Haem Ligands of the Ferricytochrome *c* of *Ustilago sphaerogena*

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The mammalian-type cytochrome *c* of the basidiomycete *Ustilago sphaerogena* contains in a single polypeptide chain of 107 residues, two histidine residues located at positions 18 and 33, and one methionine residue situated at position 80 (Bitar *et al.*, 1972). The reaction of *Ustilago* ferricytochrome *c* with bromoacetate at neutral pH resulted in the modification of histidine-33, but not of histidine-18 or of the invariant methionine residue. The activities of *Ustilago* cytochrome *c* with mitochondrial cytochrome *c* oxidase and with NADH-cytochrome *c* reductase were unaltered by the modification. The equilibrium constants for the formation of low-spin complexes of the ferrihaem octapeptide of horse cytochrome *c* (residues 14-21, including the haem bound covalently to cysteines 14 and 17) with imidazole, *N*²-acetylhistidine and monocarboxymethyl derivatives of *N*²-acetylhistidine were determined spectrophotometrically. Alkylation of the imidazole side-chain group of *N*²-acetylhistidine resulted in a marked decrease in its ability to form low-spin ferrihaem complexes. These results indicate that in *Ustilago* ferricytochrome *c* in solution histidine-33 is not involved in the central co-ordination complex. Since side-chain groups of residues other than histidine and methionine do not appear to be involved in the central complexes of other mammalian-type cytochromes *c* (Hettinger & Harbury, 1964, 1965; Myer & Harbury, 1965) it is likely that in *Ustilago* ferricytochrome *c* in solution at neutral pH, the side-chain groups of histidine-18 and methionine-80 are involved in the central co-ordination complex. The latter is stable over the pH range 2.6-8.4.

The X-ray structure analyses of horse, tuna and bonito cytochromes *c* (Dickerson *et al.*, 1971; Takano *et al.*, 1972) have demonstrated conclusively that positions 5 and 6 in the central, low-spin co-ordination complex in the crystals of both the oxidized and reduced forms of the proteins are occupied by the imidazole and the thioether side-chain groups of residues 18 and 80 respectively. It is unclear at present whether the central complex of eukaryotic cytochrome *c* remains unaltered in other states of the protein, including the solution state, and whether it remains invariant in even the most primitive eukaryotes. Some recent experimental findings suggest that caution should be exercised in extrapolating the results obtained in one phase to another. The magnetic susceptibilities of dried or freeze-dried horse cytochrome *c* in either the oxidized or the reduced form were found to be greater than of the proteins in solution (Lumry *et al.*, 1962). Electron-paramagnetic-resonance spectra of horse ferricytochrome *c* at 77°K exhibited, in addition to the lines corresponding to the low-spin components, a strong absorption line at *g* = 6, characteristic of high-spin ferrihaem proteins (Morton & Bohan,

1971). Kinetic studies of the dissolution of freeze-dried horse cytochrome *c* (Aviram & Schejter, 1972) have demonstrated that although a high-spin form of cytochrome *c* disappears on dissolution, the resulting low-spin form does not possess the 695nm absorption band characteristic of the haem iron-methionine co-ordination (Schechter & Saludjian, 1967; Eaton & Hochstrasser, 1967) and present in the native cytochrome *c* in aqueous solution.

The majority of eukaryotic cytochromes *c* contain three histidine residues located at positions 18, 26 and 33, and two methionine residues, situated at positions 65 and 80 (Dayhoff, 1972). Extensive studies of the carboxymethylation of human (Tsai & Williams, 1965), horse (Ando *et al.*, 1966; Harbury *et al.*, 1966; Stellwagen, 1968; Schejter & Aviram, 1970), ox (Tsai & Williams, 1965) and tuna (Stellwagen, 1966) cytochromes *c* under a variety of conditions have shown that, whereas modification of either histidine-33 or methionine-65 or both was innocuous, the carboxymethylation of either histidine-18 or methionine-80 or both resulted in the loss of the spectroscopic and functional properties of the native molecule. Further, it was found that the histidine residue at position 26, unlike the one at position 33, is impervious to carboxymethylation under conditions tolerant of the conformational

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integrity of native cytochrome *c* (Hettinger, 1966; Stellwagen, 1966, 1968).

Complete guanidination of the side-chain groups of the lysine residues of horse and tuna cytochromes *c* is known to leave their physical and functional properties virtually unchanged (Hettinger & Harbury, 1964, 1965; Myer & Harbury, 1965). The optical rotatory dispersion of *Ustilago* cytochrome *c* is very similar to that of the other mammalian-type cytochromes *c*, including the horse and tuna molecules (Harbury *et al.*, 1966). On the basis of similarity in conformation to other cytochromes *c* it can be argued that the side-chain groups of the lysine residues of *Ustilago* cytochrome *c* should also be discounted as haem ligands in the native molecule at neutral pH.

The *Ustilago* cytochrome *c* possesses two histidine and one methionine residues (Vinogradov *et al.*, 1972). The former are located at positions 18 and 33, and the latter is at position 80 (Bitar *et al.*, 1972). Our expectations were that carboxymethylation of the side-chain group of residue-33 of *Ustilago* cytochrome *c* under neutral conditions would be easily achieved, that it would not affect the protein's conformational or functional integrity, and that it would restrict the number of potential ligands to histidine-18 and methionine-80. The present report describes the results of the reaction of *Ustilago* ferricytochrome *c* with bromoacetate at neutral pH.

Materials and Methods

Materials

Sporidial cultures of *Ustilago sphaerogena* Burill (A.T.C.C. 12421) were grown as described previously (Bitar *et al.*, 1972). The cytochrome *c* was isolated by extraction at pH 9 and chromatography on columns of Amberlite IRC-50 (NH_4^+ form) (Vinogradov *et al.*, 1972). Horse heart cytochrome *c* was prepared by the method of Margoliash & Walasek (1967). The cytochrome *c* reductase (type I, pig heart) was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Conversion of the proteins into the ferri form was done by treatment with excess of ferricyanide followed by gel-filtration chromatography on Sephadex G-25. The haem octapeptide (14–21) of horse heart cytochrome *c* was prepared by the method of Harbury & Loach (1960). Imidazole, N^2 -acetylhistidine, bromoacetic acid and iodoacetic acid were reagent-grade chemicals (Aldrich Chemical Co., Milwaukee, Wis., U.S.A.).

Methods

Carboxymethylation of cytochrome *c*. A 1% (w/v) solution of *Ustilago* or horse heart ferricytochrome *c* in 0.05M-sodium phosphate buffer (pH 7.2) was

incubated with an equal volume of 0.64M-bromoacetic acid previously neutralized to pH 7 with 6M-NaOH. The reaction was carried out for periods of 2, 4, 8, 12, 24, 36 and 48 h. The reaction mixtures were then passed through a column (2 cm $\times$ 25 cm) of Sephadex G-25, exhaustively dialysed against water and freeze-dried.

Chymotryptic digestion of carboxymethylated ferri-cytochrome *c*. The carboxymethylated ferricytochrome *c* was dissolved in 0.1M- NH_4HCO_3 (approx. 15 mg/ml) and digested with α -chymotrypsin (0.3 mg/ml) at 40°C. The reaction was stopped after 4 h, and the product freeze-dried. The haem peptide of the modified sample was isolated by paper electrophoresis and chromatography (Margoliash & Smith, 1962).

Carboxymethylation of N^2 -acetylhistidine. Carboxymethylation of N^2 -acetylhistidine with iodoacetic acid was carried out by the method of Crestfield *et al.* (1963). When the reaction was complete, the pH was lowered to 5.5 with acetic acid. The crude carboxymethylated product was desalted on a column (12.5 cm $\times$ 2 cm) of mixed-bed ion-exchange resin (Baker AGM-610) with 2M-acetic acid as eluent. The desalted mixture of carboxymethylated N^2 -acetylhistidine was evaporated to dryness and precipitated from methanol with ether. The carboxymethylated derivatives were separated on a column (140 cm $\times$ 0.9 cm) of Dowex 1 (X2; 200–400 mesh) maintained at 29°C, by a modification of the method of Schroeder *et al.* (1962). The unchanged N^2 -acetylhistidine was eluted with 0.2M-acetic acid, the two monocarboxymethyl derivatives were eluted with 1.5M-acetic acid and the dicarboxymethylated N^2 -acetylhistidine was eluted with 10M-acetic acid.

Amino acid composition. The samples were hydrolysed in constant-boiling HCl for 24 h. The amino acid compositions were determined by the method of Spackman *et al.* (1958), with a Beckman model 120 C amino acid analyser. For methionine determinations the samples were oxidized with performic acid before acid hydrolysis (Gundlach *et al.*, 1959).

Biological activity. The oxidase activities were determined by measuring the O_2 uptake of the ferro forms of native and modified horse and *Ustilago* cytochromes *c* in the presence of coupled ox heart mitochondria (Smith & Hansen, 1962) as described previously (Vinogradov *et al.*, 1972). The rates of autoxidation of reduced native and modified cytochromes *c* were determined spectrophotometrically by the method of Tsou (1951). The NADH-cytochrome *c* reductase activities of the ferri forms of horse, *Ustilago* and modified *Ustilago* cytochromes *c* were determined by the procedure of McGowan & Stellwagen (1970).

The concentration of cytochrome *c* was determined spectrophotometrically at 550 nm after reduction with dithionite, by using an extinction coefficient of

27.6 litre·mmol⁻¹·cm⁻¹ (Margoliash & Frohwirt, 1959).

Spectroscopic properties. The absorption spectra of native and carboxymethylated *Ustilago* cytochromes *c* in 0.1M-sodium phosphate buffer (pH7) were recorded on a Cary model 15 spectrophotometer in the oxidized form and after reduction with dithionite. The variation in the visible absorption spectra of *Ustilago* ferricytochrome *c* with the pH was determined by titration with dilute HCl and NaOH solutions in the absence of extrinsic buffers.

The absorption spectra of the ferrihaem octapeptide in the Soret region were obtained at 25°C in 0.01M-sodium phosphate (pH6.96) in the presence of the following ligands: imidazole, *N*²-acetylhistidine, (π -CH₂CO₂H)-*N*²-acetylhistidine and (τ -CH₂CO₂H)-*N*²-acetylhistidine. The concentrations of the haem peptide used were less than 10 μ M. At these concentrations no deviations from Beer's law have been observed (Urry & Pettegrew, 1967).

Results

Carboxymethylation of *Ustilago* ferricytochrome *c*

A pseudo-first-order rate plot of the percentage of remaining histidine against time (Fig. 1) suggested that the reaction occurred in more than one stage. An average of one histidine residue was carboxymethylated over the initial period of about 12h, with a pseudo-first-order rate constant of 0.35h⁻¹. Polyacrylamide-gel electrophoresis carried out at pH5 in 0.05M-sodium acetate buffer revealed that no native material was left unmodified after the reaction had proceeded for 12h.

Fig. 1. Pseudo-first-order rate plot of the carboxymethylation of *Ustilago* ferricytochrome *c*

For experimental details see the text.

Location of the modified histidine residue

Table 1 presents the results of the amino acid analysis of carboxymethylated (12h treatment) *Ustilago* cytochrome *c*. On the average, one of the two histidine residues was modified. The data in Table 1 also show that the single methionine residue was converted into methionine sulphone when the modified cytochrome *c* was subjected to performic acid oxidation before acid hydrolysis. It is known that oxidation of alkylated proteins yields the acid-stable methionine sulphone only from unsubstituted methionine residues (Neumann *et al.*, 1962). Thus methionine-80 appears to be unaffected by the carboxymethylation of *Ustilago* ferricytochrome *c* at neutral pH.

The results of the amino acid analysis of the chymotryptic haem peptides of both the native and modified (carboxymethylated for 12h) *Ustilago* cytochrome *c* are shown in Table 2. They demonstrate that the histidine-18 was not modified by bromoacetate at neutral pH.

Biological activity

The carboxymethylated cytochromes *c* of *Ustilago* and horse were slightly more active than the native molecules in the ox heart mitochondrial cytochrome *c* oxidase system (Fig. 2). When autoxidation was taken into account, the activity of the modified cytochrome *c* remained equal to that of the native proteins at the higher concentrations.

The following rates of reduction by NADH-cytochrome *c* reductase relative to 100% for horse heart ferricytochrome *c* were observed: *Ustilago* ferricytochrome *c*, 105±5%; *Ustilago* ferricytochrome *c* (carboxymethylated for 12h), 115%; *Ustilago* ferricytochrome *c* (carboxymethylated for 24h), 107%. Thus carboxymethylation appears not to have affected the biological activity of *Ustilago* cytochrome *c*.

Spectroscopic properties

The carboxymethylated ferricytochromes *c* of both *Ustilago* and horse retained the characteristic visible and u.v.-absorption spectrum of cytochrome *c*.

A decrease in the intensity of the 695nm band of *Ustilago* ferricytochrome *c* occurred at pH values above 7.4. The midpoint of the titration curve was at pH8.4. The midpoint of the haem-linked ionization at low pH, characterized by the transition from low-spin to high-spin ferrihaem complex, occurred at pH2.6.

Carboxymethylated *N*²-acetylhistidine derivative

The carboxymethylated derivatives of *N*²-acetylhistidine, (π -CH₂CO₂H)-*N*²-acetylhistidine and (τ -CH₂CO₂H)-*N*²-acetylhistidine obtained from the

Table 1. *Amino acid compositions of native and carboxymethylated Ustilago cytochrome c*

For details see the text. The duration of hydrolysis was 24 h. Tryptophan and the carboxymethylated histidine derivatives were not determined.

Residue	Composition (mol/12 500 g)		
	Native	Modified	Sequence*
Lysine	12.61	12.99	13
Histidine	1.97	1.06	2
Arginine	4.70	5.08	5
Aspartic acid	11.05	11.04	11
Threonine	7.60	7.23	8
Serine	1.43	—	2
Glutamic acid	12.73	12.99	13
Proline	4.31	—	4
Glycine	13.36	13.29	13
Alanine	8.44	8.27	8
$\frac{1}{2}$ Cystine†	1.50	1.62	2
Valine	5.16	5.06	5
Methionine	1.04	—	1
Isoleucine	2.33	2.36	2
Leucine	8.53	8.77	9
Tyrosine	3.33	3.30	3
Phenylalanine	5.31	5.31	5
Methionine sulphone	—	0.86	—

* Bitar *et al.* (1972).

† Determined as cysteic acid.

Table 2. *Amino acid compositions of chymotryptic haem peptides of native and carboxymethylated Ustilago cytochrome c*

For details see the text. The duration of hydrolysis was 24 h.

Residue	Composition (mol/1688 g)		
	Native	Modified	Sequence*
Lysine	1.31	1.00	1
Histidine	0.95	0.86	1
Arginine	0.73	0.84	1
Aspartic acid	1.35	1.23	1
Threonine	1.75	1.81	2
Glutamic acid	2.00	2.03	2
Proline	0.97	—	1
Glycine	2.20	2.21	2
Alanine	1.87	2.01	2
$\frac{1}{2}$ Cystine	1.10	—	2
Leucine	0.90	0.95	1
Cysteic Acid	—	1.30	—

* Bitar *et al.* (1972).

carboxymethylation of *N*²-acetylhistidine were recrystallized from methanol-ether. Their identity was confirmed by n.m.r. spectroscopy and by the location of the corresponding carboxymethylhistidines produced by acid hydrolysis of the carboxymethylated *N*²-acetylhistidines in the elution pattern obtained by the method of Spackman *et al.* (1958).

Haem octapeptide complexes

Because the monocarboxymethylated side-chain group of a histidine residue remains a potential ligand for the haem iron, we investigated the formation of complexes of ferrihaem octapeptide with imidazole, *N*²-acetylhistidine, (π -CH₂CO₂H)-*N*²-acetylhistidine and (τ -CH₂CO₂H)-*N*²-acetylhistidine

by measuring the absorbance changes in the Soret region with increasing ligand concentration. The ligand concentrations were corrected for protonation by using the pK_a values shown in Table 3. Addition of $(\tau\text{-CH}_2\text{CO}_2\text{H})\text{-N}^2\text{-acetylhistidine}$ did not affect the Soret absorption of the ferrihaem octapeptide up to molar ratios of 4500:1. Addition of imidazole, $\text{N}^2\text{-acetylhistidine}$ and $(\pi\text{-CH}_2\text{CO}_2\text{H})\text{-N}^2\text{-acetylhistidine}$ shifted the Soret band of the ferrihaem octapeptide from 396 nm to 403 nm. This shift is characteristic of the formation of low-spin ferrihaem complexes. The plots of $\log ([\text{complexed}]/[\text{free}])$ versus $\log [\text{ligand}]$ obtained with imidazole and $\text{N}^2\text{-acetylhistidine}$ were linear with a slope of unity. The resulting $\log K$ values are given in Table 3. The $\log K$ obtained with imidazole compares well with the value of 3.92 obtained by Harbury & Loach (1960).

Fig. 2. Oxygen uptake by the ox heart mitochondrial cytochrome *c* oxidase system

For details see the text. Oxygen uptake in the presence of (▼) *Ustilago*, (▽) carboxymethylated (12h) *Ustilago*, (●) native horse heart and (○) carboxymethylated horse heart cytochromes *c*; (■) indicates the rates of autooxidation of the modified *Ustilago* protein (the dashed line).

Discussion

The imidazole side-chain group of histidine-18 that immediately follows the haem-binding cysteine residues in the amino acid sequence of cytochromes *c* has generally been assumed to be the fifth ligand in the central co-ordination complex on the basis of the following considerations: the proximity to the haem group for complexing with the haem iron atom (Ehrenberg & Theorell, 1955; Margoliash & Schejter, 1966), the resistance to chemical modification except under highly denaturing conditions (Stellwagen, 1968), and the constant location in the primary structures of the cytochromes *c* thus far examined, including the bacterial ones (Dayhoff, 1972). The compositions of the haem peptides obtained from native and carboxymethylated *Ustilago* cytochrome *c* (Table 2) showed that residue-18 was not altered. Thus an imidazole nitrogen atom of histidine-18 is one of the strong-field ligands in the central co-ordination complex of this molecule.

The susceptibility of histidine-33 to carboxymethylation at neutral pH and the absence of alterations in either the biological activities or the absorption spectra of *Ustilago* cytochrome *c* make it unlikely that the imidazole side-chain group of residue 33 is the sixth ligand. Further, the equilibrium constant for the formation of low-spin complexes of ferrihaem octapeptide is decreased by more than tenfold when the $\pi\text{-N}$ atom of the imidazole ring of $\text{N}^2\text{-acetylhistidine}$ is alkylated (Table 3). Carboxymethylation of the $\tau\text{-N}$ atom of $\text{N}^2\text{-acetylhistidine}$ appears to suppress effectively its ability to form a complex with the ferrihaem octapeptide. It is unlikely that marked alteration in the strength of the co-ordination of one of the ligands to the haem iron atom would not be reflected in either the spectra or the biological activity of the modified protein.

Recent n.m.r. evidence indicates that the changes in horse cytochrome *c* at alkaline pH that occur concomitantly with the disappearance of the absorption band at 695 nm are accompanied by the removal of the thioether side-chain group of methionine-80 from its contact with the haem iron and its

Table 3. Low-spin ferrihaem octapeptide complex-formation at pH 6.96 in 0.01 M-sodium phosphate buffer

For details see the text. —, No complex-formation. K is $[\text{complex}]/[\text{free}][\text{ligand}]$.

Ligand	Slope	$\log K$	pK_a
Imidazole*	1.01	3.98 ± 0.04	7.05†
$\text{N}^2\text{-acetylhistidine}$	1.07	4.13 ± 0.08	7.02‡
$(\pi\text{-CH}_2\text{CO}_2\text{H})\text{-N}^2\text{-acetylhistidine}$	0.94	2.72 ± 0.06	6.33‡
$(\tau\text{-CH}_2\text{CO}_2\text{H})\text{-N}^2\text{-acetylhistidine}$	—	—	5.74‡

* In 0.01 M-sodium borate buffer, pH 9.0.

† Walba & Isensee (1961).

‡ Hapner (1966). The values quoted are for the corresponding carboxymethylated histidines.

replacement by the ϵ -amino group of lysine-79 (Gupta & Koenig, 1971). The pK for the disappearance of the 695nm absorption band in *Ustilago* ferricytochrome *c* is 8.4, compared with 9.3 in horse ferricytochrome *c* (Theorell & Akesson, 1941) and 8.1 in yeast iso-1 cytochrome *c* (Aviram & Schejter, 1969). The significance of these differences in pK in such widely evolutionarily divergent molecules remains unclear.

At acid pH values, the range of stability of the central co-ordination complex of *Ustilago* ferricytochrome *c* extends to pH2.6, the mid-point of the transition from low-spin to high-spin complex. This value is 2.5 in horse cytochrome *c* (Theorell & Akesson, 1941) and 3.1 in baker's yeast iso-1 cytochrome *c* (Aviram & Schejter, 1969). Thus eukaryotic cytochromes *c* appear to possess a common low-spin (histidine)-haem-(methionine) complex that is stable over the range from pH2.5-3.0 to pH8.0-9.3. In contrast, the central (histidine)-haem-(methionine) complex of the cytochrome *c*-551 of *Pseudomonas* (Fanger *et al.*, 1967), though stable also to about pH2.4 (Vinogradov & Harbury, 1967) remains unaltered at highly alkaline pH values as indicated by the presence of the 695nm absorption band even at pH11 (Vinogradov, 1970). Thus the behaviour of *Pseudomonas* cytochrome *c*-551, which is known to be inactive towards mammalian cytochrome *c* oxidase (Horio *et al.*, 1960), provides support for the suggestion that the conformation change and the alteration in the central co-ordination complex of eukaryotic cytochrome *c* occurring at alkaline pH values plays a role in the redox mechanism of the protein (Margoliash *et al.*, 1972).

The results of the carboxymethylation of *Ustilago* ferricytochrome *c* in solution are in complete agreement with the results of X-ray diffraction studies of crystalline horse, tuna and bonito cytochromes *c* (Dickerson *et al.*, 1971; Takano *et al.*, 1972) and with the results of chemical modification of *Pseudomonas* cytochrome *c*-551 (Fanger *et al.*, 1967). In spite of the differences in the behaviour in solution of different mammalian cytochromes *c* on one hand, and of bacterial molecules on the other, all the results demonstrate the constancy of the (histidine)-haem-(methionine) central co-ordination complex over the large evolutionary distances separating the fungal from the vertebrate and the bacterial cytochromes *c*.

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