Amino Acid Composition, Heme Content, and Molecular Weight of Cytochrome c_3 of *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris**

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ABSTRACT: The cytochromes c_3 of *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* have similar molecular weights (13,000–14,700 depending upon the method of determination), partial specific volumes (0.72–0.73 ml/g), polypeptide chain lengths (106–108 residues), and contain three heme groups per mole of protein. They differ in their isoionic points and amino acid composition. The ratio of aspartic acid to glutamic acid is inverted in the two cytochromes. *D. vulgaris* cytochrome c_3 contains 1 mole of arginine/mole of protein and does not contain isoleucine whereas *D. desulfuricans* cytochrome c_3 contains 1 mole of isoleucine/mole of protein and does not contain arginine.

Previous work (Drucker and Campbell, 1969) has shown that the cytochromes c_3 of *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* are similar in their redox potentials, sedimentation constants, and calculated molecular weights. They differ, however, in their electrophoretic behavior on Geon and polyacrylamide gel electrophoresis and do not share a common precipitating antigenic determinant as judged by immunodiffusion data (Drucker and Campbell, 1969). This paper reports the amino acid composition, the heme content, and some physical properties of the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris*.

Methods and Materials

The cytochromes c_3 of *D. desulfuricans*[1] and *D. vulgaris* (strain Hildenborough) were isolated and purified to homogeneity as described by Drucker and Campbell (1969).

Amino acid analyses were performed by procedures similar to those described by Moore and Stein (1963). Prior to acid hydrolysis the heme was removed by the method of Ambler (1963). Alkylation of the cysteine residues of the apoprotein was carried out in a solution containing 0.5 M Tris-HCl (pH 8.6), 0.3 M mercaptoethanol, 6 M guanidine hydrochloride, and 0.15 M iodoacetic acid (recrystallized). The reaction mixture was degassed and placed under N_2 in the dark. The lyophilized alkylated apoprotein (3–4 mg) was dissolved in 0.1 ml of distilled water, 5 ml of 6 N HCl was added, and the tubes were sealed under vacuum. Duplicate samples were hydrolyzed at 110° for 24, 48, and 72 hr. HCl was removed by repeated rotoevaporation. The hydrolysates were dissolved in pH 2.2 buffer (Moore and Stein, 1954) and analyzed on a Beckman-Spinco amino acid analyzer. Tryptophan was estimated by the method of Spies and Chambers (1949). Horse heart cytochrome *c* (Sigma type III) and bovine serum albumin (Mann Research Laboratories) served as control proteins for the tryptophan determination.

The heme content of the two cytochromes was determined by specific extinction measurements, by iron analyses, and by extinction of the pyridine hemochromes. To determine the extinction coefficient dry desalted cytochrome c_3 (Drucker and Campbell, 1969) was dissolved in 0.02 M NH_4HCO_3 to give a stock solution containing 13 mg/ml. Samples were diluted as needed with 0.02 M NH_4HCO_3 to a volume of 1.0 ml, reduced with solid sodium dithionite and the absorbance at 552 nm read on a Zeiss spectrophotometer. Heme content was determined on the basis of an extinction coefficient (E) of 28 (Margoliash and Frohwirt, 1959). Corrections were made for ash and moisture content. In all cases where it was necessary to correct for ash and moisture content, the dry weight of the desalted (electrodialyzed) protein was determined by drying in a vacuum oven at 100° until a constant weight was reached. Ashing was carried out in a crucible at 550° in a muffle oven.

The heme content was also determined from the alkaline pyridine hemochrome spectrum. The cytochromes were placed in a solution containing 3 M pyridine and 0.075 M NaOH. After reduction with a few crystals of sodium dithionite, the absorbancy was read at 550 nm.

Iron analyses were performed by dissolving the lyophilized, desalted proteins in 0.01 M NH_4HCO_3 containing 5×10^{-4} M 4,5-dihydroxy-*m*-benzenedisulfonic acid disodium salt (Tiron, Eastman Chemical Co.), and 5×10^{-4} M 1,10-*o*-phenanthroline (Eastman Chemical Co.). The protein solution was stirred for 30 min in an ice bath. The chelators and salts were removed by layering the protein onto a column (2 × 31 cm) of Sephadex G-25 (fine bead form) that had been equilibrated with 0.1 M NH_4HCO_3. By use of a flow rate of 40

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[1] The strain of *D. desulfuricans* used was cholinicus, American Type Culture Collection 13541, referred to here as VC.

TABLE I: Amino Acid Composition of Cytochromes c_3 of *Desulfovibrio vulgaris* and *Desulfovibrio desulfuricans*.

	Type of Cytochrome[a] (moles/mole of Protein)			
	D. vulgaris		*D. desulfuricans*	
Residue	*b*	*c*	*b*	*c*
Lysine	20.31	20	17.14	17
Histidine	8.75	9	8.56	9
Arginine	1.00	1	0	0
Aspartic acid	12.00	12	6.65	7
Threonine	5.00	5	5.00	5
Serine	6.00	6	6.24	6
Glutamic acid	5.35	5	11.10	11
Proline	3.82	4	5.76	6
Glycine	9.35	9	7.54	8
Alanine	10.09	10	9.36	9
½-Cystine[d]	7.82	8	8.22	8
Valine	6.82	7	9.12	9
Methionine	3.18	3	1.03	1
Isoleucine	0	0	1.23	1
Leucine	2.27	2	6.24	6
Tyrosine	2.55	3	1.71	2
Phenylalanine	2.00	2	3.02	3
Tryptophan	0	0	0	0
Total number of residues		106		108
Molecular weight of apoprotein	11,621		11,831	
Add 3 hemes	1,845		1,845	
Molecular weight	13,466		13,676	

[a] The compositions reported are for the Hildenborough strain of *D. vulgaris* and strain VC of *D. desulfuricans*. Corrections were made for the destruction of serine and threonine by extrapolation to zero time of hydrolysis. [b] Moles/mole of protein as calculated from amino acid analysis. Recovery of amino acid residues (g/100 g of protein) was 99.71 and 100.47% for c_3 of *D. vulgaris* and *D. desulfuricans*, respectively. [c] Moles/mole of protein to nearest whole integer. [d] Determined as carboxymethylcysteine.

TABLE II: Heme Content of Cytochromes c_3.

Cytochrome	$E_{552\,nm}^{reduced}$	Heme Content[a] (moles/mole of Protein)	Iron Content (%)	Heme Content[b] (moles/mole of Protein)
D. vulgaris	84	3.0	1.21	2.92
D. desulfuricans	74	2.64	1.17	2.86
D. gigas[c]	76	2.71	1.24	2.98

[a] Heme content was estimated from the $E_{552\,nm}^{reduced}$ (28 cm^{-1}/μmole per ml) of mammalian cytochromes *c*, proteins of approximately the same molecular weight as the cytochromes c_3 and containing 1 mole of heme/mole of protein. [b] Estimated on the basis of the mammalian cytochrome *c* iron content of 0.45% (corresponding to one heme per molecule) and molecular weights of 13,466, 13,676, and 13,425 for the cytochromes c_3 of *D. vulgaris*, *D. desulfuricans*, and *D. gigas*, respectively. [c] Based on the data of Le Gall *et al.* (1965), and Bruschi-Heriaud and Le Gall (1967).

ml/hr, the cytochromes c_3 were eluted 0.5 column volumes ahead of a blue Tiron–iron complex. The protein was then lyophilized, dissolved in glass-distilled water, and lyophilized again. A stock solution of the protein (13 mg/ml) was prepared in 0.01 M NH_4HCO_3. A sample of the stock solution was placed in a 10-ml volumetric flask, lyophilized, and the iron content determined by the method of Cameron (1965).

To determine the isoionic point, the lyophilized proteins were dissolved in glass-distilled water at a concentration of 20 mg/ml. The pH was adjusted to 4.0 with 0.1 N acetic acid to displace the pH of the protein solution from its isoelectric point. The proteins were then electrodialyzed (Katz and Ellinger, 1963) until the current and pH were constant. At this point, the pH of the solution was determined using a Radiometer TTT1C pH meter and PHA630T scale expander with a Radiometer combination electrode type C. Because the values for the isoionic points of the cytochromes were high, all solutions, including the wash solutions were CO_2 free. Ion-exchange membranes (Ionics) were employed in the cell.

The partial specific volume was calculated from amino acid analyses employing the table of $\bar{V}$ given by Schachman (1957). The partial specific volume was also determined on electrodialyzed, lyophilized, salt-free protein dissolved in 0.01 M sodium phosphate buffer, pH 7.6, at a concentration of 20 mg/ml; 2- or 5-ml pycnometers were used. Dry weight and ash content were determined on the samples prior to use.

Sedimentation–equilibrium runs were performed in a Spinco Model E analytical ultracentrifuge at 20,410 rpm using 30-mm double-sector cells in an An-E rotor. The proteins were dissolved in 0.01 M ammonium phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer; the protein concentration was normally 0.15%. Schlieren optics were employed and a Kodak Wratten No. 29 filter was placed over the light source. Pictures of the schlieren pattern were recorded on Kodak type 1-N plates. Measurement of the boundaries was done with a Gaertner microcomparator according to Schachman (1957).

Results

Amino Acid Composition. Table I shows the amino acid composition of the cytochromes c_3 of *D. desulfuricans* and *D. vulgaris* as calculated by the mole ratio method. Corrections were made for the ash and moisture content of the proteins and for destruction of certain amino acids as a function of hydrolysis time. The mole ratio calculations were based on one arginine/mole for the cytochrome c_3 of *D. vulgaris* and one isoleucine/mole for the cytochrome c_3 of *D. desulfuricans*. Tryptophan was not detected in either cytochrome by the method of analysis employed.

TABLE III: Heme Content of Cytochromes c_3 from Pyridine Hemochrome Data.

Hemochrome	$E_{550\,nm}^{reduced}$	Heme Content[a] (moles/mole of Protein)
D. vulgaris	88.3	3.03
D. desulfuricans	78.9	2.71

[a] Heme content was estimated from the $E_{550\,nm}^{reduced}$ (29.1 cm^{-1}/μmole per ml) of heme *c* (Falk, 1964).

TABLE IV: Molecular Weights of Cytochromes c_3.

Method of Determination	Cytochrome c_3 Mol Wt	
	D. vulgaris	*D. desulfuricans*
Sedimentation–equilibrium	14,400	13,800
Svedberg equation[a]	14,700	13,500
Amino acid composition	13,466	13,676

[a] Data from Drucker and Campbell (1969).

Heme Content. The heme content of the two cytochromes c_3 was calculated from the extinction at 552 nm in the reduced form, on the basis of the iron content and from the absorbancy at 550 nm of the alkaline pyridine hemochromes. The data in Tables II and III strongly suggest that these cytochromes possess three heme groups per mole of protein.

Isoionic Point. The experimentally determined isoionic points were 9.91 and 8.0 for the cytochromes c_3 of *D. vulgaris* and *D. desulfuricans*, respectively. These values agree well with the values of 10.0 and 7.2 (for the corresponding cytochromes) calculated from the amino acid compositions using the method outlined by Martin (1964). In calculating the isoionic points from the amino acid compositions, it was assumed that the six propionic acid residues on the three hemes are buried in the molecule in an un-ionized form. It was also assumed that the carboxyl and amino termini of the protein were either blocked or buried. The isoionic points obtained are consistent with the electrophoretic behavior of these cytochromes (Drucker and Campbell, 1969).

Partial Specific Volume ($\bar{V}$). The $\bar{V}$ values for the cytochromes c_3 of *D. vulgaris* and *D. desulfuricans* were 0.732 and 0.725 ml per g, respectively, as determined by pycnometric determinations. The corresponding values calculated from the amino acid compositions were 0.72 and 0.73 ml per g.

Molecular Weight. Molecular weights were calculated from sedimentation–equilibrium data at two protein concentrations. All plots for the equilibrium data were linear when plotted according to the equation described by Schachman (1957). The molecular weights showed no concentration dependence over a tenfold range. Table IV presents the molecular weights obtained from sedimentation–equilibrium, amino acid composition, and sedimentation velocity data. The values obtained by the three methods are in excellent agreement and show that the two cytochromes have very similar molecular weights.

Discussion

Table I clearly shows that the cytochromes c_3 of *D. vulgaris* and *D. desulfuricans* differ markedly in their amino acid composition. The ratio of aspartic acid to glutamic acid is inverted in the two cytochromes and *D. vulgaris* cytochrome c_3 contains one residue of arginine and no isoleucine whereas *D. desulfuricans* cytochrome c_3 contains one residue of isoleucine and no arginine. The amino acid composition reported here for the cytochrome c_3 of the Hildenborough strain of *D. vulgaris* is in good agreement with that reported by Coval *et al.* (1961) and by Ambler (1968) in an abstract on the sequence of this protein. It should be noted that prior to the taxonomic revision of this species by Postgate and Campbell (1966) the Hildenborough strain was classified as *D. desulfuricans*, thus Coval *et al.* (1961) refer to this strain as *D. desulfuricans*. The amino acid composition of cytochrome c_3 of *D. gigas* (Bruschi-Heriaud and Le Gall, 1967) is different from that of *D. vulgaris* or *D. desulfuricans*. Ambler *et al.* (1969) have reported the partial sequence of *D. gigas* c_3. Forty-nine residues match those in *D. vulgaris* c_3 if five deletions in *D. vulgaris* c_3 and one in *D. gigas* are allowed. Fifty residues are not identically matched but of these only twenty-three require separation by a single mutation (Ambler *et al.*, 1969).

The values in Table II for the iron content, extinction coefficient, and the number of heme groups (Tables II and III) differ from those reported by Postgate (1956) and by Horio and Kamen (1961) for the cytochrome c_3 of the Hildenborough strain of *D. vulgaris*. These investigators reported that the cytochrome c_3 of this species contains two hemes per mole of protein instead of the three found in our study. However, these proteins are exceedingly difficult to desalt, making true dry weight determinations very difficult. Corrections were made for the ash content and moisture in the cytochromes examined in our study. The iron assay (Sandell, 1950) used by the previous investigators is not as sensitive as the assay we employed. Our attempts to use the Sandell assay gave inconsistent results. The difficulty with this assay is in the digestion of the heme groups to release the iron. The cytochrome c_3 of *D. gigas* (Le Gall *et al.*, 1965) also has an iron content and extinction coefficient at 552 nm in the reduced state consistent with a three heme protein based on a molecular weight of 13,425 (Bruschi-Heriaud and Le Gall, 1967).

How the three hemes are attached to the apoproteins is not known. The sequence reported by Ambler (1968) for the c_3 of *D. vulgaris* indicates two sites for cytochrome *c* type heme attachment, *e.g.*, the sequence Cys-x-y-CysHis. How the third heme is attached remains an interesting problem for future research.

The close agreement in the $\bar{V}$ values obtained by pycnometric determinations and by amino acid composition suggests that the $\bar{V}$ of the three heme groups does not change the overall $\bar{V}$ of the cytochrome c_3 to any significant extent. This is different from observations on mammalian cytochrome

TABLE V: Grouping of Amino Acid Residues in Cytochromes c_3.

Residue Type	Cytochromes c_3	
	D. vulgaris	*D. desulfuricans*
Acidic	17[a]	18
Basic	30	26
Sulfur containing	11	9
Proline	4	6
Small-chain aliphatic[b]	19	17
Large-chain aliphatic[c]	9	16
Total aliphatics	28	33
Alcoholics	11	11
Aromatics	5	5
Total number of residues	106	108

[a] Numbers are based on the data presented in Table I. [b] Glycine, alanine. [c] Valine, isoleucine, leucine.

c where good agreement has not been achieved by the two methods of determination (Margoliash and Schejter, 1966). Since the heme groups in the cytochromes c_3 make up about 15% of the total molecular weight of the protein, it appears that the $\bar{V}$ of the heme must be similar to the average $\bar{V}$ of the amino acids in the protein.

Drucker and Campbell (1969) suggested that conservative amino acid substitutions in the cytochromes c_3 of *D. vulgaris* and *D. desulfuricans* could be responsible for the observed differences in immunological reactivity and electrophoretic behavior. Such substitutions could result in cytochromes c_3 that have similar functions but differ in their structure. The data presented in this paper tend to support this suggestion. It is clear that the proteins have very similar molecular weights (Table IV), partial specific volumes, polypeptide chain lengths, and number of hemes even though they differ markedly in their amino acid compositions. Grouping the amino acids as in Table V, however, shows that there are certain overall similarities in the amino acid compositions of the two cytochromes. These similarities coupled with the other similarities noted here and by Drucker and Campbell (1969) suggest that the differences may result from conservative amino acid substitutions in the two cytochromes. Amino acid sequence studies now in progress by Ambler (1968) and by E. B. Trousil and L. L. Campbell (unpublished data) should give additional information on this point.

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