CARBOXYMETHYLATION OF THE HISTIDYL RESIDUES OF HORSE HEART CYTOCHROME C

Earle Stellwagen

Department of Biochemistry, University of Iowa, Iowa City, Iowa

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The characteristic hemochromogen and parahematin spectra exhibited by ferro- and ferricytochrome c, respectively, (Keilin and Hartree, 1937) indicate that the heme iron of cytochrome c is bonded to two protein nitrogen atoms in the fifth and sixth coordination positions. From extensive spectrophotometric and magnetometric studies Theorell (1941) proposed that the protein ligands were the imidazole nitrogens of two histidyl residues. This proposal was strengthened by the finding that the visible absorption spectrum of the tryptic hemopeptide of cytochrome c can be made nearly identical with that of the native protein by addition of benzoyl histidine (Tuppy and Bodo, 1954).

Imidazole nitrogens of exposed histidyl residues can be carboxymethylated rather specifically by bromoacetate at neutral pH. In the case of myoglobin, the histidyl residue coordinated with the heme iron was not carboxymethylated under these conditions (Banaszak et al., 1963). Therefore, if two of the three histidyl residues of cytochrome c are coordinated with the heme iron, only one histidyl residue should be susceptible to chemical modification. The experiments described here demonstrate that only one histidyl residue in the native protein can be carboxymethylated by bromoacetate at pH 6.9. The two remaining residues can be carboxymethylated in urea producing a marked change in the visible absorption spectrum of the heme protein.

Experimental Procedures. Sigma type III horse heart cytochrome c, lot 114B-7235, was dissolved in 0.2 M bromoacetate and 1.0 M phosphate buffer, pH 6.9, to give a final concentration of 10 mg/ml and incubated at room

temperature. Aliquots of the reaction mixture were removed at 24 hour intervals, dialyzed overnight against distilled water at 2°, and hydrolyzed in 6 N HCl at 110° for 24 hours. The amino acid composition of the hydrolyzate was then determined by the method of Spackman et al. (1958) using a Spinco Model 120C amino acid analyzer. The number of histidyl residues per molecule of protein was calculated from the ratio of μmoles of histidine to arginine, assuming the arginine content represented two residues per molecule of cytochrome c (Margoliash et al., 1962). The pH 3.28 citrate buffer was adjusted to pH 3.21 with HCl to separate 1- and 3-carboxymethylhistidine from the adjacent amino acids in the elution sequence. The elution positions of 1-carboxymethylhistidine, 3-carboxymethylhistidine and 1,3-dicarboxymethylhistidine were determined using a mixture of these three forms prepared from α-N-acetylhistidine as described by Crestfield et al. (1963). The number of carboxymethylhistidyl residues per protein molecule was calculated from the ratio of μmoles of carboxymethylhistidine to valine, assuming that carboxymethylhistidine had the same ninhydrin color value as alanine and that the valine content represented three residues per molecule of cytochrome c.

Carboxymethylation of ferrocytochrome c was done in 0.1 M ascorbate in an evacuated Thunberg tube. The cyanide derivative of ferricytochrome c was obtained by addition of 0.1 M NaCN to the reaction mixture. Spectral characteristics of both ferrocytochrome c and the cyanide derivative of ferricytochrome c were constant through the extended reaction with bromoacetate.

Absorption spectra were measured at room temperature with a Cary Model 14 recording spectrophotometer. Protein concentrations were calculated from the absorption at selected wavelengths in the visible region, using the extinction coefficients of Margoliash and Frohwirt (1959).

Results and Discussion. The decrease in the number of histidyl residues of cytochrome c upon continued exposure to bromoacetate is shown in Figure 1. After three days, both ferri- and ferrocytochrome c underwent a maximal loss of one histidyl residue. This loss was complemented by the appearance of

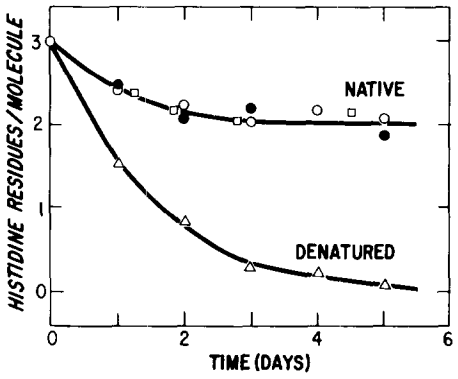

Figure 1. Kinetics of the reaction of bromoacetate with histidyl residues. ○, ferricytochrome c; ●, ferrocytochrome c in 0.1 M ascorbate; □, ferricytochrome c in 0.1 M NaCN; △, ferricytochrome c in 8 M urea. All reactions were done in 0.2 M bromoacetate-1.0 M phosphate buffer, pH 6.9, at room temperature.

1-carboxymethyl- and 1,3-dicarboxymethylhistidine, the former predominating. Carboxymethylation of the histidyl residue caused only minor changes in the visible absorption spectrum of the protein, as shown in Table I. It was

TABLE I

COMPARISON OF SPECTRAL CHARACTERISTICS

Cytochrome c	Oxidized		Reduced		
	Soret max	$\frac{A_{Soret}}{A_{528}}$	Soret max	$\frac{A_{Soret}}{A_{550}}$	$\frac{A_{550}}{A_{520}}$
	(mμ)		(mμ)		
Native	410	9.46	416	4.66	1.74
Exposed to 0.2 M BrAA, 3 days	407	11.0	416	5.16	1.74
Exposed to 8 M urea	407	10.9	416	6.18	1.68
Exposed to 0.2 M BrAA in 8 M urea, 5 days	390	---	415	----	----
Above, in 0.1 M imidazole	403	14.0	412	7.12	1.60
Native, in 0.1 M imidazole	408	10.4	415	5.14	1.72

All solutions contained 0.1 M phosphate buffer, pH 7.0. Solutions of cytochrome c exposed to bromoacetate (BrAA), urea or both were dialyzed overnight against 0.1 M phosphate buffer, pH 7.0, prior to spectrophotometric measurements.

therefore concluded that this histidyl residue lies on the surface of the protein and is not intimately associated with the heme moiety.

If the two remaining histidyl residues serve as ligands for the heme iron, formation of the cyanide derivative of ferricytochrome c should expose an additional histidyl residue to bromoacetate (George and Lyster, 1958). As shown in Figure 1, no additional histidine became reactive under these conditions, suggesting that the substituted ligand was not a histidyl residue. Alternatively, it is possible that substitution of a histidyl-heme coordinate-covalent bond by cyanide caused a conformational change which shifted the liberated histidyl residue to a sterically unreactive position.

In the presence of 8 M urea, all three histidyl residues were converted to the 1,3-dicarboxymethyl derivatives by bromoacetate. No significant changes in the other amino acids were observed under these reaction conditions. Carboxymethylation of the three histidyl residues produced marked spectral changes, as shown in Table I and Figure 2.

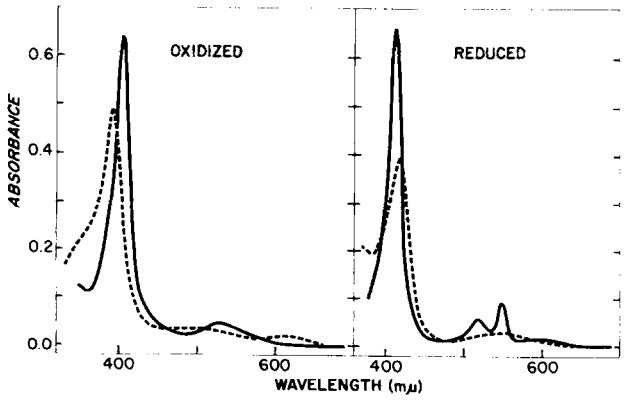

Figure 2. Visible absorption spectra of cytochrome c containing three carboxymethylated histidyl residues. The protein was reacted with 0.2 M bromoacetate in 8 M urea and 1.0 M phosphate buffer, pH 6.9, for five days at room temperature and then dialyzed overnight against 0.1 M phosphate buffer, pH 7.0, at 2°C. — — — —, protein diluted in 0.1 M phosphate buffer, pH 7.0; ————, protein diluted in 0.1 M phosphate buffer-0.1 M imidazole, pH 7.0. Solid sodium dithionite was added to reduce the heme iron.

These changes cannot be attributed solely to exposure of cytochrome c to 8 M urea since the spectral characteristics of the native protein were largely regained after removal of the urea by dialysis, as shown in Table I. Addition of imidazole to the protein containing three carboxymethylhistidine residues sharpened the absorption spectrum of the protein substantially (Figure 2). It is not surprising that the spectral characteristics of the native protein are not completely regained by the addition of imidazole, since carboxymethylation of a ligand would alter its steric and electrostatic properties, and probably prevent the relocation of the heme in the crevice structure.

The reactivity of the histidyl residues of cytochrome c with bromoacetate and the resultant spectral changes are consistent with the view that at least one of the protein ligands is an imidazole nitrogen. A similar conclusion has been reached by Horinishi et al. (1965) using the reagent diazonium-1-H-tetrazole.

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