The Primary Structure of Cytochrome *c* from the Rust Fungus *Ustilago sphaerogena*

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1. The complete amino acid sequence of cytochrome *c* from the basidiomycete *Ustilago sphaerogena* was determined from the amino acid compositions and sequences of either tryptic or chymotryptic peptides, and in homology with at least thirty other established sequences of cytochrome *c*. 2. The primary structure of the molecule bears all of the characteristics of a mammalian-type cytochrome *c*, showing the typical clustered distribution of hydrophobic and basic residues with a single polypeptide chain of 107 residues. 3. Like all other fungal cytochromes *c*, it possesses a free *N*-terminus, and one less residue at the *C*-terminus than vertebrate cytochromes *c*. The region of residues 70–80 is strictly conserved, as is histidine at position 18. Position 26 is occupied by an asparagine residue, in contrast to histidine which occurs at this location in most of the known sequences of mammalian-type cytochromes *c*. 4. In contrast to some other fungal and plant cytochromes *c* of known primary structures, the *Ustilago* cytochrome *c* molecule does not contain trimethyl-lysine. 5. The sequence of *Ustilago* cytochrome *c* differs from the sequences of human, horse, chicken, tuna, wheat, and baker's yeast proteins at loci 47, 43, 44, 44 and 38 respectively.

The primary structures of the cytochrome *c* from a variety of species have provided considerable information about the extent of variability of primary structure compatible with a unique set of functions, and the relationship of protein structure to the evolution of the species (Margoliash *et al.*, 1968). However, in considering the biological origin of the available sequences, it is evident that there exists a substantial disparity between the number of cytochromes *c* derived from higher organisms, mammals and other vertebrates on the one hand, and that from the lower forms on the other. Among the fungi, the species that have been studied so far, namely *Saccharomyces* (Yaoi, 1967), *Neurospora* (Heller & Smith, 1966), *Candida* (Narita & Titani, 1968), and *Debaryomyces* (Sugeno *et al.*, 1971), all belong to one class, the Ascomycetes. The amino acid sequence of the cytochrome *c* isolated from the basidiomycete *Ustilago sphaerogena* (rust fungus), a common wheat pathogen, was undertaken because of the phylogenetic position of this micro-organism, and because of the unique presence, in this mammalian-type molecule, of only two histidine residues and a single methionine residue (Vinogradov *et al.*, 1972).

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Materials and Methods

Growth of organism and isolation of cytochrome c

Sporidial cultures of *Ustilago sphaerogena* Burrill (ATCC 12421) were grown in 2% (w/v) yeast extract (Difco), 2% (w/v) dextrose, and 1% (w/v) potassium phosphate buffer, pH 7.2, in 2-litre shaker flasks with vigorous aeration at 25°C. The cells were harvested by centrifugation at 6000*g* and washed. The cytochrome *c* was isolated and purified as described previously (Vinogradov *et al.*, 1972).

Enzymic digests, isolation of peptides and nomenclature

Digestion employing trypsin treated with TPCK (1-chloro-4-phenyl-3-tosylamidobutan-2-one), α -chymotrypsin and carboxypeptidase A were carried out essentially as described by Nolan & Margoliash (1966).

The chymotryptic digest (16.7 μ mol) was initially chromatographed on a column (3.8 cm $\times$ 240 cm) of Sephadex G-50 (from Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A.) equilibrated in 0.1 M-NH₄HCO₃. Six major fractions were obtained

by measuring the absorbance at 220nm. These were designated as: U-C-I, U-C-II, U-C-III, U-C-IV, U-C-V and U-C-VI. Each of these fractions, with the exception of U-C-I, was further fractionated on a column (1.9cm×150cm) of Dowex 50 (X2) (from Bio-Rad Laboratories, Richmond, Calif., U.S.A.) with volatile buffers essentially as described by Margoliash & Smith (1962). The resin was a mixture of equal parts of 80–200 and 250–325 wet-mesh beads; elution was done at 40°C under a linear gradient established between 0.2M-pyridine–acetate buffer, at pH3.1, and 2.0M-pyridine–acetate buffer, at pH5.0. Peptide U-C-I was further fractionated on a column (1.9cm×150cm) of Sephadex G-50.

The tryptic hydrolysate (16.5μmol) was initially treated with triethylamine to pH6.9. Supernatant 1 and precipitate 1 resulted. To supernatant 1, 0.1M-HCl was added to give pH4.6. The resultant precipitate was washed with 50% (v/v) acetic acid. The supernatant obtained from such a treatment was concentrated to 1 ml. To this fraction 2 ml of 50% (v/v) acetic acid, 2 ml of glacial acetic acid and 0.16 ml of pyridine were added. The resultant supernatant was combined with supernatant 1, freeze-dried and chromatographed on a column (3.6cm×150cm) of Dowex 50 (X2) employing the conditions described before.

Peptide fractions were further purified, when necessary, by chromatography on columns (0.9cm×150cm) of Dowex 50 (X2) (Schroeder *et al.*, 1962), by gel filtration on columns (1.9cm×150cm) of Sephadex G-25 (superfine grade; Pharmacia Fine Chemicals Inc., Piscataway, N.J., U.S.A.), by preparative paper electrophoresis in pyridine–acetic acid–water (25:1:225, by vol.), pH 6.4, at 14 V/cm (Margoliash & Smith, 1962), and by preparative paper chromatography in the solvent system butan-1-ol–acetic acid–water (15:10:3:12, by vol.). In the case of one peptide, U-C-III-1, further purification was carried out by using the technique of free-flow electrophoresis (Hannig, 1964) with the Brinkman free-flow-electrophoresis apparatus, model FF-1 (Brinkman Instruments, N.Y., U.S.A.). The electrode buffer was pyridine–acetic acid–water (5:0.2:95, by vol.), pH6.5; the plate buffer was the same buffer diluted with an equal volume of water. Electrophoresis was at 1900V and 5°C with a sample retention-time of 60min. Fractions (3ml) were obtained, and 0.2ml samples were used for the detection of the peptide with the ninhydrin reagent (Margoliash & Smith, 1962).

Peptide ‘mapping’ (Ingram, 1958) on either Whatman 3MM paper, (46cm×57cm), or Brinkman thin-layer sheets (20cm×20cm) pre-coated with cellulose was used to monitor the purification steps. The ‘maps’ were developed with the ninhydrin reagent and, in some cases, with the Pauly, Sakaguchi, Ehrlich and iodoplatinate reagents for the detection of tyrosine and histidine, arginine, tryptophan, and

methionine, respectively, as described by Nolan & Margoliash (1966) and Toennies & Kolb (1951).

Peptides isolated and purified from chymotryptic and tryptic hydrolysates of cytochrome *c* are given the prefix C and T, respectively. They are preceded by the letter U denoting *Ustilago*, and followed by a numeral number indicating the number of the chromatographic peak in which they emerged. The prefix S refers to Sephadex.

Amino acid analysis and sequence determination

Amino acid analyses were carried out by the method of Spackman *et al.* (1958) by using the Beckman Spinco model 120C amino acid analyser. Performic acid oxidation was done by the method of Hirs *et al.* (1956).

Sequences of the purified peptides were determined by a modification of the Edman procedure (Edman & Begg, 1967), as described below.

A portion (0.1–1 μmol) of the peptide was taken in 100 μl of the coupling buffer [0.4M-dimethylallylamine in propan-1-ol–water (3:2, by vol.), pH adjusted to 9.5 with trifluoroacetic acid]. Then 5 μl of phenylisothiocyanate was added, and the contents were flushed with N₂ and incubated at 50°C for 20min. Then 200 μl of benzene was added, and the aqueous phase dried under a stream of N₂, and 100 μl of trifluoroacetic acid was added. The contents were flushed with N₂ and incubated at 50°C for 7min. The acid was evaporated under a stream of N₂ and the thiazolinones were extracted with 1 ml of peroxide-free ether, and converted into the hydantoin by heating for 8 to 10min at 80°C in 1M-HCl. The phenylthiohydantoin derivative was extracted with 1.5 ml of ethyl acetate, and identified qualitatively by t.l.c. on Eastman chromatogram silica-gel sheets pre-coated with a fluorescent indicator. The solvents IV and V and reference standards as described by Jeppsson & Sjöquist (1967) were used. The chromatograms were sprayed with 0.1% ninhydrin in 95% (v/v) ethanol and heated at 110°C for 2min and each phenylthiohydantoin amino acid developed a characteristic colour that was an aid in its identification. The water-soluble derivatives namely those of arginine, histidine and cysteine, were identified by direct amino acid analysis, or by amino acid analysis of the residual peptide.

Results

Amino acid composition and characterization of chymotryptic and tryptic peptides

Tables 1 and 2 include a summary of the composition as well as characterization of some of the chymotryptic and tryptic peptides that were used in

deriving the amino acid sequence of *Ustilago* cytochrome *c*.

Derivation of the amino acid sequence of Ustilago cytochrome c

Residues -4 to 7 (*H₂N-Gly-Phe-Glu-Asp-Gly-Asp-Ala-Lys-Lys-Gly-Ala*). The amino acid sequence of this segment was established from two steps of Edman degradation on peptide U-C-V-9 (S-2) and eleven steps of the same procedure on the whole molecule that was previously denatured with performic acid by the method of Hirs *et al.* (1956).

Residues 5 to 10 (*Lys-Gly-Ala-Arg-Ile-Phe*). Chymotryptic specificity makes it very likely that phenylalanine is the C-terminal residue of peptide U-C-IV-2(1) (S-1). Four steps of the Edman procedure followed by amino acid analysis of the residual peptide placed arginine at position 8. By difference isoleucine was placed next to the C-terminal phenylalanine.

—Haem—

Residues 11 to 27 (*Lys-Thr-Arg-Cys-Ala-Gln-Cys-His-Thr-Leu-Gly-Ala-Gly-Glu-Pro-Asn-Lys*). Three steps of Edman degradation on peptide U-C-IA-S2 followed by amino acid analysis of the residual peptide established the sequence of the first three residues. The rest of the sequence was established from the tryptic haem peptide U-T-XXVIII-9; 1 μ mol of the peptide was denatured with performic acid (Hirs *et al.*, 1956) and eleven steps of the Edman procedure established the sequence of residues 14–24. Phenylthiohydantoin cysteine-14 was identified by amino acid analysis after hydrolysis in 6M-HCl. Phenylthiohydantoin cysteine-17 was identified by electrophoresis, and phenylthiohydantoin histidine-18 was identified by spraying with Pauly's reagent. Tryptic specificity makes it very likely that lysine is the C-terminal residue. Through an overlap based on composition only of the tryptic and chymotryptic haem peptides, proline was placed in position 25, and asparagine at 26.

Residues 27 to 36 (*Lys-Val-Gly-Pro-Asn-Leu-His-Gly-Leu-Phe*). Five steps of Edman degradation on peptide U-C-IV-7a, and one step of the same procedure on peptide U-C-V-16 followed by amino acid analysis of the residual peptide established the sequence of residues 27–33. Peptide U-C-V-12 was subjected to two steps of the Edman procedure, which established the sequence of the remainder of the segment. The complete amino acid sequence of residues 27–36 was confirmed by an overlap (based on composition) with the tryptic peptide U-T-XXXII.

Residues 37 to 46 (*Gly-Arg-Lys-Ser-Gly-Thr-Val-Glu-Gly-Phe*). Nine steps of the Edman procedure on peptide U-C-IV-7 established the sequence of residues 37–45. The position of arginine at 38 was established through an overlap with peptide U-T-XXXII since tryptic specificity makes it probable that arginine is

the C-terminal residue of this peptide. Chymotryptic specificity makes it very likely that phenylalanine is at position 46.

Residues 47 to 48 (*Ser-Tyr*). Two steps of the Edman procedure on peptide U-C-V-11 established the above sequence.

Residues 49 to 59 (*Thr-Asp-Ala-Asn-Lys-Lys-Ala-Gly-Gln-Val-Trp*). Nine steps of the Edman procedure on peptide U-C-VI-1 established the sequence of residues 49–57. Incubation with carboxypeptidase A for 3 min showed tryptophan to be the C-terminus and valine to be the next residue.

Contamination of peptide U-C-VI-1 by serine and glycine made it necessary to run the appropriate blanks. Thus amino acid analysis on 0.50 μ mol of the peptide not subjected to acid hydrolysis revealed 5.30 residues of serine and 4.24 residues of glycine. The values included in Table 1 for serine and glycine are those obtained when the respective values of blanks are subtracted from those values, 5.66 and 5.33 respectively, obtained for a comparable sample of peptide U-C-VI-1 previously hydrolysed in 6M-HCl for 24h.

Residues 60 to 65 (*Glu-Glu-Glu-Thr-Phe-Leu*). Four steps of the Edman procedure on peptide U-C-III-1 gave the sequence of the first four residues. Incubation with carboxypeptidase A for 7 min showed Phe-Leu to be the C-terminal fragment.

Residues 66 to 74 (*Glu-Tyr-Leu-Glu-Asn-Pro-Lys-Lys-Tyr*). Three steps of the Edman procedure on peptide U-C-IV-5(1), and three steps of the same procedure on peptide U-C-V-18, established the sequence of the first six residues. One step of the Edman degradation procedure on peptide U-T-XXVII, and four steps on peptide U-T-XXVI, showed that Lys-Tyr are the last two residues of the segment. Lysine-72 was placed by difference. Analysis of a 24h acid hydrolysate of peptide U-C-V-18 by the method of DeLange *et al.* (1970) failed to show the presence of trimethyl-lysine at position 72. A similar digest of the whole protein did not reveal any trimethylated lysine.

Residues 75 to 80 (*Ile-Pro-Gly-Thr-Lys-Met*). Six steps of the Edman procedure on U-C-IV-3 (S-1) established the above sequence. The sequence was confirmed through an overlap with U-T-XXVII and U-T-XXIX.

Residues 80 to 85 (*Met-Ala-Phe-Gly-Gly-Leu*). Six steps of Edman degradation on peptide U-T-XXIX, together with the composition of peptide U-C-V-10, established the sequence of the above segment.

Residues 86 to 97 (*Lys-Lys-Glu-Lys-Asp-Arg-Asn-Asp-Leu-Val-Thr-Tyr*). Ten steps of the Edman procedure on peptide U-C-III-2b-4 established the sequence of the first ten residues. Tryptic specificity makes it very likely that arginine is the C-terminal residue of peptide U-T-XIX, and thus correctly places it at position 91. Digestion with carboxypeptidase A

Table 1. *Amino acid composition and characterization of chymotryptic peptides*

The numbers given in parentheses following the analytical values are the stoichiometric numbers of residues per molecule of pure peptide. Peptides which gave a yellow colour with ninhydrin are indicated as Yellow; no notation is made if ninhydrin colour was the normal blue; positive reactions to residue specific reagents are marked by the name of the reaction. Mobility, in cm, was determined by paper electrophoresis and chromatography as described by Margoliash & Smith (1962). Abbreviations: El, electrophoresis; Ch, chromatography; +, cathodic migration; -, anodic migration; 0, no electrophoretic movement; PE, paper electrophoresis; FFE, free-flow electrophoresis.

Amino acid residue		U-C-V-9 (S-2)	U-C-IV-2(1) (S-1)	U-C-IA-S2	U-C-IV-7a	U-C-V-16
Peptide ...						
Trp			1.23 (1)	1.31 (1)	1.06 (1)	
Lys				0.95 (1)	0.96 (1)	1.01 (1)
His			1.22 (1)	0.73 (1)		
Arg				1.35 (1)	1.01 (1)	
Asp				1.75 (2)		
Thr						
Ser						
Glu				2.00 (2)		
Pro				0.97 (1)	0.85 (1)	
Gly		0.93 (1)	0.98 (1)	2.20 (2)	2.13 (2)	
Ala			0.91 (1)	1.87 (2)		
Cys				1.10 (2)*		
Val					0.85 (1)	
Met						
Ile			0.99 (1)		1.81 (2)	0.98 (1)
Leu						
Tyr						
Phe		1.06 (1)	0.88 (1)		0.75 (1)	
Yield (%)	...	3.08	2.57	6.42	2.70	1.34
Mobility (cm)	...	El, 0;	El, -4.9;	El, 0;	El, -7.5;	El, -7.1;
	...	Ch, 23.6	Ch, 15.6	Ch, 12.5	Ch, 23.2	Ch, 9.4
Colour reaction	...	Yellow	Sakaguchi	Haem colour	Pauly	Pauly
Purification method	...	Sephadex G-25 superfine	Sephadex G-25 superfine	Sephadex G-50	PE	Dowex 50 (X2)
Amino acid residue		U-C-V-12	U-C-IV-7	U-C-V-11	U-C-VI-1 (1)*	U-C-III-1
Peptide ...					1.83 (2)	
Trp			1.05 (1)			U-C-IV-5 (1)
Lys						2.05 (2)
His						
Arg			0.80 (1)			
Asp					1.88 (2)	1.02 (1)
Thr			0.95 (1)		1.28 (1)	
Ser			1.08 (1)	0.96 (1)	0.36 (0)	
Glu			1.01 (1)		0.98 (1)	3.14 (3)
						2.02 (2)

Pro	1.02 (1)	3.06 (3)		1.09 (1)	1.13 (1)
Gly				2.08 (2)	
Ala					
Cys					
—					
Val		0.95 (1)		0.93 (1)	
Met					
Ile					
Leu	1.00 (1)				1.04 (1)
Tyr	0.98 (1)				1.77 (2)
Phe					
Yield (%)	...	0.95 (1)			
Mobility (cm)	35.80	45.38			15.04
	El, -1.3;	El, -5.8;			El, -1.2;
	Ch, 30	Ch, 8.2			Ch, 18.1
Colour reaction	...	Sakaguchi			Pauly
Purification method	...	Dowex 50 (X2)			Dowex 50 (X2)
Amino acid residue					
Peptide	...	U-C-V-18	U-C-IV-3 (S-1)	U-C-V-10	U-C-IV-4b
Trp					
Lys	1.90 (2)	2.11 (2)	1.08 (1)		1.02 (1)
His					
Arg					
Asp	1.21 (1)	1.03 (1)	1.00 (1)		0.93 (1)
Thr					1.01 (1)
Ser					
Glu	0.91 (1)	0.92 (1)			2.08 (2)
Pro	1.21 (1)	0.71 (1)	0.63 (1)		
Gly			0.99 (1)		
Ala				0.92 (1)	
Cys					
—					
Val					
Met					
Ile					
Leu	0.76 (1)		0.95 (1)		
Tyr	0.91 (1)		0.96 (1)		
Phe		0.64 (1)			0.88 (1)
Yield (%)	2.01	3.32	12.51	1.08 (1)	68.63
Mobility (cm)	...	El, -3.7;	El, -7.7;	33.05	El, -1.2;
	...	Ch, 7.9	Ch, 16.8	El, -1.3;	Ch, 10
Colour reaction	...	Pauly	Iodoplatinate	Ch, 26.5	Sakaguchi
Purification method	...	Dowex 50 (X2)	Sephade G-25 superfine	Yellow Dowex 50 (X2)	PE

* Assumed value.

Table 2. *Amino acid composition and characterization of tryptic peptides*

The abbreviations, conditions and symbols are described in the legend to Table 1.

Amino acid residue	Peptide	...	U-T-XXVIII-9	U-T-XXXII	U-T-XXVII	U-T-XVI	U-T-XXIX	U-T-XIX
Trp								
Lys			1.04 (1)		2.00 (2)	1.00 (1)	2.00 (2)	1.05 (1)
His			0.96 (1)	1.00 (1)				
Arg				1.00 (1)				0.86 (1)
Asp			1.01 (1)	1.07 (1)				1.05 (1)
Thr			0.95 (1)		1.29 (1)	1.12 (1)		
Ser								
Glu			1.95 (2)					1.05 (1)
Pro			1.18 (1)	1.02 (1)	0.65 (1)	1.05 (1)		
Gly			2.01 (2)	3.17 (3)	1.45 (1)	1.01 (1)	2.18 (2)	
Ala			1.78 (2)				1.12 (1)	
Cys			1.18 (2)*					
—								
Val				0.93 (1)			(1)*	
Met								
Ile					0.81 (1)	0.91 (1)		
Leu			0.83 (1)	1.92 (2)			1.00 (1)	
Tyr					0.97 (1)	0.91 (1)		
Phe				0.88 (1)			0.83 (1)	
Yield (%)	...	13.87		9.30	1	20.39	8.70	2.88
Mobility (cm)	...	El, +0.8; Ch, 10.3	El, -3.3; Ch, 21.5	El, -6.2; Ch, 12.3	El, -3.7; Ch, 19.7	El, -6.5; Ch, 19.5	Iodoplatinate Dowex 50 (X2)	El, -0.5; Ch, 3.5
Colour reaction	...	Haem colour						
Purification method	...	Sephadex G-25	Dowex 50 (X2)	Dowex 50 (X2)	Dowex 50 (X2)	Dowex 50 (X2)	Dowex 50 (X2)	Dowex 50 (X2)

Amino acid residue		U-T-XIV	U-T-XXI-5	U-T-IX	U-T-XXII	U-T-XXI-2	U-T-XI
Peptide	...						
Trp							
Lys		1.07 (1)		1.04 (1)	1.34 (1)	1.00 (1)	
His							
Arg			1.04 (1)		1.96 (2)	0.88 (1)	1.76 (2)
Asp					2.91 (3)		3.25 (3)
Thr				1.00 (1)	1.38 (1)	0.83 (1)	1.07 (1)
Ser							
Glu		0.93 (1)		2.00 (2)	1.09 (1)	1.62 (2)	
Pro							
Gly							
Ala							
Cys							
—							
Val					1.32 (1)		0.98 (1)
Met							
Ile							
Leu			0.95 (1)		1.85 (2)	1.00 (1)	0.90 (1)
Tyr					0.77 (1)		1.02 (1)
Phe							
Yield (%)	...	2.67	12.54	15.96	2.58	10.12	16.96
Mobility (cm)	...	El, -0.5; Ch, 6.5		El, +3.5; Ch, 6.5	El, -0.5; Ch, 11.2	El, -1.0; Ch, 5.7	El, -1.5; Ch, 12.5
Colour reaction	...						
Purification method	...	Dowex 50 (X2)	Sephadex G-25	Dowex 50 (X2)	Dowex 50 (X2)	Sephadex G-25	Dowex 50 (X2)

* Assumed value.

Fig. 1. Amino acid sequence of *Ustilago sphaerogena* cytochrome *c*

Abbreviations are as follows: $\rightarrow$, sequence was determined by the modified Edman method as described in the Materials and Methods section; $\leftarrow$, sequence was determined by using carboxypeptidase A. The positions of enzymic cleavage are shown by: $\downarrow$, tryptic; $\uparrow$, chymotryptic.

for 30 min established the sequence of the four residues at the C-terminal end of the segment 86–97. Overlap based on composition only with peptides U-T-XIV, U-T-XIX, U-T-XXII and U-T-XI confirmed the above sequence.

Residues 98 to 103 (*Leu-Arg-Glu-Glu-Thr-Lys-CO₂H*). This sequence was established from five steps of Edman degradation on peptide U-C-IV-4b. The position of arginine was determined through an overlap with peptides U-T-IX and U-T-XXII. It is noteworthy that the compositions of peptides U-T-XXI-2 and U-T-XXI-5 indicate perhaps an unusual tryptic split at tyrosine-97. Incubation of peptide U-C-IV-4b with carboxypeptidase A for 48 h released only lysine, the C-terminal residue of this molecule.

Based on the preceding results, and in homology with at least thirty other established sequences of cytochrome *c*, the complete amino acid sequence of *Ustilago sphaerogena* cytochrome *c* was deduced and is shown in Fig. 1.

Discussion

The cytochrome *c* from *Ustilago sphaerogena*, like all known fungal cytochromes *c*, contains additional residues at the N-terminus, which is free, and one residue less at the C-terminus compared with the cytochromes *c* obtained from vertebrates (for a summary of these sequences see Dickerson *et al.*, 1971). However, *Ustilago* cytochrome *c* shows the same clusters of hydrophobic and basic residues as most other mammalian-type cytochromes *c*. It retains the invariant residues such as the cysteines at positions 14 and 17, the histidine at position 18, the tyrosine at position 48, the tryptophan at position 59, as well as the constant region 70–80 including the single

methionine residue. Although most of the cytochromes *c* whose sequences have so far been determined contain a histidine residue at position 26, this position is occupied by an asparagine residue in *Ustilago*, and a glutamine residue in *Neurospora* (Heller & Smith, 1966).

A noteworthy difference between *Ustilago* cytochrome *c* and other fungal and plant cytochromes *c* is the absence of trimethyl-lysine in the sequence. Although the cytochromes *c* of *Neurospora* and *Saccharomyces* were found to contain a single residue of trimethyl-lysine at position 72 (DeLange *et al.*, 1969, 1970), the plant cytochromes *c* appear to contain two residues at positions 72 and 86 (Thompson *et al.*, 1971). Scott & Mitchell (1969) have demonstrated the presence of two molecular forms of cytochrome *c* in the *mi-1* (poky) mutant of *Neurospora* and shown that the only difference occurred at position 72, occupied by lysine in one molecule, and by trimethyl-lysine in the other. The absence of electrophoretic heterogeneity in the case of *Ustilago* cytochrome *c* (Vinogradov *et al.*, 1972) argues against the occurrence of a similar situation.

The isoelectric point of *Ustilago* cytochrome *c* occurs at pH 9.40 (Vinogradov *et al.*, 1972) and is significantly lower than that of other mammalian-type cytochromes *c*, including the fungal ones, with the exception of that of *Neurospora* cytochrome *c* which occurs at pH 9.34 (Heller & Smith, 1966). The difference between the isoelectric points of horse heart and *Ustilago* cytochrome *c* can be rationalized on the basis of their amino acid compositions. Horse heart cytochrome *c* (Margoliash, 1962) contains 19 lysine, 2 arginine, 3 aspartic acid and 9 glutamic acid residues plus the free C-terminal carboxylate, giving a net excess of 8 positive charges. *Ustilago* cytochrome

c contains 14 lysine, 5 arginine, 11 glutamic acid and 5 aspartic acid residues, plus the free amino and carboxylate groups of the *N*- and *C*-termini, giving a net excess of 3 positive charges.

Ustilago cytochrome *c* reveals 32 differences in primary structure relative to *Candida*, 37 relative to *Neurospora*, 38 relative to *Saccharomyces iso-1*, 43 relative to horse heart, and 46 relative to wheat cytochrome *c*. On the other hand, the *Neurospora*, *Candida*, and *Saccharomyces* proteins differ by 46, 51 and 46 residues, respectively, from the horse heart cytochrome *c*. Thus, the *Ustilago* protein appears to be equidistant in evolutionary terms from the plant cytochromes *c* and the vertebrate proteins.

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