A Comparison of the Physical and Chemical Properties of Four Cytochromes *c* from *Azotobacter vinelandii*

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1. A modified method for the separation and purification of four cytochromes *c* from *Azotobacter vinelandii* is described. Two new cytochromes *c* have been purified and are designated cytochromes *c*(551) and *c*(555). 2. Additional evidence is presented to establish the dihaem nature of cytochrome *c*₄. Ultracentrifugation data indicated similar molecular weights for the native and the denatured protein. Cleavage with CNBr yielded seven peptides; the amino acid compositions of the purified peptides were determined. Only one haem peptide was recovered. 3. Cytochromes *c*(551) and *c*(555) were characterized as acidic proteins of molecular weights about 12000. The spectral properties, isoelectric points, 'maps' of peptides from CNBr cleavage and amino acid compositions were determined for these two proteins. 4. The spectral properties, isoelectric points, molecular weights, CNBr peptide 'maps', amino acid compositions, relative oxidation-reduction potentials and e.p.r. (electron-paramagnetic-resonance) spectra of the four cytochromes *c* were compared. Cytochrome *c*₄ and cytochrome *c*(551) appear to be distinct proteins. The distinction between cytochromes *c*₅ and *c*(555) was not as clear, and our data are inadequate to establish firmly that they are distinct proteins. 5. The dihaem nature of cytochrome *c*₄ is evident in its e.p.r. spectrum. The e.p.r. spectra are similar to the spectra of mammalian cytochromes *c*.

Mammalian-type cytochromes *c* have been investigated intensively (Margoliash & Schejter, 1966). Elucidation of the three-dimensional structure of horse heart cytochrome *c* has provided information on the relationship between the structure and function of cytochrome *c* (Dickerson *et al.*, 1971).

Although the extent of understanding of bacterial cytochromes *c* has not reached that of mammalian cytochromes *c*, amino acid sequences have been reported for a few bacterial cytochromes *c* and the three-dimensional structure of *Rhodospirillum rubrum* cytochrome *c* has been completed at low resolution (Kamen & Horio, 1970). Relative neglect of bacterial cytochromes can be attributed to their ill-defined homology and the multiplicity of cytochromes *c* that can be isolated from one bacterial species. Kamen *et al.* (1971) have reported that photosynthetic bacteria contain seven forms of cytochrome *c*, representing four classes. Four different cytochromes *c* have been isolated from *Desulfovibrio* species (Le Gall *et al.*, 1971), rather than the single cytochrome *c*₃ previously found (Postgate, 1956). A more thorough discussion of bacterial cytochromes *c* can be found in recent reviews on the subject (Bartsch, 1968; Kamen & Horio, 1970; Horio & Kamen, 1970; Kamen *et al.*, 1971).

Cytochromes of the *c* type were observed in *Azotobacter vinelandii* by Hyndman *et al.* (1952), and a crude cytochrome *c* fraction was isolated by Vernon

& Kamen (1954). The cytochromes *c*₄ and *c*₅ were isolated in a purified form by Tissières & Burris (1956) and Tissières (1956). The report of Tissières (1956) contains the details of purification and properties of the cytochromes *c*₄ and *c*₅. Neumann & Burris (1959) crystallized the two cytochromes and reported additional information on their properties.

Swank & Burris (1969) isolated a third cytochrome *c* component from *A. vinelandii* and designated it minor cytochrome *c*₄. They reported that cytochrome *c*₄ has a molecular weight of 24000. Cytochrome *c*₅ was isolated as a dimer of 24400 molecular weight from a DEAE-cellulose column; however, under denaturing conditions the molecular weight was 12800. The minor cytochrome *c*₄ was reported to have a spectrum identical with that of the major cytochrome *c*₄ but a molecular weight of 12000.

A molecular weight of 23 500 was assigned to cytochrome *c*₄ on the basis of Sephadex gel filtration (van Gelder *et al.*, 1968). Tissières (1956) had found a minimum molecular weight of 12000 by iron analysis; therefore it has been suggested that cytochrome *c*₄ is a dihaem protein (van Gelder *et al.*, 1968; Swank & Burris, 1969).

After assignation of subscripts for distinguishing cytochromes *c*₄ and *c*₅, it became apparent that such a nomenclature would become increasingly cumbersome. No completely satisfactory system of nomenclature for cytochromes has been established, but the

International Union of Biochemistry (Webb *et al.*, 1965) has proposed a nomenclature that would suggest a designation for minor cytochrome c_4 (Swank & Burris, 1969) as cytochrome $c(551, A. vinelandii)$ and minor cytochrome c_5 (Campbell, 1972) as cytochrome $c(555, A. vinelandii)$. Because of extensive prior use we will retain the designation of cytochromes c_4 and c_5 , and because all cytochromes described in this paper are from *A. vinelandii*, we will omit the name of the organism and designate the cytochromes as cytochromes c_4 , c_5 , $c(551)$, and $c(555)$.

The investigations reported here describe further studies of cytochromes c_4 and c_5 and the purification and properties of two additional cytochromes c from *A. vinelandii*, cytochromes $c(551)$ and $c(555)$.

Materials and Methods

Methods

Organism and growth conditions. *A. vinelandii* strain OP was grown under N_2 -fixing conditions on a modified Burk's medium at 30°C (Newton *et al.*, 1953; DerVartanian *et al.*, 1969). The bacteria were grown in a 1900-litre fermenter with a 10% (v/v) inoculum. The yield was 13 kg of cell paste (2860 g dry wt.), which was divided into portions and stored at -20°C.

Extraction and purification. The cytochromes c were extracted with butanol and purified by the procedure of Swank & Burris (1969), with the following modifications. The precipitation with $(NH_4)_2SO_4$ was replaced by a batch absorption of the crude extract on to DEAE-cellulose. Cell paste (500 g) was extracted with butanol-water (1:2, v/v), centrifuged and the extract was mixed for 3-4 h at 4°C with 350 g of Whatman DE-52 cellulose which had been equilibrated with 5 mM-Tris-HCl buffer, pH 7.6. The DEAE-cellulose was washed on a 24 cm diam. Büchner funnel and poured into a 7.5 cm diam. glass chromatographic column (Glenco, Houston, Tex., U.S.A.). The cytochromes c were eluted with 100 mM-Tris-HCl buffer, pH 7.6, in a volume of 400-600 ml. The volume was decreased to 60 ml with a Diaflo model 401 ultrafilter (Amicon Corp., Cambridge, Mass., U.S.A.) with a UM-2 membrane. The concentrated extract was dialysed overnight against 5 mM-Tris-HCl buffer, pH 7.6. The cytochromes c were separated by procedures described by Swank & Burris (1969). All dialysis tubing was treated with EDTA before use to remove traces of copper.

Isoelectric focusing. This was performed with the LKB 8101 Ampholine column (Vesterberg & Svensson, 1966). The column was used to separate cytochromes $c(551)$ and $c(555)$. A variety of pH gradients were used (pH 3-10, 3-5 and 4-6); the grad-

ients were prepared from commercial Ampholine carrier ampholytes (LKB Instrument Inc., Rockville, Md., U.S.A.).

Spectral determinations. Cary 14 and 15 spectrophotometers were used to record the absorption spectra of the cytochromes c . The low-temperature reflectance spectra were measured with a modified Cary 14 spectrophotometer (Palmer & Beinert, 1964). The procedure of Flatmark (1964) was used for testing combination of CO with the cytochromes c .

Molecular-weight determinations. Ultracentrifugation of cytochrome c_4 was done with a Spinco model E ultracentrifuge equipped with a temperature-control (RTIC) unit set for 20°C. The meniscus-depletion method of Yphantis (1964) was used to determine the molecular weight and homogeneity of the cytochrome c_4 . The cytochrome c_4 was oxidized and dissolved in a buffer composed of 0.035 M-veronal and 0.065 M-NaCl, pH 7.6. The protein was centrifuged at 29500 and 33450 rev./min. for about 20 h each. A second solution of the cytochrome c_4 was dialysed against 6 M-guanidine-HCl buffer containing 0.5% 2-mercaptoethanol, pH 7.2, for 48 h at 4°C. The protein was centrifuged at 35600 rev./min. for 22 and 46 h. The partial specific volume of cytochrome c_4 was calculated from the amino acid composition by the method of Cohn & Edsall (1943).

The molecular weights of cytochromes $c(551)$ and $c(555)$ were determined by using the sodium dodecyl sulphate-polyacrylamide-gel technique of Shapiro *et al.* (1967) as modified by Dunker & Rueckert (1969).

The molecular weight of cytochrome $c(551)$ also was determined on Sephadex G-100 by the method of Andrews (1964). The column (2.5 × 85 cm) of Sephadex G-100 used in this determination was eluted with 0.05 M-sodium phosphate buffer, pH 7.4.

Amino acid analyses. Samples of the cytochromes c were hydrolysed in three-times-distilled 6 M-HCl (constant-boiling) in tubes which were sealed under vacuum and kept at 110°C for 12, 24 or 72 h. The analyses were performed on a Beckman automatic amino acid analyser 120 B or C by the method of Spackman *et al.* (1958). Performic acid oxidation of cytochrome c_4 was done by the method of Moore (1963). Performic acid oxidation of cytochromes $c(551)$ and $c(555)$ was not done and assumed values of $\frac{1}{2}$ Cys were used based on the presence of one haem. Losses of serine, threonine and tyrosine during hydrolysis were corrected for by extrapolation to zero time. Analyses for tryptophan were not done and assumed values are based on data of Swank & Burris (1969) and the similarity of u.v. spectra of the cytochromes c .

CNBr cleavage. The method of Gross & Witkop (1961) for CNBr cleavage of proteins was used with modifications (Steers *et al.*, 1965; Gross, 1967).

CNBr peptides of cytochrome *c*₄ were initially separated on a column (2.5 cm × 500 cm) of Sephadex G-75 developed with formic acid–propan-1-ol–water (3:2:5, by vol.). Whatman CM-52 and DE-52 cellulose were used for further separation and purification of the CNBr peptides. Column fractions were analysed by the quantitative ninhydrin procedure of Hirs *et al.* (1956) and by 'mapping' as described below.

Trypsin digestion. Denatured cytochrome *c*₄ (12 mg) (Margoliash *et al.*, 1962) was digested for 2 h with trypsin (1%, by wt.) as described by Heller & Smith (1966).

Peptide 'mapping'. Peptides from either the CNBr cleavage or trypsin digestion of the cytochromes *c* were 'mapped' on Whatman 3MM paper by descending chromatography; the chromatograms were developed for 18 h at room temperature with butan-1-ol–acetic acid–water (40:6:15, by vol.). Compounds on the dried papers were separated electrophoretically at 30 V/cm for 60 min in 2 M-acetic acid–0.7 M-formic acid buffer, pH 1.9. The dried papers were stained with ninhydrin–collidine (Margoliash & Smith, 1962).

N-Terminal residue. Fraenkel-Conrat *et al.* (1955) described the methods used for determination of N-terminal residues of proteins with fluorodinitrobenzene and phenylisothiocyanate. The paper-chromatography system of Blackburn & Lowther (1951) was used to analyse the products of the fluorodinitrobenzene reaction with cytochrome *c*₄. Paper chromatography and t.l.c. of the phenylisothiocyanate-N-terminal derivative were performed by the technique of Edman & Sjöquist (1956); solvent F was used for paper chromatography and solvent D for t.l.c. After chromatography, the papers or plates were dried and stained with fresh iodine–azide stain (Sjöquist, 1953). The phenylisothiocyanate N-terminal derivative was hydrolysed in 6 M-HCl at 150°C for 20 h and quantitative amino acid analysis performed on the hydrolysate.

E.p.r. (electron-paramagnetic-resonance) spectra. A modified X-band e.p.r. instrument with a helium boil-off gas temperature-control device was used to take the spectra of the cytochromes *c* (Beinert & Palmer, 1964; Palmer, 1967). It was necessary to oxidize the cytochromes *c* with K₃Fe(CN)₆ before the spectra were taken and to remove the excess of oxidant by either dialysis or gel filtration on Sephadex G-25. In general, the field positions of the observed e.p.r. resonances were determined with the Fieldial, but the proton probe was used for more accurate magnetic-field determinations (Palmer, 1967). Instrument conditions and sample concentrations are given in the legends of the Figures.

Relative oxidation–reduction potentials. The cytochromes *c* were oxidized with K₃Fe(CN)₆ and dialysed to remove excess of oxidant. Exact volumes of cyto-

chromes *c*₄ and *c*₅ of known concentration were pipetted into an anaerobic cuvette, and a weighed amount of sodium dithionite was added anaerobically (Orme-Johnson & Beinert, 1969). The amount of sodium dithionite was calculated to approximately half-reduce the mixture of cytochromes *c*. Spectra were recorded with the Cary 14 spectrophotometer before and after partial reduction and after excess of sodium dithionite was added.

Analytical determinations. The iron and copper analyses were done by the methods described by Van de Bogart & Beinert (1967) and nitrogen determinations by the method of Johnson (1941).

Sources of chemicals

Standard proteins used for molecular-weight determinations with Sephadex and sodium dodecyl sulphate–polyacrylamide gels were: Sigma Chemical Co. (St. Louis, Mo., U.S.A.) crystallized and freeze-dried bovine serum albumin (mol. wt. 66 500), grade V ovalbumin (mol. wt. 45 000), type II chymotrypsinogen (bovine pancreas) (mol. wt. 25 000), type III horse heart cytochrome *c* (mol. wt. 12 400), and Mann (Orangeburg, N.Y., U.S.A.) crystallized and freeze-dried sperm-whale myoglobin (mol. wt. 17 000). Coomassie Brilliant Blue R250 was a product of Imperial Chemical Industries Ltd., London, U.K. Ninhydrin, phenyl isothiocyanate, fluorodinitrobenzene and CNBr were products of Pierce Chemical Co., Rockford, Ill., U.S.A. The trypsin, from Worthington Biochemical Corp. Freehold, N.J., U.S.A., was twice crystallized and treated with L-1-chloro-4-phenyl-3-tosylamidobutan-2-one. Bathophenanthroline and the sulphonated salt of bathocuproine were products of G. Frederick Smith Chemical Co., Columbus, Ohio, U.S.A.

Results

Isolation and purification

The modification of the procedure of Swank & Burris (1969) provided a more rapid purification of the cytochromes *c*. The cytochrome *c*₄ could be obtained in the fully purified state in 3 days; 4 days were required for the other three cytochromes *c* because of an additional purification step. A typical elution pattern of the four cytochromes *c* from the DEAE-cellulose column is shown in Fig. 1. The overlap of the *c*(551) and *c*(555) peaks required that they be separated by isoelectric focusing to obtain the purified cytochromes *c*. The elution profile of a typical separation on an isoelectric-focusing column is shown in Fig. 2. The final purification of cytochromes *c*(551) and *c*(555) was performed by gel filtration on Sephadex G-75 or G-100. The recoveries of these four cytochromes *c* are summarized in Table 1.

Extraction of the cytochromes *c* from the cell paste

Fig. 1. Chromatographic separation of *A. vinelandii* cytochromes *c* on DEAE-cellulose

The extract from 99 g dry wt. of *A. vinelandii* was purified by batch absorption and then was dialysed and concentrated to 60 ml; it contained 75 mg of cytochrome *c* and was applied to a column of DEAE-cellulose (3.4 cm diam. $\times$ 10 cm high), which had been equilibrated with 5 mM-Tris-HCl buffer, pH 7.6, at 4°C. The extract was washed into the column with 5 mM-Tris-HCl buffer, then 10 ml of this same buffer was added and a linear gradient was formed from 500 ml of 0.01 M-Tris-HCl, pH 7.6, and 500 ml of 0.10 M-Tris-HCl, pH 7.6. The flow rate was about 1.2 ml/min and 5 ml fractions were collected. —, E_{270} ; ---, E_{550} ; ····, E_{554} . *c*(551), *c*(555), *c*₅ and *c*₄ represent the various cytochromes.

by sonication, as described by Swank (1969), yielded an increased proportion of cytochromes *c*(551) and *c*(555) compared with cytochromes *c*₄ and *c*₅; however, the total yield was lower. The butanol extraction procedure may preferentially extract cytochromes *c*₄ and *c*₅; Swank & Burris (1969) reported a similar observation.

Purity of the isolated cytochromes *c* generally was expressed by the purity ratio *R* (Table 1). When cytochrome *c*(551) or *c*(555) was examined electrophoretically on sodium dodecyl sulphate-polyacrylamide gels only one band was apparent when each gel was stained for protein (Dunker & Rueckert, 1969).

Additional properties of the cytochromes c₄ and c₅

Ultracentrifugation of cytochrome *c*₄. The pure cytochrome *c*₄ was centrifuged as both the native protein and the protein denatured in 6 M-guanidine-hydrochloride. The linear plots, which resulted from plotting the logarithm of the protein concentration in fringe units against the square of the distance of each fringe from the centre of rotation, i.e. log *c* versus x^2 , indicated that the preparation of cytochrome *c*₄ was homogeneous (Yphantis, 1964). Calculations of the molecular weights, from these plots and a calculated partial specific volume $\bar{v}$ = 0.715, indicated that the molecular weight of the

native protein was dependent on protein concentration. These molecular weights were extrapolated to infinite dilution, and a molecular weight of 20000 was found for the native protein. The molecular weight of denatured protein was not dependent on protein concentration and was calculated as 20000 ± 1000 .

Amino acid composition of cytochrome *c*₄. Amino acid analyses were performed on duplicate samples from three separate preparations of the protein. These were hydrolysed for 24 and 72 h. Performic acid oxidation was used to determine cysteic acid. The results of these analyses are shown in Table 2. No analyses were done for tryptophan.

CNBr peptide 'maps' of cytochromes *c*₄ and *c*₅. Cytochromes *c*₄ and *c*₅ were cleaved with CNBr for 24 h at room temperature. The resulting peptides were 'mapped' and located by ninhydrin staining. A diagrammatic representation of peptide locations on the papers is shown in Fig. 3. The haem-peptides could be identified by their orange colour before staining.

The CNBr peptides of cytochrome *c*₄ were separated initially by column chromatography on Sephadex G-75 developed with formic acid-propanol-water. The peptides were purified further by a combination of techniques, including paper chromatography, paper electrophoresis and column chromatography on CM- and DEAE-cellulose.

Fig. 2. Separation of cytochromes *c*(551) and *c*(555) by isoelectric focusing

A sample (2.2ml; 4mg) of cytochromes *c*(551) and *c*(555), purified on a DEAE-gradient column, was added in place of the light ampholyte solution (pH range 4–6) at the centre of the sucrose gradient. Isoelectric focusing was performed at 475 V and 15°C for 69 h. The column was drained by pumping with a Milton Roy Minipump at a rate of 1 ml/min. Fractions (2 ml) were collected and the pH was taken with the tubes maintained at 15°C. After dilution of each fraction with 1 ml of water the E_{270} was read. —, E_{270} ; ----, pH. Peaks as follows: (A) reduced *c*(555); (B) oxidized *c*(555); (C) reduced *c*(551); (D) modified reduced *c*(551); (E) oxidized *c*(551).

Fig. 3. Peptide 'maps' of the products of CNBr cleavage of cytochromes *c*₄ and *c*₅

Products of CNBr cleavage of the cytochromes *c* were 'mapped' by the procedures described in the text. The spots found on the paper are represented in the Figures by open ovals and are shown in their approximate positions. The darkened spot in the right-hand corner of each peptide 'map' marks the origin where the material was spotted on the paper. The 'H' next to one spot had an orange colour before the paper was stained and was assumed to be the haem peptide(s) spot. Chromatography was in butanol-acetic acid-water, as described in the Materials and Methods section. Peptide map (a) is from the CNBr cleavage of cytochrome *c*₄. The Roman numerals were used to identify the spots during separation and purification of the peptides. Peptide 'map' (b) is from the CNBr cleavage of cytochrome *c*₅.

Table 1. Summary of purification and yields of cytochromes *c*

Details are given in the Materials and Methods section.

	Amount of cytochrome <i>c</i> (mg/100g dry wt. of cells)	% recovery	Purity ratio <i>R</i> *
Butanol extract	141	100	
Batch absorption	91	65	0.38
Cytochrome <i>c</i> ₄			
DEAE-gradient	45	32	0.83
Sephadex G-100	42	30	1.25
Cytochrome <i>c</i> ₅			
DEAE-gradient	19	13	0.55
Isoelectric focusing	16	11	0.70
Sephadex G-100	14	10	1.15
Cytochrome <i>c</i> (551) plus <i>c</i> ₅			
DEAE-gradient	7	5.0	0.85
Cytochrome <i>c</i> (551)			
Isoelectric focusing	4	2.8	1.05
Sephadex	2	1.4	1.2–1.4
Cytochrome <i>c</i> (555)			
Isoelectric focusing	2	1.4	0.90
Sephadex	1	0.7	1.21

* *R* = absorbance at α peak (reduced)/absorbance at 270 nm (oxidized).

Table 2. *Amino acid compositions of cytochromes c₄, c(551) and c(555)*

The details of these amino acid analyses are given in the Materials and Methods section. The actual numbers of residues measured are given in parentheses.

	Cytochrome c ₄		Cytochrome c(551)		Cytochrome c(555)	
	(g of amino acid/ 100 g of protein)	(residues/ molecule)	(g of amino acid/ 100 g of protein)	(residues/ molecule)	(g of amino acid/ 100 g of protein)	(residues/ molecule)
Lys	5.4	11 (11.0)	9.0	8 (8.20)	9.2	8 (7.69)
His	2.1	4 (4.00)	2.3	2 (2.04)	1.3	1 (1.00)
Arg	3.4	6 (5.80)	1.2	1 (1.02)	2.8	2 (2.00)
Asp	11.7	26 (25.5)	7.9	8 (8.14)	16.8	15 (15.1)
Thr	4.0	10 (10.4)	3.6	8 (8.00)	3.2	3 (3.33)
Ser	4.5	11 (11.4)	3.7	7 (6.90)	4.0	5 (4.49)
Glu	10.9	21 (21.2)	13.9	13 (13.1)	4.0	4 (3.36)
Pro	5.3	13 (12.8)	4.4	5 (5.26)	3.2	3 (3.39)
Gly	7.1	28 (28.4)	5.6	10 (10.1)	9.0	15 (14.7)
Ala	11.5	34 (33.6)	7.9	12 (12.0)	13.0	18 (17.6)
½Cys	—	4 (2.79)	—	2*	—	2 or 4*
Val	2.3	6 (5.84)	3.9	5 (4.72)	3.4	4 (3.53)
Met	2.9	6 (6.08)	0.6	1 (1.08)	2.0	2 (1.67)
Ile	2.6	6 (5.68)	3.5	4 (3.71)	1.0	1 (1.18)
Leu	7.2	15 (15.4)	5.8	6 (6.00)	11.2	10 (9.53)
Tyr	3.6	6 (5.92)	2.5	3 (2.83)	1.0	1 (0.73)
Phe	3.3	5 (5.00)	1.6	1 (1.26)	0	0
Trp	—	0*	—	0*	—	1*
	87.8	212	77.4	96	85.1	95 or 97

* Not determined; assumed value.

Table 3. *Amino acid composition of CNBr peptides of cytochrome c₄*

The designations for the peptides are taken from Fig. 3. The peptides were separated and purified by gel filtration on Sephadex G-75 and by ion-exchange chromatography on DEAE- or CM-cellulose. Values are presented as whole residues per peptide.

Peptide ...	Ia-b	II	III	IV	V	VI	VII	Total
Lys	1	1	3	2	2	3	1	13
His	2-3	0	0	1	0	0	0	4
Arg	2	1	1	1	1	0	2	8
Asp	14	3	1	2	2	1	1	24
Thr	6	1	1	0	2	0	0	10
Ser	3	1	1	3	2	2	2	14
Glu	12	3	3	1	3	2	2	26
Pro	7	2	2	0	2	0	0	13
Gly	10	4	4	1	6	4	4	33
Ala	16	5	2	4	3	1	1	32
Val	4	1	1	0	1	0	0	7
Met	0	0	0	0	0	0	0	0
Ile	1	0	1	4	1	0	0	7
Leu	7	2	1	2	1	0	0	13
Tyr	2			1	0	0	0	3
Phe	4			0	0	0	0	4
Homoserine and homoserine lactone	2	1	1	0	1	1	1	7
Total	96	25	22	22	27	14	14	218

The amino acid compositions of these purified peptides are shown in Table 3. We were unable to resolve fully the haem peptides; hence the existence of two haem peptides was not established.

The peptide 'map' of the tryptic digest of cytochrome *c*₄ had only one haem peptide spot and 14 other ninhydrin-positive spots. This is a smaller number of peptides than expected from the amino acid composition; however, the high content of proline could influence the action of the enzyme.

*N-Terminal residue of cytochrome c*₄. Both the Sanger and Edman determinations (Fraenkel-Conrat *et al.*, 1955) for the *N*-terminal residue of cytochrome *c*₄ failed to yield an *N*-terminal amino acid. These results suggest that the *N*-terminal residue is blocked so that no amino group is available to react.

Fig. 4. Low-temperature reflectance spectra of ferrocytochromes *c*₄ and *c*₅

The spectra were taken with a modified Cary 14 spectrophotometer. The samples were in 5 mM-Tris-HCl buffer, pH 7.8, and were reduced with sodium dithionite before the spectra were taken. The absorbance scale is not given for these spectra, because direct quantitative measurement is not possible (Palmer & Beinert, 1964). (a) Spectrum of 0.1 mM-ferrocytochrome *c*₅ at -181°C; (b) spectrum of 0.1 mM-ferrocytochrome *c*₄ at -181°C.

Low-temperature reflectance spectra. The low-temperature reflectance spectra of the reduced cytochromes *c*₄ and *c*₅ are shown in Fig. 4. Similar spectra have been reported by Estabrook (1961) for low-temperature absorption spectra of cytochromes *c*₄ and *c*₅. The α peak of cytochrome *c*₄ is broad and may actually be split into two nearly equal maxima; however, the α peak of cytochrome *c*₅ is narrower and has a single maximum. The β peak of cytochrome *c*₅ has greater fine structure with a secondary peak at 530 nm; this region of the β peak of cytochrome *c*₄ only has a shoulder. Some difference also appears in the β -band region of the room-temperature absorption spectra of these two cytochromes *c* (Tissières, 1956; Neumann & Burris, 1959).

Properties of cytochromes *c*(551) and *c*(555)

Cytochrome *c*(555) was discovered during the determination of the isoelectric point of cytochrome *c*(551) by column isoelectric focusing. The similarity of the spectra of these cytochromes *c* to the spectra of cytochromes *c*₄ and *c*₅ suggests the possibility that these components are artifacts. The properties of cytochromes *c*(551) and *c*(555) were investigated to determine whether they were artifacts or distinct proteins.

Spectral properties of cytochromes c(551) and c(555). The similarity of the visible spectra of cytochromes *c*₄ and *c*(551) was discussed by Swank & Burris (1969). However, when the visible spectra of these two purified cytochromes are compared, a shoulder at 530 nm is present in the spectrum of cytochrome

Fig. 5. Spectrum of ferrocytochrome *c*(551)

A solution of the cytochrome *c*(551) in 0.05 M-sodium phosphate buffer, pH 7.4, was reduced with sodium dithionite crystals and the spectrum taken at room temperature with a Cary 15 spectrophotometer.

Fig. 6. Low-temperature reflectance spectra of ferrocytochromes *c*(551) and *c*(555)

The spectra were taken as described in Fig. 4. (a) Spectrum of 0.01 mM-ferrocytochrome *c*(555) at -181°C ; (b) spectrum of 0.1 mM-ferrocytochrome *c*(551) at -179°C .

c(551) (Fig. 5) but absent from the spectrum of cytochrome *c*₄ (compare with the spectrum of cytochrome *c*₄, Tissières, 1956). This shoulder at 530 nm is present in the spectra of cytochromes *c*₅ and *c*(555).

The low-temperature reflectance spectra of cytochromes *c*(551) and *c*(555) show this shoulder as a well separated secondary peak on the β peak (Fig. 6). The low-temperature spectra of cytochromes *c*₄ and *c*(551) have a dissimilar appearance; however, the low-temperature spectra of cytochromes *c*₅ and *c*(555) are quite similar.

The spectral properties of the four cytochromes *c* are presented in Table 4. Quantitative differences between the spectra are apparent. Spectra of the cytochromes *c*(551) and *c*(555) in the region 650–750 nm are given in Fig. 7. These spectra of the *A. vinelandii* cytochromes *c* do not have as clear a band at 695 nm as reported for mammalian cytochrome *c* (Theorell & Akesson, 1939; Schejter & George, 1964), but this band is temperature-dependent and might be more prominent in the spectra of the *A. vinelandii* cytochromes *c* at lower temperatures. The only major difference between the spectra of cytochromes *c*₅ and *c*(555) was in the 695 nm region where cytochrome *c*₅ has no significant absorption (Swank, 1969). The spectrum of cytochrome *c*(551) in Fig. 7 differs from that presented by Swank (1969) for the partially purified cytochrome.

Molecular-weight determinations of cytochromes c(551) and (555). The protein concentration of the sample was determined from nitrogen analysis on three samples. Cytochrome *c*(551) contained 0.46% iron, which corresponds to one iron atom per molecule of 12000 molecular weight.

Table 4. Comparison of the spectral properties of *Azotobacter vinelandii* cytochromes *c*

Values for ϵ_{mM} are in litre $\cdot$ mmol $^{-1} \cdot$ cm $^{-1}$. References: (1) Tissières (1956); (2) Swank & Burris (1969); (3) Neumann & Burris (1959); (4) Calculated from Swank (1969).

Cytochrome ...	<i>c</i> ₄ ϵ_{mM}	<i>c</i> ₅ ϵ_{mM}	<i>c</i> (551) ϵ_{mM}	<i>c</i> (555) ϵ_{mM}
α band (reduced)	23.8 (1)	26.1	28.2	22.7
α band (oxidized)	7.7 (1)	9.5	6.9	8.2
β band (reduced)	17.6 (1)	18.6	14.9	16.5
γ band (reduced)	157.2 (1)	157	140	152
γ band (oxidized)	115.8 (1)	132	106	126
NIR band (oxidized)*	0.46 (4)	0.05 (4)	0.61	0.57
270 nm (oxidized)	20.5 (1)	22.5	21.7	18.6
<i>R</i> (γ/α) (reduced)	6.6 (1)	6.0 (3)	5.0	6.7
	6.5 (3)			
<i>R</i> (γ/β) (reduced)	1.35 (1)	1.4 (3)	1.9	1.4
	1.3 (3)			
<i>R</i> ($\alpha/270$) (reduced)	1.16 (1)	1.19 (3)	1.30	1.22
	1.27 (3)	1.16 (2)		
	1.30 (2)			

* NIR, near-infrared band (695–700 nm).

Cytochrome *c*(551) was eluted from the Sephadex G-100 column at 308ml in comparison with 305ml for horse heart cytochrome *c*. The molecular weight of cytochrome *c*(551) was estimated to be 12000. The molecular weight of cytochrome *c*(555) was not determined by Sephadex-gel-filtration methods; however, cytochrome *c*(555) was eluted together with cytochrome *c*(551) from a column of Sephadex G-100.

In 10% sodium dodecyl sulphate–polyacrylamide gels, cytochromes *c*(551), *c*(555) and horse heart cytochrome *c* had mobilities relative to myoglobin

of 1.20, 1.23, and 1.20 respectively. The molecular weights of cytochromes *c*(551) and *c*(555) were estimated to be 12400 and 11800 respectively.

Amino acid compositions of cytochromes c(551) and *c*(555). The results of the amino acid analyses of cytochrome *c*(551) are given in Table 2. One 12h and two 24h acid hydrolyses were performed. The results of the amino acid analyses of the cytochrome *c*(555) are given in Table 2. Three 24h acid hydrolyses were performed. No analyses were done for half-cystine or tryptophan.

Isoelectric points were determined for the cytochromes *c*(551) and *c*(555) when these proteins were separated by isoelectric focusing. The results from five separate preparations are given in Table 5. On several occasions secondary peaks were observed for cytochrome *c*(551) but not for *c*(555); similar secondary peaks were found during the isoelectric focusing of *R. rubrum* cytochrome *c*₂ and were attributed to deaminated forms of the cytochrome (Sletten & Kamen, 1968). No secondary components arising from electrofocusing were reported by Swank & Burris (1969) for cytochromes *c*₄ and *c*₅.

The major fraction of cytochrome *c*(551) was isolated in the reduced form, whereas the major fraction of cytochrome *c*(555) was in the oxidized form. When the time between isoelectrofocusing of the cytochromes *c*(551) and *c*(555) and their separation from cytochromes *c*₄ and *c*₅ was minimized, more of the reduced forms of cytochromes *c*(551) and *c*(555) could be isolated. Not only did cytochrome *c*(555) exhibit autooxidation but it also showed a 15% decrease in the reduced α peak combination with CO.

CNBr peptide ‘maps’. Cytochromes *c*(551) and *c*(555) were cleaved with CNBr and the peptides

Fig. 7. Spectra of ferricytochromes *c*(551) and *c*(555) in the 650–750nm region

Spectra were taken at room temperature (22°C) with a Cary 15 spectrophotometer after oxidation of the cytochrome *c* solutions with potassium ferricyanide. (a) Spectrum of 0.16mm-ferricytochrome *c*(551); (b) spectrum of 0.27mm-ferricytochrome *c*(555).

Table 5. Isoelectric points as determined by isoelectric focusing at 15°C

Isoelectric focusing was performed in the pH ranges indicated with Ampholine carrier ampholytes and the pI of the eluted protein was determined by measuring the pH at 15°C. The cytochromes were identified by their visible spectra. Mean values are given $\pm$ S.D.

Cytochrome	Ampholyte pH range	pI	
		Reduced	Oxidized
<i>c</i> (551)	3–5	4.50	—
	3–10	4.75	5.30
	4–6	4.70 (4.95)*	5.11 (5.38)*
	4–6	4.55 (4.90)*	—
	4–6	4.64 (4.92)*	5.10
	Mean values	4.63 $\pm$ 0.13	5.17 $\pm$ 0.13
<i>c</i> (555)	3–5	—	4.22
	3–10	—	4.25
	4–6	—	4.28
	4–6	4.04	4.23
	4–6	4.10	4.32
	Mean values	4.07 $\pm$ 0.03	4.26 $\pm$ 0.06

* Secondary peaks or shoulders.

were 'mapped'. After the 'maps' were stained with ninhydrin, the location of the CNBr peptides was established. The 'map' of cytochrome $c(551)$ had two ninhydrin-positive spots, whereas the 'map' of cytochrome $c(555)$ had three ninhydrin-positive spots. When the CNBr peptide 'maps' of cytochromes c_4 and $c(551)$ were compared no similarity was apparent [only two spots for $c(551)$ as compared with Fig. 3a]. However, when the CNBr peptide 'maps' of cytochromes c_5 and $c(555)$ were compared, a similarity was apparent. Both 'maps' had three spots. Two of these spots had similar locations, however, the third spot had quite different locations. Limitation in the amounts of cytochromes $c(551)$ and $c(555)$ available prevented further investigation of the CNBr peptides.

Relative oxidation-reduction potentials

The relative oxidation-reduction potentials of the *A. vinelandii* cytochromes c were determined by partially reducing a mixture of two of the cytochromes c . The known spectral properties of the four cytochromes c and the Nernst equation were used to

determine the relative oxidation-reduction states of the components of the various mixtures. The relative potentials of the four cytochromes c are given in Table 6. The fully purified cytochromes c were in a solution of 0.1M-KCl-0.01M-Tris-HCl buffer, pH 7.3 (at 25°C), and determinations were done at or near 25°C.

E.p.r. spectra

Representative e.p.r. spectra of the four *A. vinelandii* cytochromes c are shown in Figs. 8 and 9. Despite all attempts to exclude copper during the extraction and purification procedure, the cytochromes c retained the copper (II) e.p.r. signals. Dialysis of the cytochromes c against EDTA solutions to remove the copper appeared to cause partial denaturation, and after dialysis of cytochromes $c(551)$ and $c(555)$ the e.p.r. spectra still showed copper(II) signals. After EDTA treatment, cytochromes c_4 and c_5 revealed no detectable copper; however, the cytochromes $c(551)$ and $c(555)$ had 0.23 and 0.09 atom of copper/atom of iron respectively.

Table 6. *Relative potentials of Azotobacter cytochromes c*

Equimolar mixtures of the two ferricytochromes c were partially reduced with sodium dithionite in an anaerobic atmosphere. The visible spectra before and after partial reduction were used to determine the difference in potential.

Cytochrome mixture	E_{551}	E_{555}	Concentrations (μ M)				Potential difference (mV)	
			c_4 (reduced)	c_4 (oxidized)	c_5 (reduced)	c_5 (oxidized)		
c_4 and c_5	0.216	0.220	4.2	3.3	3.8	3.7	c_4	5 +
$c(551)$ and c_5	0.230	0.260	3.5	2.5	6.7	0.8	$c(551)$	50 -
c_4 and $c(555)$	0.210	0.205	3.7	3.8	4.3	3.2	$c(555)$	8 +
c_4 and c_5^*							c_5	20 +

* From Tissières (1956).

Table 7. *Apparent g values from e.p.r. spectra of ferricytochromes c*

These values summarize the data of Figs. 8, 9 and 10 and were obtained under the conditions given in those Figures. Values for two mammalian cytochromes c are included for comparison. N.O., not observed.

Cytochrome	g_x	g_y	g_z
c_4	3.19; 3.58	2.30*	N.O.
c_5	3.28	2.30*	N.O.
$c(551)$	3.23	2.30*	N.O.
$c(555)$	3.31	N.O.	N.O.
Horse heart ferricytochrome c	3.05	2.26*	N.O.
Ox heart ferricytochrome†	3.06	2.24	1.24

* Peak maximum, not the crossover point, which lies toward higher field, but which could not be unambiguously determined.

† From Salmeen & Palmer (1968).

Fig. 8. E.p.r. spectra of ferricytochromes *c*₄ and *c*₅

The e.p.r. spectra were taken with a modified X-band Varian e.p.r. instrument with a temperature-control device. The spectra were taken at 12–13°K. The microwave frequency was approx. 9.2 GHz for all experiments, the power 3 mW, modulation 10 G, the time-constant 0.25 s and the scanning rate 1000 G/min. The abscissa indicates magnetic field increasing linearly to the right and the ordinate the first derivative of the microwave absorption in arbitrary units. All samples were in 5 mM-Tris-HCl buffer, pH 7.8. (a) Spectrum of 0.9 mM-ferricytochrome *c*₄ (gain 200); (b) spectrum of 0.75 mM-ferricytochrome *c*₅ (gain 200). The *g* values of peaks are shown.

Passage of cytochrome *c*(551) through Sephadex G-100 decreased the copper(II) signal relative to the haem iron e.p.r. signal; apparently the copper is easily dissociated.

In a further attempt to remove copper, approximately equimolar quantities of bathocuproine (sulphonated salt, G. Frederick Smith Chemical Co.) were added to the solutions of cytochromes *c*. The sulphonated bathocuproine binds copper(I), and in excess this chelator should convert all the copper(II) into the chelated copper(I) form, which is diamagnetic and has no e.p.r. signal. Fig. 10 gives the e.p.r. spectra of three of the cytochromes *c* treated for 16 h at 4°C with sulphonated bathocuproine. The copper(II) resonance peaks were decreased substantially and the haem iron resonance signals were not altered. Although the e.p.r. spectra do not show the sharp signal observed for mammalian cytochrome *c* (Salmeen & Palmer, 1968), the middle *g* value resonance peak appeared near *g* = 2.3; it is most prominent in the spectra of cytochrome *c*₄ (Fig. 10).

The *g* values determined for *A. vinelandii* cytochromes *c* are recorded in Table 7, together with two mammalian cytochromes *c* for comparison.

Discussion

Tissières & Burris (1956) extracted two cytochromes *c* from *A. vinelandii* and designated them

cytochromes *c*₄ and *c*₅. Neumann & Burris (1959; Fig. 2) accounted for about 90% of the cytochromes as *c*₄ and *c*₅ but had additional peaks from CM-cellulose with absorption at 550 nm. Swank & Burris (1969) partially purified a third cytochrome *c* from this organism. We have fully purified this third cytochrome *c* [cytochrome *c*(551)] and have separated and purified a fourth cytochrome *c* [cytochrome *c*(555)]. The roles of cytochromes *c*(551) and *c*(555) in *A. vinelandii* are unknown.

The spectrum of cytochrome *c*(551) is very similar to that of cytochrome *c*₄ (Swank & Burris, 1969); however, the spectrum of cytochrome *c*(551), unlike that of cytochrome *c*₄, has a shoulder at 530 nm. The molecular weight of cytochrome *c*(551) is 12200, whereas cytochrome *c*₄ has a molecular weight of 24000 for a single polypeptide chain which has two covalently bound haem groups (Tissières, 1956; van Gelder *et al.*, 1968; Swank & Burris, 1969).

We have given additional evidence for the dihaem nature of cytochrome *c*₄. (1) Ultracentrifugation of the protein under denaturing conditions indicated a molecular weight similar to that found for the native protein. (2) The peptide 'map' and amino acid compositions of the CNBr cleavage peptides suggested that the protein may be a covalently joined dimer with two very similar haem peptides. (3) The e.p.r. spectrum of cytochrome *c*₄ has two resonance signals which could arise from two haem iron centres

Fig. 9. E.p.r. spectra of ferricytochrome *c*(551) and *c*(555)

The instrument conditions for these spectra were the same as those given in Fig. 8. (a) Spectrum of 1.1 mM-ferricytochrome *c*(555) (gain 125); (b) spectrum of 0.4 mM-ferricytochrome *c*(551) (gain 250). The *g* values of peaks are shown.

in differing environments. Other spectral studies of cytochrome *c*₄ had revealed no difference in the two haem centres postulated for the protein (van Gelder *et al.*, 1968; Swank & Burris, 1969).

Comparison of the properties of cytochromes *c*₄ and *c*(551) suggests that cytochrome *c*(551) is not a subunit of the cytochrome *c*₄, but that cytochrome *c*(551) is a distinct protein. However, the amino acid sequences of these two proteins may reveal a closely related genetic origin.

A copper signal appears in the e.p.r. spectrum of cytochrome *c*(551). Copper, if active in native cytochrome *c*(551), could confer a different function from that of cytochrome *c*₄. No extra function has been found, and the copper probably is inactive; the oxidation-reduction potential of cytochrome *c*(551) was about 50 mV more negative than that of cytochrome *c*₄.

The similarity of the visible spectra (including low-temperature spectra) of cytochromes *c*₅ and *c*(555) suggests that these two proteins have very similar structures. However, their molecular weights differ; native cytochrome *c*₅ exists as a dimer of 13100-molecular-weight monomers (Swank & Burris, 1969), and cytochrome *c*(555) is a monomer. One might attribute differences observed in the properties of cytochrome *c*₅ and *c*(555) which have been found (isoelectric points, visible and near-i.r. spectral properties, e.p.r. spectra) to the fact that these properties of cytochrome *c*₅ were determined with the dimer form, as monomers and dimers of cytochrome *c* have been reported to differ in certain of these properties (Schejter *et al.*, 1963; Margoliash

Fig. 10. E.p.r. spectra of sulphonated bathocuproine-treated ferricytochromes *c* from *A. vinelandii*

The instrument conditions for these spectra were the same as those given in Fig. 8. A portion (10 μ l) of 0.1 M-sulphonated bathocuproine was added to each sample, and the samples were stored overnight at 4°C before the spectra were taken. The gain setting was 200 for these spectra. Spectrum (a) 0.9 mM-ferricytochrome *c*₄; spectrum (b) 0.75 mM-ferricytochrome *c*₅; spectrum (c) 0.4 mM-ferricytochrome *c*(551). The *g* values of peaks are shown.

& Lustgarten, 1962; Henderson & Rawlinson, 1961). However, the peptide 'maps' of the CNBr cleavage products of cytochromes c_5 and $c(555)$ are different. The amino acid compositions that we observed and those reported by Swank & Burris (1969) differ, but if the compositions are normalized to a similar total number of amino acids the differences are small.

Although cytochromes c_5 and $c(555)$ exhibit differences, cytochrome $c(555)$ may be a monomer derived from cytochrome c_5 , and our data are inadequate to establish unequivocally that they are distinct proteins.

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