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*OPTICAL ROTATORY DISPERSION OF A HEME PEPTIDE FROM CYTOCHROME C**

BY DAVID D. ULMER†

BIOPHYSICS RESEARCH LABORATORY, DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, AND DIVISION OF MEDICAL BIOLOGY, DEPARTMENT OF MEDICINE, PETER BENT BRIGHAM HOSPITAL, BOSTON

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The optical rotatory properties of horse heart cytochrome *c* have been reported recently,[1, 2] and have suggested new approaches for relating the structure of this molecule to its function.[3–6] Since the prosthetic group of cytochrome *c* is covalently bonded to the protein through cysteinyl side chains, heme peptides consisting of only about 10 per cent of the total residues present in the parent molecule may be obtained by means of proteolytic digestion.[7] It seemed of interest to examine the spectropolarimetric characteristics of such a peptide in terms of its possible structure and as a guide for the interpretation of the origin of the optically active transitions observed in the intact cytochrome. The observations here reported suggest that the limited segment of the peptide chain to which the heme is bonded largely determines the magnitude and general form of the extrinsic Cotton effects in the native protein.

Methods and Materials.—Horse heart cytochrome *c*, Type III, was obtained commercially (Sigma Chemical Co.), as was twice-crystallized pepsin (Worthington Biochemical Co.). Heme peptide *c* was prepared by peptic digestion,[8] and its composition determined by amino acid analysis.[9] Oxidation and reduction of the peptide were performed as described previously for cytochrome *c*.[1] Optical rotatory

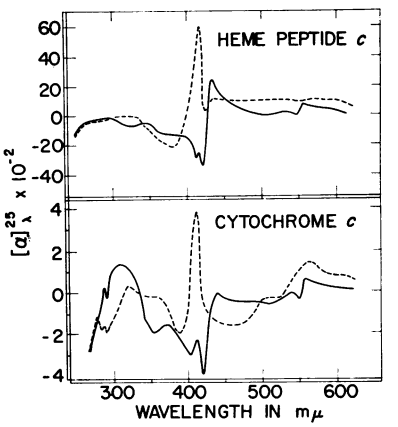

FIG. 1.—Extrinsic Cotton effects of oxidized (- - -) and reduced (——) heme peptide *c* and cytochrome *c*. Specific rotation at 25°, $[\alpha]^{25}_{\lambda}$, is plotted against wavelength. Both in the oxidized peptide and in ferricytochrome, the Soret band, near 400 mμ, generates a marked positive Cotton effect, while in the reduced peptide and ferrocytochrome, the principal deflection of this effect is negative. In addition, the α-absorption band of the reduced peptide, at 550 mμ, gives rise to a sharply defined positive Cotton effect virtually identical to that observed in the reduced cytochrome. Based on specific rotation, the amplitude of these Cotton effects is more than ten times larger in the peptide than in the protein. While the cytochrome exhibits Cotton effects between 270 and 300 mμ, associated with the chromophores of the aromatic amino acids, the rotatory dispersion of the heme peptide, which has no aromatic residues, is plain in this spectral range. Conditions: 0.4–1 mg per ml peptide and 2–3 mg per ml cytochrome in 0.1 *M* sodium phosphate, pH 6.8. (Data for cytochrome *c* are from ref. 1.)

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- to 10-mm pathlength were employed. Heme peptide concentrations varied from 0.1 to 1 mg per ml. The slit width of the instrument was programed to yield maximal and constant light intensities at all wavelengths. In areas of high absorbance, absolute values for the specific rotation were confirmed at several protein concentrations or pathlengths, eliminating the possibility of spurious Cotton effects.[10] Specific rotations were calculated on the basis of the dry weight of the heme peptide.

Results.—The heme peptide of cytochrome *c* retains much of the spectral detail observed in the native protein, including the characteristic changes in hemochrome absorption bands upon oxidation-reduction.[7, 8] Similarly, many *spectropolarimetric* properties of the native cytochrome are preserved in the peptide. The optical rotatory dispersion of oxidized and reduced heme peptide *c* is compared with that of oxidized and reduced cytochrome *c* in Figure 1. Notably, in the heme peptide, the principal deflections arising from the optically active absorption bands of the iron-porphyrin moiety assume the same general form and direction as those observed in cytochrome *c*. A marked positive extrinsic Cotton effect[11] is associated with the Soret band of the ferripeptide, at 400 mμ,[12] while in the reduced peptide the principal deflection of this Cotton effect is negative. In addition, the characteristic α-absorption band of the ferropeptide, at 550 mμ, generates a remarkably sharp positive Cotton effect very similar to that in the reduced protein itself. The ferripeptide exhibits less rotatory detail than the intact protein in this spectral range, although the possibility of anomalous dispersion at still longer wavelengths cannot be eliminated. On the basis of *specific rotation*, the extrinsic Cotton effects of the peptide are more than ten times greater in amplitude than those of the intact protein; on a *molar* basis, however, these rotations are of the same order of magnitude, the peptide having only 11 of the 104 residues found in horse heart cytochrome *c*.

The similarities in rotatory dispersion of cytochrome *c* and its heme peptide are not limited to optically active heme transitions. Figure 2 compares the intrinsic Cotton effect of ferricytochrome *c* with that of the ferripeptide. The peak and

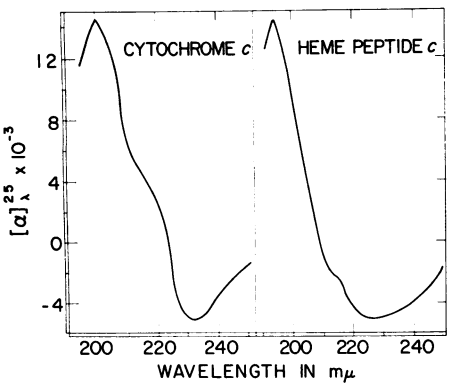

FIG. 2.—Intrinsic Cotton effects of heme peptide *c* and cytochrome *c*. Specific rotation at 25°, $[\alpha]_\lambda^{25}$, is plotted against wavelength. The Cotton effect of the cytochrome is characteristic of a helical protein with a trough at 233 mμ, a peak at 200 mμ, and a crossover to positive rotations at 223 mμ. In the peptide, all these values lie at shorter wavelengths. Conditions: 0.1–0.6 mg per ml peptide and 0.3–1 mg per ml cytochrome in 0.1 *M* sodium phosphate, pH 6.8. (Data for the cytochrome are from ref. 1.)

trough of the peptide Cotton effect, at 195 and 225 mμ, respectively, lie at somewhat shorter wavelengths than in the intact protein, and the dispersion of the peptide displays an unusually marked inflection at 212 mμ; however, the magnitude of specific rotation and general form of this intrinsic effect are the same as in native cytochrome.[18]

In contrast, in the spectral range 250–300 mμ, the optical rotatory dispersion of the heme peptide is *dissimilar* to that of native cytochrome. In the intact protein, the rotatory dispersion in this region is strongly influenced by optically active absorption bands of the aromatic amino acids.[1] The heme peptide, however, does not contain aromatic amino acids. Its rotatory dispersion is flat in this spectral range.

Discussion.—The heme peptide of cytochrome *c* consists of amino acid residues 11 to 21 in the sequence Val-GluNH_2-Lys-Cys-Ala-GluNH_2-Cys-His-Thr-Val-Glu. The heme is covalently linked through thio-ether bonds to cysteines nos. 14 and 17 and it is the stability of this structure which permits ready isolation of the peptide by means of proteolytic digestion.[14] The physical chemical properties of the peptide, its conformation, and the orientation of heme in relation to the protein chain have long been of interest, particularly in regard to the possible ligands of the fifth and sixth coordination sites of the iron atom. Studies of molecular models indicate that if the peptide is α-helical, either the imidazole group of histidine no. 18 or the ε-amino group of lysine no. 13 can be made to coordinate with the heme iron, but not both groups simultaneously.[15, 16] It was postulated early, therefore, that histidine no. 18 forms one coordination site for the heme iron and that the other site is outside the peptide segment, and likely either histidine no. 26 or no. 33. It has been pointed out, however, that both the imidazole of histidine no. 18 and ε-amino group of lysine no. 13 can be made to coordinate with the central heme iron atom on opposite sides of the heme plane if the peptide chain is fully extended, rather than formed into an α helix.[8] The choice between these ligand pairs, and perhaps others,[17–20] remains uncertain but the spectropolarimetric studies of the peptide are of special interest in this regard.

The optical rotatory dispersion of heme peptide *c*, like that of cytochrome *c*, reflects contributions both from the heme and from the peptide backbone. At longer wavelengths, rotation is largely controlled by the heme, which generates extrinsic Cotton effects; at shorter wavelengths, rotatory dispersion is dominated by an intrinsic Cotton effect thought to arise principally from conformational orientation of peptide amide bonds. *Both* types of Cotton effect provide a basis for comparison of heme peptide *c* with its parent molecule.

The extrinsic Cotton effects of the heme peptide, and their alterations upon oxidoreduction, closely resemble in appearance those of intact cytochrome *c* (Fig. 1). This indicates that the steric orientation of the heme in relation to the peptide chain must be very similar in both instances. Such similarity would seem most unlikely unless the conformation of the heme peptide sequence, as it exists in the native cytochrome, is preserved virtually unchanged in the isolated peptide.

Comparison of the intrinsic Cotton effects of the peptide and intact protein also reveals remarkable similarities (Fig. 2), particularly when considering the disparities in size and composition of the molecules from which the effects originate. Native cytochrome *c* exhibits an intrinsic Cotton effect typical of a helical protein; the estimated helix content, on the basis of rotatory dispersion, is about 30 per cent.[2, 21] Such estimates are quite problematical, however, due to the uncertain contribution of the heme to the total rotation in this spectral range. A rotational contribution from heme is potentially of even greater significance for the much smaller peptide; conceivably, this could account for the fact that the peak, trough, and inflection of the intrinsic Cotton effect of the peptide vary slightly from those in native cytochrome. In view of this uncertainty, the conformation of the heme peptide cannot be discerned unambiguously at present; the data do not exclude the possibility that the conformation of the isolated peptide is unchanged from that of the heme peptide segment in the intact cytochrome. Circular dichroism measurements in this spectral range will doubtless help in further resolution of the problem.

In the spectral region of the *intrinsic* Cotton effects, the heme peptide accounts for about one tenth of the total rotation of the protein, on a molar basis. This contribution is in direct proportion to the size of the peptide and is, therefore, as expected. At longer wavelengths, however, the peptide accounts for nearly the entire rotation of the protein, on a molar basis. The magnitudes of the extrinsic Cotton effects of the peptide are actually ten times larger than would be expected from its size. Hence, this indicates that the asymmetric chromophores indigenous to the heme peptide segment of cytochrome *c* have sufficient rotatory power to generate the extrinsic Cotton effects observed in the entire protein.

Native cytochrome *c* exhibits striking Cotton effects in the region of absorption of its aromatic amino acids.[1, 2] These effects, like those at longer wavelengths due to heme, are altered upon oxidoreduction of the iron atom, implying a possible interdependence. However, in heme peptide *c* which contains no aromatic residues, the rotatory dispersion between 250 and 300 mμ is essentially plain, in either oxidation state of iron (Fig. 1). Thus cytochrome heme transitions, of themselves, do not appear to contribute significantly to anomalous dispersion in the region of the aromatic side chain chromophores. By the same token, it is clear that the presence of aromatic residues is not prerequisite to the occurrence of characteristic, heme-induced Cotton effects.

Like heme peptide *c*, neither the apoprotein nor hematohemin[22, 23] from cytochrome *c* exhibit anomalous dispersion between 250 and 300 mμ.[6] This suggests that the aromatic Cotton effects in the native cytochrome, and their changes with oxidoreduction, must arise from *interaction* of the heme group with more distant portions of the peptide chain. Oxidoreduction appears to induce a conformational change in the protein affecting this interaction and thereby masking and un-

masking aromatic amino acid residues and altering their asymmetric environments.[24]

Finally, the present results are directly pertinent to interpretation of the origin of extrinsic Cotton effects of proteins. These effects have now been studied in many heme proteins, other metalloproteins, protein-dye complexes, and the complexes of enzymes with coenzymes, substrates, and inhibitors.[26] Investigation of the binding of dyes or hemin to synthetic polypeptides indicated early that the helical conformation is necessary to produce such Cotton effects.[21, 27, 28] However, the nature of binding to the helix, the relationship of the Cotton effect to over-all protein conformation, and the length of protein chain required to induce an extrinsic effect could not be discerned. Studies of metalloproteins first suggested that very limited areas of the protein, e.g., metal-protein ligand binding sites, might generate extrinsic Cotton effects.[29, 30] The isolation and study of a limited segment which retained the Cotton effect of the native protein was not possible in any of these systems. Heme peptide *c* represents such a discrete segment of protein structure. Thus, examination of its spectropolarimetric properties appears to have general implications. It seems clear that the 11-amino-acid peptide generates the rotatory power and, in a large measure, controls the characteristics of the extrinsic Cotton effects exhibited by the native cytochrome.

Since the covalently bonded heme undoubtedly stabilizes the conformation of the peptide, it may be that an even shorter sequence could exhibit the characteristic cytochrome Cotton effects.[32] Demonstration of the critical length of chain required will be of interest. On the other hand, in heme proteins where the prosthetic group is not covalently bonded, it is likely that larger portions of the molecule are required to preserve the orientation of the heme. In either instance, spectropolarimetry appears to provide a distinctive approach to the study of *limited areas* of protein structure on the basis of their asymmetry.

Summary.—The oxidized and reduced peptic heme peptide of cytochrome *c* generates *extrinsic* Cotton effects which closely resemble those of the intact protein. The *intrinsic* Cotton effect of the peptide is also similar in appearance to that of native cytochrome, but differs slightly in spectral position. The Cotton effects which arise from aromatic amino acid chromophores in the intact protein are absent in the peptide, which contains no aromatic residues.

The data suggest that both the magnitude and the general form of the extrinsic Cotton effects of cytochrome *c* are determined by a small portion of the molecule—the heme peptide segment. The conformation of this segment, as it exists in native cytochrome, is apparently largely preserved in the isolated peptide.

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[11] The terminology employed is as suggested by Blout:[31] *intrinsic* Cotton effects are those which arise from optically active absorption bands of the polypeptide backbone, or the side chains of the common amino acids. *Extrinsic* Cotton effects are generated by a local chromophoric site on the protein or by a chromophoric molecule, such as a coenzyme, interacting with an asymmetric site of the protein.[26]

[12] The heme peptide has a marked tendency to aggregate,[8, 16] but at neutral pH, in the presence of imidazole, or at alkaline pH it depolymerizes and the Soret band shifts to 408 mμ, typical of the Soret band in native ferricytochrome *c*. Imidazole does not appear to affect the *spectropolarimetric* properties of the heme peptide significantly. At pH 10.5, however, the peptide undergoes changes in rotatory dispersion analogous to those of the native ferricytochrome at alkaline pH.[4-6]

[13] Because of the high absorption of reagents such as dithionite, necessary to maintain the reduced state, it has not yet proved possible to establish with certainty the rotatory dispersion of the reduced peptide at these short wavelengths. Observations to date, however, suggest that the position of the trough in the ferripeptide may be at 233 mμ, indicating the presence of an α helix. If confirmed, a conformational change in the peptide may underlie the reported differences in the intrinsic Cotton effect of ferri- and ferrocytochromes *c*.[1, 2]

[14] Hematohemin *c* isolated from cytochrome *c* by splitting with silver salts[22] is optically active.[23] In contrast to the heme peptide, however, hematohemin *c* exhibits a plain rotatory dispersion curve without discernible Cotton effects in the presently accessible spectral range.[6]

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