residue acetylated. Modification with *N*-bromosuccinimide (NBS)[1] was carried out as described by Witkop (1961) at 23°, in 0.1 M sodium acetate, pH 4.0, followed by dialysis for 18 hr at 4° *vs.* several changes of 0.1 M Tris–HCl, pH 8.5. pH was determined with a Radiometer pH meter using a general purpose glass electrode.

Optical rotatory dispersion was measured in the Cary Model 60 recording spectropolarimeter over the spectral range 190–600 mμ at a temperature of 25°. Cells with fused-quartz end plates and 0.1–20-mm path length were employed using protein concentrations which varied from 0.4 to 3 mg/ml. The appropriate dialysates were used as blanks. The slit width of the instrument was programmed to yield maximal and constant light intensities at all wavelengths. In areas of high absorbance, absolute values for specific rotation were confirmed at two or more protein concentrations or path lengths, eliminating the possibility of spurious Cotton effects (Urnes and Doty, 1961). The reported dispersion curves are ordinarily based upon the average value obtained from three or more separate scans under varying settings for sensitivity, period, and scan speed. Specific rotations were calculated on the basis of protein concentration and are not corrected for the refractive index of the solvents employed.

[1] Abbreviations used: NBS, *N*-bromosuccinimide; DPNH, reduced diphosphopyridine nucleotide.

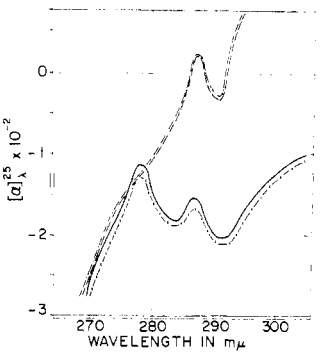

FIGURE 1: Aromatic side-chain Cotton effects of native and acetylated ferri- and ferrocytochrome *c*. Specific rotation $[\alpha]_\lambda^{25}$, is plotted *vs.* wavelength. Ferricytochrome (——); acetylferricytochrome (×—×—×); ferrocytochrome (— —); acetylferrocytochrome (—·—·). In the oxidized protein, peaks at 278 and 287 mμ are associated with the chromophores of the aromatic amino acids while only one peak, that at 287 mμ, is present in the reduced protein. Acetylation with acetylimidazole does not alter these Cotton effects in either ferro- or ferricytochrome. Conditions: 1–2 mg/ml of protein in 0.1 M sodium phosphate, pH 6.8. Acetylation was carried out with a 30-fold molar excess of acetylimidazole (see text).

Results

Spectropolarimetry of cytochrome *c* suggests that alterations in the asymmetric environment of aromatic amino acid residues accompany changes in the valence of the heme iron atom (Ulmer, 1965; Urry and Doty, 1965). While ferricytochrome *c* exhibits two maxima

TABLE I: Acetylation of Oxidized and Reduced Horse Heart Cytochrome *c* with Acetylimidazole.

Molar Excess of Acetylimidazole	Moles of *O*-Acetyltyrosine/Mole of Protein	
	Ferricytochrome	Ferrocytochrome
15	1.53	0–0.2
30	2.00	0.43
45	2.00	0.60
80	2.12	0.48
200	4.15[a]	...

[a] In the presence of a 200-fold molar excess of acetylimidazole cytochrome undergoes spectrophotometric and spectropolarimetric changes indicative of denaturation.

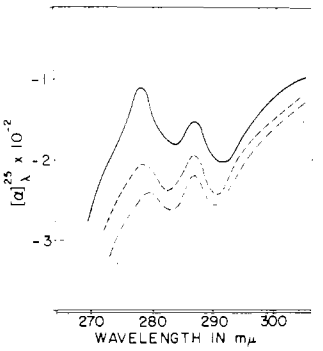

FIGURE 2: Effect of *N*-bromosuccinimide (NBS) on aromatic side-chain Cotton effects of ferricytochrome *c*. Specific rotation, $[\alpha]_\lambda^{25}$, is plotted *vs.* wavelength. Native ferricytochrome (——) exhibits peaks at 278 and 287 mμ, associated with chromophores of the aromatic amino acids. The peak at 278 mμ is obliterated progressively upon exposure to 0.76-fold (— —), 1.5-fold (—·—·), and 3.1-fold molar excess NBS (. . . .). Conditions: 1–3 mg/ml of protein in 0.1 M Tris–Cl, pH 8.5.

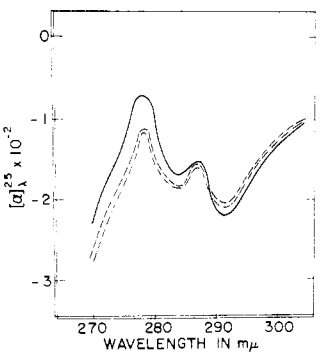

FIGURE 3: Comparison of aromatic side-chain Cotton effects of tuna (——), horse (— —), and human (—·—·) heart ferricytochromes *c*. Specific rotation, $[\alpha]_\lambda^{25}$, is plotted *vs.* wavelength. The tuna cytochrome generates a Cotton effect peak at 278 mμ nearly twice the magnitude of those observed in the horse and human proteins. Conditions: 1–3 mg/ml of protein in 0.1 M sodium phosphate, pH 6.8.

in the region of absorption of the aromatic amino acids, at 278 and 287 mμ, only the peak at 287 mμ is preserved in the reduced protein (Figure 1). Thus, the asymmetric chromophore which gives rise to the peak at 278 mμ appears to be influenced by the oxidation state of the iron atom. The nature of this chromophore was investigated by means of chemical modification.

Acetylation of ferricytochrome *c* with 30-fold molar excess of acetylimidazole results in modification of two tyrosines, while less than one-half the tyrosine of ferrocytochrome *c* reacts (Table I). Based upon the change in ninhydrin color, 3–4 lysyl residues are also modified by this procedure, but the number does not appear to differ between the two oxidation states. Notably, acetylation of either the oxidized or reduced protein does *not* change the rotatory dispersion in the regions of absorption of the aromatic amino acids (Figure 1), in the Cotton effect arising from the Soret band or in the intrinsic Cotton effect (Ulmer, 1965). Thus, while oxidation–reduction appears to alter the environment of tyrosyl residues, such that two tyrosines are exposed to acylation in ferricytochrome and none in ferrocytochrome, these chromophores do not appear to generate the oxidation–reduction-dependent Cotton effect at 278 mμ.

Acetylation with higher concentrations of acetylimidazole, *i.e.*, 150–200 molar excesses, modifies all four tyrosyl residues of horse heart ferricytochrome *c* (Table I) but is also accompanied by denaturation of the protein, as evidenced by loss of the characteristic cytochrome spectrum, a marked decrease in the magnitude of the trough of the intrinsic Cotton effect at 233 mμ, and obliteration of the extrinsic Cotton effects at longer wavelengths. At such concentrations of acetylimidazole, ferrocytochrome quickly becomes autoxidizable and a variable number of tyrosines are modified.

Titration of ferricytochrome *c* with *N*-bromosuccinimide modifies one tryptophyl residue (Table II) and,

TABLE II: Titration of Ferricytochrome *c* with *N*-Bromosuccinimide.

Moles of NBS/ Mole of Protein	Moles of Tryptophan Modified/Mole of Protein	Amplitude of Cotton Effect Peak at 278 mμ (in deg)[a]
0	0	50
0.76	0.40	30
1.50	0.64	20
3.10	1.01	0

[a] Data taken from Figure 2.

concomitantly, obliterates the Cotton effect at 278 mμ (Figure 2). Although exposure to *N*-bromosuccinimide slightly decreases the magnitude of the Cotton effect at the Soret band, under the conditions employed there is no change in the magnitude of the trough of the intrinsic Cotton effect of the protein. Thus, this chemical modification does not alter protein conformation as revealed by optical rotatory dispersion. These data suggest that it is the single tryptophyl residue of the horse heart protein which generates the oxidation–reduction-dependent Cotton effect with a peak at 278 mμ.

Both acylation with acetylimidazole and modification

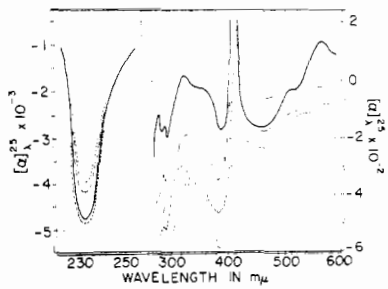

FIGURE 4: Optical rotatory dispersion of ferricytochrome *c* at pH 2.7 (----), pH 3.5 (— · — ·), pH 4.5–8.5 (——), pH 9.6 (— —), and pH 10.5 (—×—×). At both acid and alkaline pH the Cotton effect generated by the Soret band decreases in magnitude and becomes asymmetric; concomitantly, the peak of the Cotton effect at 278 mμ is obliterated. Based upon the magnitude of the trough of the intrinsic Cotton effect, on the left, protein conformation is unchanged at alkaline pH but markedly altered at acid pH. Conditions: 0.4–3 mg/ml of protein in 0.1 M sodium citrate–phosphate, pH 2–6.5; 0.1 M sodium phosphate, pH 6.5–8.0; 0.1 M glycine–NaOH, pH 9–10.5.

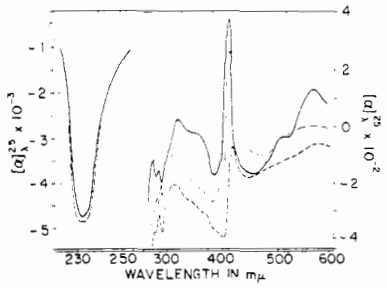

FIGURE 5: Effect of cyanide and azide on optical rotatory dispersion of ferricytochrome *c*. Specific rotation, $[\alpha]_\lambda^{25}$, is plotted *vs.* wavelength. Both the heme-induced Cotton effects and the peak at 278 mμ, arising from an aromatic side-chain chromophore, are markedly altered upon binding of ligands to the iron atom. However, the intrinsic Cotton effect (on the left), reflecting protein conformation, is virtually unchanged in the cyanide complex. The intrinsic effect of the azide complex could not be measured due to the intense absorption of this ligand at shorter wavelengths. Conditions: 0.4–3 mg/ml of ferricytochrome *c* in 0.1 M sodium phosphate, pH 6.8 (——); 0.1 M phosphate–1 M sodium azide, pH 6.8 (— · — ·); 0.1 M phosphate–0.05 M potassium cyanide pH 6.8 (— —).

with *N*-bromosuccinimde render cytochrome *c* autoxidizable and interfere with its functional role in electron transfer as measured in the DPNH–cytochrome *c* reductase system (Strittmatter and Ball, 1954).

The optical rotatory dispersions of cytochromes *c*, isolated from different species, and of varying aromatic amino acid content, lend further support to the view that tryptophan gives rise to the Cotton effect with a peak at 278 mμ. Thus, human heart cytochrome *c* contains five tyrosyl residues, one more than the horse heart protein, but exhibits the same rotatory dispersion (Figure 3). However, tuna ferricytochrome, having both five tyrosyl and *two* tryptophyl residues, generates a Cotton effect at 278 mμ nearly twice the magnitude of that observed in the horse or human protein (Figure 3) (Vallee and Ulmer, 1965; Myer and Harbury, 1965). As with the horse cytochrome, *N*-bromosuccinimide obliterates the maximum at 278 mμ of the tuna protein, although larger molar excesses are required than for the horse heart protein.

The optical rotatory dispersion of cytochrome *c* is remarkedly sensitive to hydrogen ion concentration. Spectropolarimetry of ferricytochrome was performed over a range of from pH 2.5 to 11 (Figure 4).[2] At a pH <4 or >9, the Cotton effect generated by the Soret band decreases in magnitude and becomes asymmetric; concomitantly, the peak of the Cotton effect at 278 mμ is obliterated. Notably, the maximum at 287 mμ is lost on the acid, but not on the alkaline side.

At pH 2.7 and 3.5, the magnitude of the trough of the intrinsic Cotton effect, at 233 mμ, decreases (Figure 4), suggesting changes in protein conformation. When the pH is increased from neutral to 9.6 or 10.5, however, the magnitude of the trough remains constant (Figure 4), indicating that protein conformation is unaltered. Thus, at acid pH, changes in the Cotton effects of heme and aromatic chromophores appear to be caused, at least partly, by protein denaturation, but at alkaline pH denaturation is not evident; rather, the changes in rotatory dispersion suggest a difference in the manner of *heme–peptide interaction*.

The optical rotatory dispersion of ferricytochrome *c* in the presence of cyanide is remarkably similar to that observed at alkaline pH (Figure 5). The principal Cotton effect at the Soret band is decreased in amplitude and slurred while the extrinsic Cotton effects associated with the absorption bands at 528 and 360 mμ are also decreased in amplitude and shifted, in line with the known spectral changes (Horecker and Kornberg, 1946). Cyanide virtually obliterates the peak of the Cotton effect at 278 mμ while that at 287 mμ remains unaltered, changes similar to those observed at alkaline pH. The trough of the *intrinsic* Cotton effect of the cyanide complex of cytochrome *c* is identical with that of the native protein, suggesting that binding of the CN ligand does not significantly alter the over-all conformation of protein. Similar changes in optical rotatory dispersion accompany the binding of azide to the oxidized protein although these are less marked than with cyanide (Figure 5).

Neither the apoprotein of cytochrome *c*, the isolated heme group prepared by silver salt treatment, nor the

[2] While this investigation was in progress, the results of similar studies were reported by Urry (1965) and Myer and Harbury (1965).

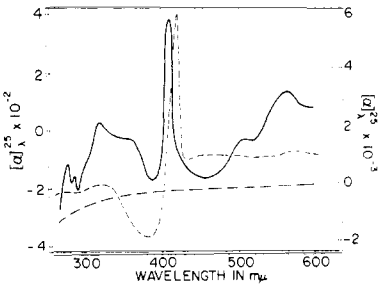

FIGURE 6: Optical rotatory dispersion of ferricytochrome *c* (——), the peptic heme peptide of cytochrome *c* (— —), and heme-free apocytochrome *c* (—·—·). Specific rotation, $[\alpha]_\lambda^{25}$, of the ferricytochrome, the left ordinate, and of the heme peptide and apocytochrome, the right ordinate, are plotted *vs.* wavelength. While the rotatory dispersion of the apocytochrome is essentially plain in this spectral range, the peptide exhibits heme-induced extrinsic Cotton effects very similar to those of the native protein. The pronounced Cotton effects arising from the aromatic side-chain chromophores of the native cytochrome are absent both in the peptide, which lacks aromatic amino acids, and in the apocytochrome, which lacks the iron–porphyrin moiety. Conditions: 0.3 to 3 mg/ml of protein or peptide in 0.1 M sodium phosphate, pH 6.8.

heme-peptide prepared by peptic digestion, which does not contain aromatic amino acids, exhibits anomalous rotatory dispersion between 250 and 300 mμ (Figure 6). Both the heme moiety, "hematohemin *c*," and the apocytochrome are levorotatory, but exhibit plain rotatory dispersions over the spectral range 240–600 mμ. In contrast, the 11 amino acid "heme peptide *c*" generates a pronounced Soret band Cotton effect similar to that of native ferricytochrome *c* (Figure 6) and an intrinsic Cotton effect suggesting the presence of considerable secondary structure (see also Ulmer, 1966).

Discussion

Cytochrome *c* has proven particularly attractive for the elucidation of many current problems in protein chemistry because of its small size, relative stability, the covalent binding of the prosthetic group to the protein, its prominent spectral, magnetic, and oxidation–reduction properties, and its ready availability and easy preparation from a wide variety of species for which the primary structure has now been determined. Recently, efforts relating the structure of the protein to its function have received increasing attention and, in this regard, spectropolarimetry appears to be of particular interest. Cytochrome *c* exhibits remarkable extrinsic Cotton effects which vary with the oxidation state of the heme iron atom. Moreover, optical rotatory dispersion suggests that oxidation–reduction alters the environment of aromatic amino acid residues (Ulmer, 1965).

The present investigation attempts to distinguish the aromatic groups involved in the spectropolarimetric changes by virtue of differences in their susceptibility to modification in the oxidized and reduced proteins and by examination of the rotatory dispersion of cytochrome derivatives and cytochromes from different species which vary in their content of aromatic amino acids. The results indicate that both tyrosyl and tryptophyl residues may be affected by the oxidation–reduction changes and that modification of these groups interferes with electron-transfer function of the protein.

In ferricytochrome, two tyrosines are acetylated with acetylimidazole and both of these groups are inaccessible to modification when the heme iron is reduced. Acetylation interferes with the oxidation–reduction function and renders the protein autoxidizable, but it does not alter the optical rotatory dispersion. Thus, the *tyrosyl residues which are modified by acetylation* may be essential for the functional integrity of the prosthetic group, but they do not appear to contribute to the aromatic side-chain Cotton effects. A functional role for tyrosine in cytochrome *c* was suggested previously on the basis of iodination (Ishikura *et al.*, 1959) and, recently, it has been shown that in guanidinated cytochrome *c* two tyrosines are acetylated by acetylimidazole, but only one appears to be associated with altered function (Cronin and Harbury, 1965).

There is some ambiguity at present concerning the position of the tyrosines in the three-dimensional arrangement of cytochrome *c*. Spectrophotometric titrations of the ionization of the phenolic hydroxyl groups have been interpreted as showing that all four (Stellwagon, 1964), only three (Rupley, 1964), or only two (Flatmark, 1964) of the tyrosyl residues of horse heart cytochrome *c* ionize at an abnormally high pH, and are, therefore, "buried." It seems pertinent, in this regard, that in most proteins studied to date, acetylimidazole modifies only "free" or "surface" tyrosines (Riordan *et al.*, 1965). By this criterion, ferricytochrome *c* exhibits two "free" and two "buried" tyrosines, while all four residues are buried in the ferrocytochrome. A contribution from buried tyrosines to generation of the aromatic side-chain Cotton effects, particularly the peak at 287 mμ, cannot be eliminated on the basis of the present observations (Simpson, 1966).

The location of the *O*-acetyltyrosyl residues in the primary sequence is, as yet, uncertain. It is of interest, however, that the sequence of amino acids from 70 to 80 is invariant in all species of cytochrome studied so far (Smith and Margoliash, 1964), suggesting that the precise structure of this segment of the chain is highly critical to function. Matsubara *et al.* (1965) have reported recently that methionine no. 80 can be carboxymethylated in ferricytochrome, with resultant loss of functional activity, but that this group is unmodified in the reduced protein. Methionine no. 65 is attacked in both oxidation states but without alteration in function. Analogous results have been reported by Tsai and Williams (1965). Moreover, trinitrophenylation of either lysine no. 72 or 73 appears to interfere with cytochrome function (Okunuki *et al.*, 1965). This suggests that the critical sequence of amino acids from the

70th to the 80th residues may participate directly in the oxidation-linked conformational change. On this basis it seems quite possible that tyrosine no. 74 is one of those modified by acetylimidazole in the oxidized, but protected in the reduced protein. Studies to test this postulate are in progress.

One tryptophyl residue in ferricytochrome *c* is titrated with *N*-bromosuccinimide with concomitant disappearance of the rotatory dispersion peak at 278 mμ. This chemical modification does not change the intrinsic Cotton effect, reflecting protein conformation, and there is but a very slight decrease in magnitude of the Soret band and its Cotton effect, reflecting the integrity of the heme moiety. Thus, it would appear that the single tryptophan of the horse heart protein, residue no. 59, generates the 278-mμ peak. In the reduced protein the accessibility to modification of this residue cannot readily be determined in the same manner as in ferricytochrome because of the oxidizing properties of the reagent.

The investigation of the physicochemical properties of cytochrome *c* from a wide variety of species, differing in their amino acid sequence, provides still another general approach to determining critical relationships of protein structure and function (Smith and Margoliash, 1964). Such genetically determined variations in sequence help to delineate the limits of functionally "acceptable" changes in primary structure and uniquely complement chemical modification experiments. Thus, the spectropolarimetric examination of *tuna* cytochrome *c* provides further support for the role of tryptophan in generating the anomalous dispersion: the magnitude of the Cotton effect peak at 278 mμ in the tuna cytochrome, having two tryptophyl residues, is just about double that in the horse heart cytochrome, having only one (Figure 3). In both species, this effect is obliterated on reduction of the protein, indicating an altered asymmetric environment of tryptophan consequent to the valence change of the heme iron atom.[3]

Other residues are known to be essential to the function of cytochrome *c*, although differences in their reactivity in the oxidized and reduced proteins apparently have not been discerned. Thus, it has been suggested on the basis of diazotization (Ishikura *et al.*, 1959) and photooxidation (Nakatani, 1960) that at least one histidyl residue is essential for function of cytochrome *c*. Histidine, of course, is also thought to occupy the fifth and perhaps the sixth coordination sites of the heme iron (Theorell and Åkesson, 1941; Ehrenberg and Theorell, 1955a,b; Schejter and George, 1965). Trinitrophenylation of critical lysyl residues also can result in functional change (Takemori *et al.*, 1962; Okunuki *et al.*, 1965); however, the mechanism of this alteration is uncertain since completely guanidinated horse heart cytochrome *c* does not appear to differ functionally in any important way from the native protein at neutral pH (Hettinger and Harbury, 1964). Lysine also has been considered a possible iron ligand (Margoliash *et al.*, 1959), but the metal-binding groups are currently the subject of intensive reexamination (Hettinger and Harbury, 1965; Myer and Harbury, 1965; Heller and Smith, 1965).

The optical rotatory dispersion of the peptic heme-peptide of cytochrome *c* is of special interest since this short segment of the peptide chain, containing only 11 amino acid residues, generates an extrinsic Cotton effect very similar to that of native ferricytochrome *c* (Figure 6). Reduction of the heme-peptide iron with dithionite strikingly alters this extrinsic effect quite analogous to the changes which occur upon reduction of the intact protein. Moreover, the heme-peptide gives rise to an intrinsic Cotton effect of the same general form, although of lesser magnitude, than that of the parent molecule, suggesting the presence of a well-organized secondary structure. Neither the heme-peptide, which contains no aromatic residues, nor "hematohemin *c*" exhibits anomalous dispersion in the wavelength region 250–300 mμ. Thus, a contribution to the Cotton effects in this spectral range from an iron–porphyrin group transition seems unlikely. Since the apocytochrome also lacks these Cotton effects but contains, of course, all the aromatic residues of the intact protein it would appear that the anomalous dispersion arises through an *interaction* of the heme with more distant sections of the peptide chain which contain aromatic amino acids. The oxidation-linked conformational change likely affects this interaction, thereby masking and unmasking aromatic amino acid residues and altering their asymmetric environments and chemical reactivity.

The exposure of horse heart ferricytochrome to heme-binding ligands such as cyanide and azide decreases or obliterates the Cotton effect peak at 278 mμ (Figure 5). Similar changes in this peak are observed in the pH range 9–9.5 (Figure 4), and both the iron ligands and alkaline pH also alter the heme-induced Cotton effects, in agreement with the results reported recently by Urry (1965) and Myer and Harbury (1965). Several observations are consistent with an ionization of cytochrome with a pH of *ca.* 9–9.5 including a drop in oxidation–reduction potential of the ferri–ferrocytochrome *c* couple (Paul, 1947; Theorell and Åkesson, 1941), a change in temperature dependence of the magnetic moment (George *et al.*, 1963), and a decreased rate of formation of ferricytochrome–ligand complexes about pH 9 (George and Tsou, 1952). Thus, it would appear that there is an important relationship between fundamental properties of the prosthetic group and ionization of ferricytochrome *c* in the pH range 9–9.5. The observed alterations in optical rotatory dispersion of the protein in this pH range, and in the presence of cyanide and azide, presumably reflect this sensitive relationship. Like oxidation–reduction, these conditions appear to affect the heme–protein interaction so as to alter the asymmetric

[3] Studies of the optical rotatory properties of cytochromes isolated from a large number of different species are currently in progress (D. D. Ulmer, B. L. Vallee, and E. Margoliash, unpublished data).

environments or chemical reactivity of the aromatic chromophores.[4]

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