# Optical Rotatory Dispersion of Carboxymethylated Cytochrome *c**

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ABSTRACT: The optical rotatory dispersion spectra of mono- and dicarboxymethylated horse heart cytochrome *c* were examined in the wavelength range 220–650 mμ. The optical rotatory dispersion spectrum of the monocarboxymethyl derivative is very similar to that of the native horse heart protein in both oxidized and reduced oxidation states.

The optical rotatory dispersion spectrum of the dicarboxymethyl derivative differs from that of the native cytochrome in the reduced form and in the oxidized form at pH 4, but at pH 7 the oxidized form shows many similarities to the native cytochrome.

Carboxymethylation of horse, human, and beef heart cytochrome *c* under specified conditions results in a derivative which has no activity in the mitochondrial electron transport system. The modification of the methionine residue in position 80 in the amino acid sequence causes this loss of activity and also the changes seen in the absorption spectrum (Tsai and Williams, 1965a,b; Ando *et al.*, 1966; Schejter and George, 1965). Schejter and George (1965) showed that this derivative gives normal hemochrome- and hemichrome-type spectra above pH 6, although the extinction coefficient at 550 mμ in the reduced form does not reach that of cytochrome *c* until pH 10.5. Moreover, this derivative does not possess the 695-mμ absorption band which is characteristic of native ferricytochrome *c* at pH 7. At pH 4 the derivative has a metmyoglobin type of spectrum.

Since optical rotatory dispersion (ORD) studies of cytochrome *c* show that optical rotation in the 270–700-mμ range reflects changes in the environment of the heme (Ulmer, 1965; Urry, 1965; Myer and Harbury, 1965, 1966; Mirsky and George, 1966), we thought that by comparing the ORD spectra of carboxymethylated cytochrome *c* at pH 4 and 7 with the ORD spectra of cytochrome *c* we could obtain information about some of the structural changes occurring around the heme group when methionine 80 is carboxymethylated. Our results show that at pH 4 the ORD spectrum of the dicarboxymethyl derivative in the 260–650-mμ range is quite different from that of the native cytochrome. At pH 7 the ORD spectrum of the ferri derivative resembles that of cytochrome *c* in the pH range 2.8–3.5 and 8.5–10, where it is slightly modified but can still function in the mitochondrial system. The ORD of the ferro form of the dicarboxymethyl derivative shows substantial differences from that of ferrocytochrome *c*.

## Methods and Materials

Carboxymethylated cytochrome *c* was made by the method of Schejter and George (1965) from purified horse heart cytochrome made by the procedure of Margoliash and Walasek (1967). Tris buffer was substituted for phosphate buffer since it promotes carboxymethylation. Derivatives were made with and without cyanide in the solution, so that di- and monocarboxymethyl derivatives were obtained (A. Schejter, private communication).

ORD spectra were run on a Jasco ORD/UV 5 recording spectropolarimeter using 5- and 10-mm cells at a room temperature of 25°. pH was measured with a Beckman GS pH meter and absorption spectra were run on Cary 14 and Zeiss PMQ II spectrophotometers.

Concentrations of solutions were measured using $\epsilon_{mm}$ 11.2 at 530 mμ for oxidized cytochrome *c* and its derivatives at pH 7 (Margoliash and Frohwirt, 1959). This value may not be absolutely correct for derivatives but provides a reasonable basis for comparison.

## Results

Figures 1 and 2 show the ORD spectra of cytochrome *c* carboxymethylated in the presence and absence of cyanide. Rotations are plotted as $[\alpha]$, the specific rotation, and are uncorrected for the refractive index of the solvent.

Cytochrome *c* which is carboxymethylated in the absence of cyanide is modified only at methionine residue 65 (Tsai and Williams, 1965b; A. Schejter, private communication). The ORD of the oxidized derivative is very similar to the ORD spectrum of ferricytochrome *c* at about pH 3 (*e.g.*, Mirsky and George, 1966). The trough at 590–600 mμ is still

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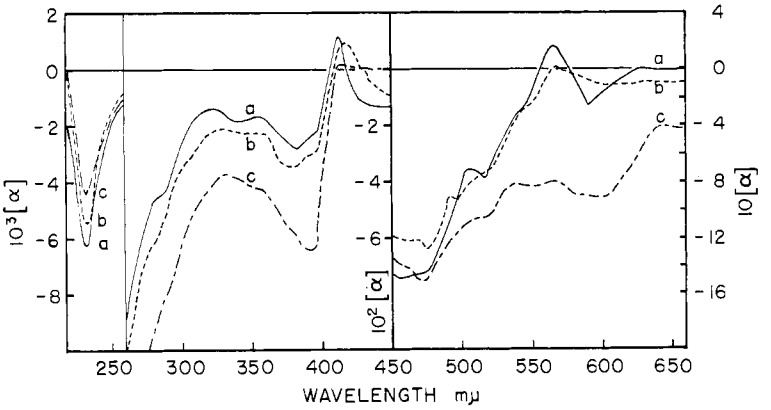


FIGURE 1: ORD of mono- and dicarboxymethyl ferricytochrome *c*. (a) Monocarboxymethyl ferricytochrome *c*, pH 7, phosphate buffer. (b) Dicarboxymethyl ferricytochrome *c*, pH 7, phosphate buffer. (c) Dicarboxymethyl ferricytochrome *c*, pH 4.2, phosphate plus HCl.

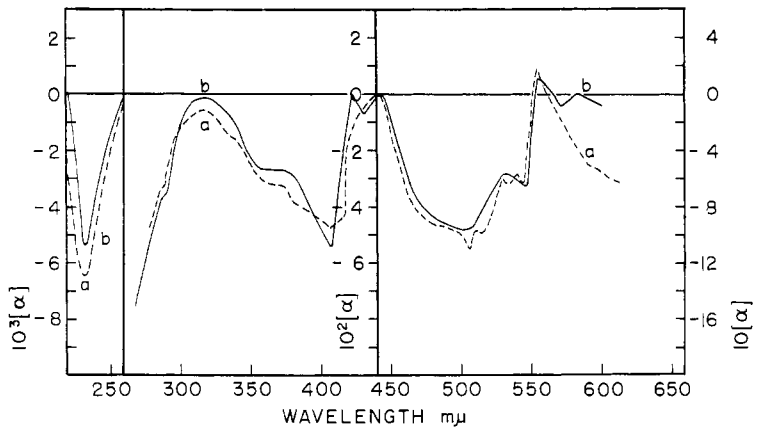


FIGURE 2: ORD of mono- and dicarboxymethyl ferrocytochrome *c*. (a) Monocarboxymethyl ferrocytochrome *c*, pH 7, phosphate buffer, minimal amount of sodium dithionite or borohydride. (b) Dicarboxymethyl ferrocytochrome *c*, pH 7, phosphate buffer, minimal amount of sodium dithionite.

present, but the sharp tryptophan and tyrosine effects at 278 and 287 mμ are present only as a shoulder at about 280 mμ. Rotation in the 300–400-mμ region is considerably more negative than ferricytochrome *c* at pH 7 but similar to that in the native cytochrome *c* at both more acid and more alkaline pH.

Samples which are carboxymethylated in the presence of cyanide have two modified methionine residues at positions 65 and 80, and it is the carboxymethylation at position 80 which leads to the loss of activity in the electron transport system and the metmyoglobin-type spectrum at pH 4. At pH 7 this derivative forms hemichrome- and hemochrome-type spectra in the oxidized and reduced forms. Figure 1 shows that in the oxidized form at pH 7 the ORD spectrum of the dicarboxymethyl form is rather similar to that of the monocarboxymethyl derivative although the Cotton effects are less well defined. At pH 4 the ORD spectrum is entirely changed. There is a Cotton effect centered around 620 mμ, where there is a strong absorption band, and structure in the 650–450-mμ region is poorly defined. In the Soret region there is one large Cotton effect centered at 402 mμ similar to those seen in both ferrihemoglobin and ferrimyoglobin (Beychok and Blout, 1961), in ferricytochrome *c* cyanide (Myer and Harbury, 1965), and at pH values lower than 2 (Mirsky and George, 1966). The value of the 233-mμ trough in the monocarboxymethyl derivative is the same as in the native cytochrome, and in the dicarboxymethyl derivative it is less both at pH 7 and 4, particularly at the latter, indicating that the carboxymethylation of methionine 80 does cause some changes in over-all protein conformation as well as in the neighborhood of the heme group.

Figure 2 shows the reduced cytochrome derivatives at pH 7. The dicarboxymethyl derivative is so autoxidizable at pH 4 that we could not get a reduced ORD spectrum. However, at pH 7 the monocarboxy-

methyl derivative shows the typical ferrocytochrome *c* spectrum except in the 300–350-mμ region where the rotation is less positive than in the native cytochrome. In the dicarboxymethyl derivative there are several changes. Rotation in the 600–560-mμ region is more positive, and in the Soret region in addition to the peak at 440 mμ a sharp peak appears at 421 mμ. This spectrum is very similar to that obtained with the undecahemepeptide of cytochrome *c* at pH >7 and also to the spectrum of reduced yeast iso-1 cytochrome *c* at pH 10 (Ulmer, 1966; R. Mirsky and P. George, unpublished results).

The reduced monocarboxymethyl derivative has the same value for the 233-mμ trough as native cytochrome *c*. No difference could be detected between the values of the 233-mμ trough for the oxidized and reduced forms of the dicarboxymethyl derivative.

## Discussion

Experiments on the binding of methionine to a heme octapeptide from cytochrome *c* have shown that a sulfur bonded to heme iron can form hemochrome- and hemichrome-type spectra (Harbury *et al.*, 1965). This, in conjunction with the work of Tsai and Williams (1965a,b) and Ando *et al.* (1966) on carboxymethylation of methionine residues in cytochrome *c*, led to the suggestion that methionine 80 provides the sixth ligand to the heme iron in cytochrome *c*.

Previous ORD experiments on cytochrome *c* have shown that changes in the ORD in the 260–700-mμ range reflect both small and large changes in the environment of the heme. Small changes are observed on changing the pH within the range 2.5–12, on raising the temperature to 50° (Urry, 1965), and on dimerization (Mirsky and George, 1966), and larger changes occur at pH extremes, on replacing the sixth ligand to the heme by cyanide, azide, or other anions (Myer and Harbury, 1965), on denaturing with guanidine, and on making the heme undecapeptide and octapeptide (Ulmer, 1966; Myer and Harbury, 1966).

The main product of the carboxymethylation of methionine with iodoacetate, and presumably with bromoacetate as well, is the carboxymethylsulfonium salt of methionine. This salt is destroyed by acid hydrolysis and is, therefore, not detectable in amino acid analyses of proteins (Gundlach *et al.*, 1959). The existence of a bond between the heme iron and the sulfur in this compound is possible since the sulfur atom still possesses a lone pair of electrons for coordination with the iron. The similarities between the ORD spectra of native ferricytochrome *c*, monocarboxymethyl ferricytochrome *c*, and dicarboxymethyl ferricytochrome *c* are such that it seems improbable that ligand replacement has occurred in the dicarboxymethyl derivative. It is more likely that steric hindrance due to carboxymethylation of methionine 80 causes weakening of the normal heme iron–ligand bond which is either the methionine or positioned very near it in space. This could also explain the fact that the protein ligand is replaced by a water molecule (or some similar small ion such as chloride) below pH 5 in the dicarboxymethyl derivative while such a change does not occur until pH 2.2 in the native cytochrome. The ORD spectra of these two species are fairly similar and both show a decrease in value of the 233-mμ trough from the values at pH 7.

The ORD of the reduced dicarboxymethyl derivative at pH 7 shows enough differences from native ferrocytochrome *c* to suggest the possibility that in the reduced form the normal ligand is from methionine and that this is replaced by another ligand in the dicarboxymethyl derivative, especially since the ORD spectrum of the derivative is so similar to that of the undecaheme peptide at alkaline pH (R. Mirsky and P. George, unpublished results) in which there are no methionine residues. However, the possibility exists that changes in the ORD spectrum of the reduced form are caused not by a change in ligand, but by a change in conformation around the ligand due to carboxymethylation of methionine 80, as suggested for the dicarboxymethyl ferricytochrome *c*. This change might have a larger effect on the ORD of the reduced cytochrome because the Fe–S bond is stronger in the reduced form (Harbury *et al.*, 1965) and the conformation around the heme seems to be much tighter.

The amino acid sequence between residues 70 and 80 is invariant in all of the cytochromes *c* so far examined (Margoliash and Schejter, 1966); this suggests that this segment of the protein has a highly specific function. This function is disrupted by carboxymethylation of methionine 80, but from the ORD spectra it seems that in the modified ferricytochrome *c* the same ligand is attached to the iron in the sixth position as in the native cytochrome *c*. In ferrocytochrome *c* there is some evidence for a change of ligands on carboxymethylation of the methionine.

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## CORRECTION

In the paper "The Amphoteric Behavior of Bovine Plasma Albumin. Evidence for Masked Carboxylate Groups in the Native Protein," by Kamala Kant Vijai and Joseph F. Foster, Volume 6, April 1967, page 1152, there is an unfortunate error in the labeling of the abscissa of Figure 1. There should be a constant interval of 0.5 pH unit between the reference marks with the first mark, somewhat in from the left-hand side of the abscissa, being pH 3.0 and the extreme right-hand limit of the axis being pH 6.0. The mark at pH 5.5 is partially obscured by the experimental points. In addition the labeling of the ordinate, which is partially blurred in some copies, should read "$\bar{h}_{\text{total}}$ or $\Delta\text{OD} \times 10^3$."