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CYTOCHROME c_3.

A CLASS OF ELECTRON TRANSFER HEME PROTEINS FOUND IN BOTH PHOTOSYNTHETIC AND SULFATE-REDUCING BACTERIA

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SUMMARY

Cytochromes *c* with absorption spectra and oxidation–reduction potentials very similar to those of *Desulfovibrio vulgaris* cytochrome c_3 were isolated from the photosynthetic bacteria *Chloropseudomonas ethylicum*, *Rhodopseudomonas spheroides* and *Rps. palustris*, as well as from the blue-green alga *Anacystis nidulans*. Analysis of the highly purified *C. ethylicum* cytochrome *c*-551.5 indicates that this protein contains three heme groups per molecule, as compared to Desulfovibrio cytochrome c_3, which has four hemes. It is suggested that a special class of cytochromes *c* be recognized which has absorption spectra and oxidation–reduction potentials similar to those of Desulfovibrio cytochrome c_3.

INTRODUCTION

The low redox potential cytochrome c_3 found in the sulfate-reducing bacteria Desulfovibrio has been considered unique to this genus. However, *c*-type cytochromes having properties similar to Desulfovibrio cytochrome c_3 have been isolated from a number of photosynthetic bacteria.

Desulfovibrio vulgaris cytochrome c_3 was first isolated by POSTGATE[1] who characterized the cytochrome as a probable diheme protein with the very low redox potential $E_{m,7} = -205$ mV. Subsequently HORIO AND KAMEN[2] crystallized the cytochrome for which again two hemes were determined per molecule of $11.3 \cdot 10^3$ molecular weight. A study by DRUCKER *et al.*[3] suggests that the heme content of cytochrome c_3 is closer to three than to two. The amino acid sequence determined by AMBLER[4] included four possible heme binding sites. From this sequence one may calculate the formula weight of $14.1 \cdot 10^3$ with 107 amino acids, if four hemes are assumed present.

Cytochrome *c*-549 isolated from the blue-green alga *Anacystis nidulans* by HOLTON AND MYERS[5,6] has a redox potential of — 260 mV at pH 7.0 and absorption spectra similar to those of cytochrome c_3. Unlike Desulfovibrio cytochrome c_3,

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Anacystis cytochrome *c*-549 binds CO (refs. 5 and 6). Cytochromes *c*-551.5 isolated from the green bacterium *Chloropseudomonas ethylicum* 2K by OLSON AND SHAW[7] and from the purple bacterium *Rhodopseudomonas spheroides* ATCC 17023 by MEYER *et al.*[8] also have absorption spectra similar to Desulfovibrio cytochrome c_3. Similarly, cytochrome *c*-551.5 isolated from *Rps. palustris* ATCC 17007 by BARTSCH, HORIO AND KAMEN[9] was found to have absorption spectra like Desulfovibrio cytochrome c_3 and a redox potential of — 150 mV.

The extent of similarity or difference between the low redox potential photosynthetic bacterial cytochromes *c* and Desulfovibrio cytochromes c_3 may be important in terms of function. By analogy with the mitochondrial cytochromes *c*, if the cytochrome c_3 proteins are not only similar in the immediate area surrounding the heme, but are homologous in amino acid sequence as well, then the possibility of having similar electron transport functions is strengthened.

This work was pursued with the intention of further characterizing the photosynthetic bacterial cytochromes and determining the extent of similarity to the Desulfovibrio cytochromes c_3.

EXPERIMENTAL AND RESULTS

C. ethylicum cytochrome 551.5

C. ethylicum 2K, a pure culture of which was provided by Dr. R. C. Fuller, was grown on the modified medium of KONDRAT'EVA (see ref. 10) in 20-l carboys at 30°. 0.5 kg wet weight packed cells were suspended in 3 l 0.1 M potassium phosphate buffer (pH 7.0) and stirred overnight. The cell suspension was then processed in the Sorvall–Ribi cell fractionator operated at 20000 lb/inch^2 and about 20°. The suspension was centrifuged for 40 min at 30000 × *g* in a Sorvall RC-2B centrifuge and then in the Spinco centrifuge using the Type 42 rotor for 90 min at 205700 × g_{max}.

The supernatant solution was fractionated on a DEAE-cellulose column (Brown Co., DEAE Selectacel Standard, 5.6 cm × 14 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 7.0). Ferredoxin and rubredoxin[11] were adsorbed on the top three quarters of the column, which was then washed with 2 l of the extraction buffer. The effluent and washings of this column were desalted with buffer change to 1 mM Tris–HCl buffer (pH 7.3) on Sephadex G-25-C. This cytochrome solution was then adsorbed on a DEAE-cellulose column (Brown Co., Type 20, 5.6 cm × 20 cm) equilibrated with 1 mM Tris–HCl buffer (pH 7.3). Cytochrome *c*-555 (ref. 7) was adsorbed in a sharp band about one-third of the way down the column and eventually was eluted with 20 mM Tris–HCl buffer (pH 7.3) *plus* 40 mM NaCl to yield about 4 μmoles of the cytochrome. A flavoprotein was eluted as a sharp band behind the cytochrome *c*-555 with 20 mM Tris–HCl buffer (pH 7.3) *plus* 60 mM NaCl. Only one-fourth of the cytochrome *c*-551.5 was adsorbed on the column and was then eluted at all salt concentrations between 1 mM Tris–HCl buffer (pH 7.3) and 20 mM Tris–HCl buffer (pH 7.3) *plus* 0.5 M NaCl.

To recover cytochrome *c*-551.5, the unadsorbed solution *plus* the cytochrome *c*-551.5 eluted from the DEAE-cellulose column was fractionated with $(NH_4)_2SO_4$. Most of the particle-bound carotenoid and chlorophyll was precipitated with 40% saturated solution $(NH_4)_2SO_4$, while only a small fraction of the cytochrome precipitated. Most of the cytochrome precipitated between 40–100% saturation, and that

which remained in suspension was filtered out by passage through a small DEAE-cellulose column from which it was eluted with water. The total yield at this state was about 40 μmoles of cytochrome *c*-551.5 heme. Once separated from the bulk of the extracted proteins the cytochrome could be chromatographed on DEAE-cellulose from which most of the cytochrome was eluted with approx. 0.2 M NaCl in 20 mM Tris–HCl buffer (pH 7.3). The cytochrome was desalted with a Sephadex G-25-C column, adsorbed on a small DEAE-cellulose column and eluted with 20 mM Tris–HCl buffer (pH 7.3) *plus* 0.5 N NaCl. The concentrated solution was then chromatographed on a Sephadex G-100 column (8 cm × 110 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.3) *plus* 0.2 M NaCl. The main cytochrome *c*-551.5 band was fractionated with $(NH_4)_2SO_4$ between 80 and 100 % saturation. On a Sephadex G-25-F column, the purified protein separated into two bands, which had nearly identical absorption spectra. For the best fractions, the ratio $A_{280\,nm,\,oxidized}/A_{418\,nm,\,reduced}$ ($A_{280\,nm,\,ox.}/A_{418\,nm,\,red.}$) = 0.04 was obtained. The absorption spectra are given in Fig. 1.

Rps. spheroides cytochrome c-551.1

Rps. spheroides ATCC 17023 cytochrome *c*-551.5 was prepared by the method of MEYER *et al.*[8]. The procedure was quite similar to that described for preparation of cytochromes of *C. ethylicum*. 1 kg wet weight of packed cells, grown in 1-l bottles in a medium essentially that of COHEN-BAZIRE *et al.*[10,12], was suspended in 0.1 M Tris–HCl

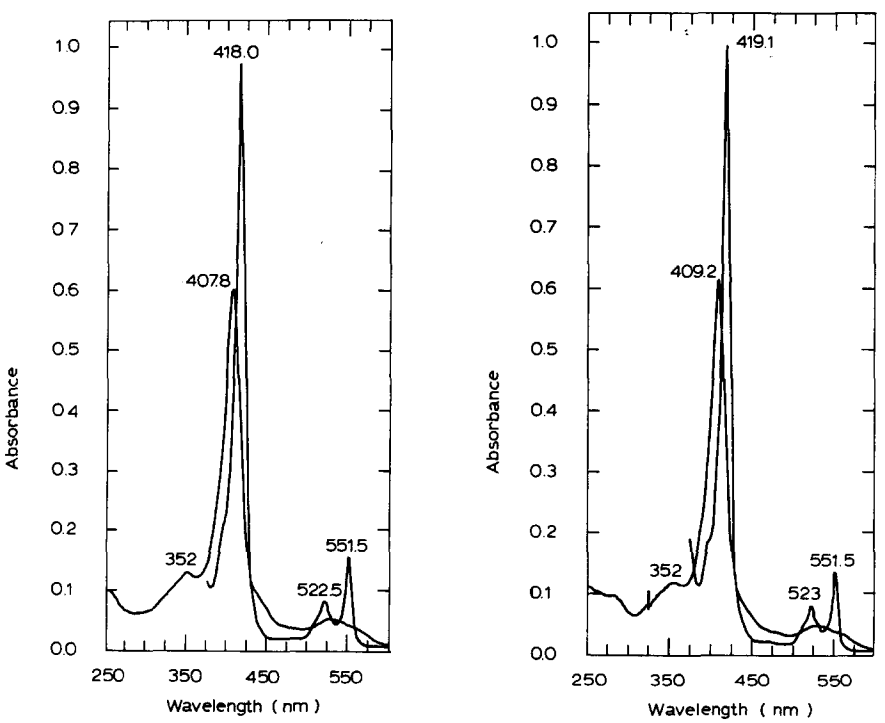

Fig. 1. Absorption spectra of *C. ethylicum* cytochrome *c*-551.5 in 0.1 M potassium phosphate buffer (pH 7.0). The spectrum of the reduced form was traced following addition of solid $Na_2S_2O_4$.

Fig. 2. Absorption spectra of *Rps. spheroides* cytochrome *c*-551.5 in 0.1 M potassium phosphate buffer (pH 7.0). The spectrum of the reduced form was traced following addition of solid $Na_2S_2O_4$.

buffer (pH 7.3) and treated as for *C. ethylicum*. Most of the cytochrome *c* mixture was adsorbed on the top half of a DEAE-cellulose column (Type 20, 5.6 cm × 30 cm, equilibrated with 1 mM Tris–HCl buffer (pH 7.3)). The cytochromes were eluted with a stepwise gradient of Tris–HCl buffer (pH 7.3) from 1 to 20 mM, and then increasing in steps of 20 mM NaCl. The band containing cytochrome *c*-551.5 appeared between the cytochromes *c*. and *c*-554 bands. It was eluted with 20 mM Tris–HCl buffer (pH 7.3) *plus* 0.12 M NaCl. The yield was about 4 μmoles heme. This cytochrome was purified by a combination of $(NH_4)_2SO_4$ fractionation (60–90 % satn.), DEAE-cellulose chromatography, and Sephadex G-100 chromatography. The best ratio of $A_{280\,nm}/A_{419\,nm}$ was 0.08. The absorption spectra of this preparation are given in Fig. 2.

Rps. palustris cytochrome c-551.5

Cytochrome *c*-551.5 was prepared from *Rps. palustris* ATCC 17007 by a modification of the technique of BARTSCH *et al.*[9] The bacteria were grown in the same manner as *Rps. spheroides*. This cytochrome was difficult to prepare for two reasons — it was present in small concentrations and was eluted from a DEAE-cellulose column over a wide range of buffer concentrations. About 4 kg wet weight cells were suspended in 0.1 M Tris–HCl buffer (pH 7.3) and broken 1 kg at a time with the Sorvall–Ribi cell fractionater, and centrifuged as described for the other bacteria. The clear supernatant solution from the ultracentrifuge was desalted with buffer change to 1 mM Tris–HCl buffer (pH 8.0) on Sephadex G 25-C. Acidic proteins were then adsorbed on a DEAE-cellulose column (Brown Co., Type 20, 8.0 cm × 15 cm). The effluent contained several basic cytochromes. The acidic proteins were eluted with 20 mM Tris–HCl buffer (pH 7.3) *plus* 0.5 M NaCl. The solution was concentrated by $(NH_4)_2SO_4$ precipitation (50–90 % satn.) and chromatographed on a Sephadex G-100 column (8 cm × 110 cm). The yield of cytochrome *c*-551.5 heme at this stage was about 1.5 μmoles/kg cells. The cytochrome was concentrated with the aid of DEAE-cellulose and rechromatographed on Sephadex G-100. The best fractions were then chromatographed on DEAE-cellulose, using a gradient linear from 1 mM Tris–HCl buffer (pH 7.3) to 20 mM Tris–HCl buffer (pH 7.3) *plus* 0.1 M NaCl. Cytochrome *c*-551.5 separated into two bands. Most of the cytochrome was in the band eluted at the maximum salt concentration (approx. 0.1 M NaCl). The best fractions in this band were pooled and rechromatographed. Again, the cytochrome separated into two bands. The best ratio obtained for $A_{280\,nm}/A_{418\,nm} = 0.18$. The cytochrome was probably still impure as compared with the other preparations and because the quantity available was very small the purification was not continued. The absorption spectra for *Rps. palustris* cytochrome *c*-551.5 are given in Fig. 3.

A. nidulans cytochrome c-549

A. nidulans (obtained through the courtesy of Drs. A. A. Benson and R. F. Lee) was grown on Medium C of KRATZ AND MYERS[13] at 40° in 20-l carboys with illumination by tungsten lamps and aeration by 5 % CO_2 in air. These were not ideal conditions for growth, and the cell yield was only about 1 g wet weight cells per l of medium. The cells (647 g) were suspended in 2 l of 0.1 M Tris–HCl buffer (pH 7.3) broken in the Ribi cell fractionator as described for the photosynthetic bacteria, and centrifuged in the Sorvall for 40 min, giving a loose pellet. The supernatant solution was then centrifuged in the Type 42 rotor in a Spinco L-2 centrifuge for 90 min at 205700 × *g*. The algal

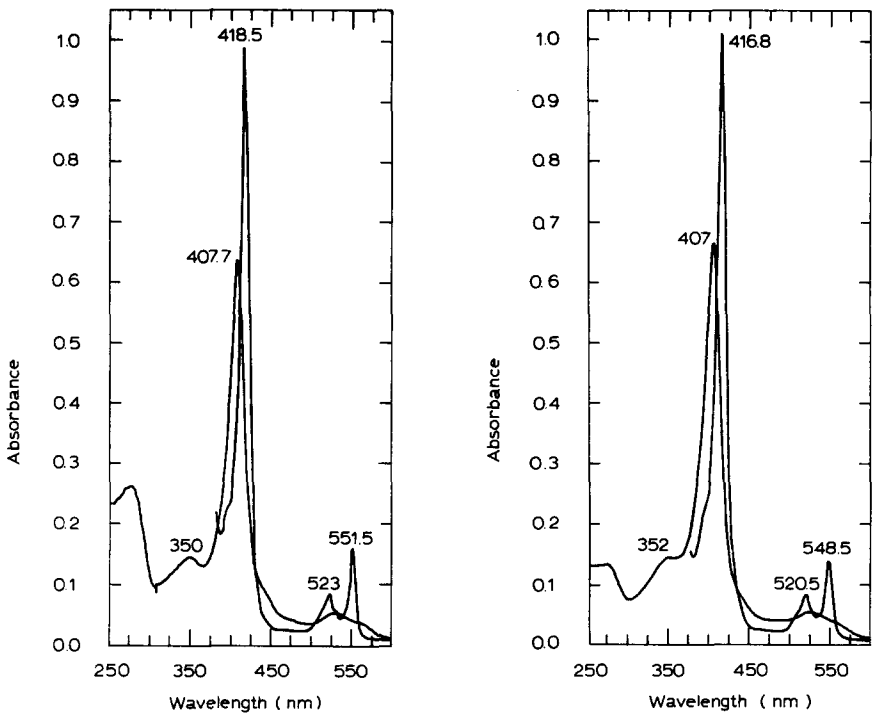

Fig. 3. Absorption spectra of *Rps. palustris* cytochrome *c*-551.5 in 0.1 M potassium phosphate buffer (pH 7.0). The spectrum of the reduced form was traced following addition of solid $Na_2S_2O_4$.

Fig. 4. Absorption spectra of *A. nidulans* cytochrome *c*-549 in 0.1 M potassium phosphate buffer (pH 7.0). The spectrum of the reduced form was traced following addition of solid $Na_2S_2O_4$.

ferredoxin was adsorbed from the crude extract by a DEAE-cellulose column (Selectacel Standard, 2.8 cm × 10 cm) equilibrated with the extraction buffer.

The unadsorbed proteins from the DEAE-cellulose column were fractionated with $(NH_4)_2SO_4$, and the precipitate obtained between about 20 and 60% saturation was applied to a Sephadex G-100 column (8 cm × 100 cm). Cytochromes *c*-549 and *f* (ref. 6) were retarded relative to large amounts of phycocyanin. The two cytochromes were chromatographed on a DEAE-cellulose column (Type 20, 5.6 cm × 15 cm). Cytochrome *f* was eluted behind the residual phycocyanin with 20 mM Tris–HCl buffer (pH 8.0) *plus* 30 mM NaCl, and cytochrome *c*-549 was eluted with 20 mM Tris–HCl buffer (pH 8.0) *plus* 50 mM NaCl. The cytochrome *f* was combined with that obtained from 60 to 100% saturated $(NH_4)_2SO_4$ precipitate. The yield of cytochrome *f* at this stage was about 0.4 μmole.

The yield of cytochrome *c*-549 heme after Sephadex G-100 and DEAE-cellulose chromatography was about 8 μmoles. This cytochrome was then purified by $(NH_4)_2SO_4$ precipitation (20–60% satn.), Sephadex G-100 chromatography, and DEAE-cellulose chromatography with stepwise gradient of NaCl. It was eluted from the DEAE-cellulose column with 20 mM Tris–HCl buffer (pH 7.3) *plus* 50 mM NaCl. For the best fractions the ratio $A_{280\ nm}/A_{417\ nm} = 0.10$ was obtained. The absorption spectra of cytochrome *c*-549 are shown in Fig. 4.

Molecular size of the cytochromes as determined by Sephadex G-75 chromatography

The molecular size of the proteins was estimated by the method of ANDREWS[14] with a Sephadex G-75 column calibrated with *Chromatium* flavocytochrome *c*-552, sperm whale myoglobin (Mann Research Laboratory), horse heart cytochrome *c* (Sigma Type II), and *Ch. thiosulfatophilum* cytochrome *c*-555 used as standards. Blue Dextran was used to measure the void volume, and each protein was run separately on the 1.4 cm × 50 cm column. Although deviation from regular behavior in gel permeation columns has been reported for proteins which are relatively asymmetric in shape[15], no independent test was made to determine if the various low potential cytochromes behaved ideally. The experimentally determined elution volumes and estimated molecular sizes are listed in Table I. The molecular weight of the photosynthetic bacterial cytochromes has not been measured by ultracentrifuge methods, nor has the size of Desulfovibrio cytochromes c_3 been determined by Sephadex chromatography. Therefore any comparison of numbers obtained by the two methods must be viewed as approximate.

TABLE I

MOLECULAR SIZE OF LOW REDOX POTENTIAL PHOTOSYNTHETIC BACTERIAL CYTOCHROMES *c*

Protein	V/V_0*	*Mol. wt. or formula wt.*** ($\times 10^{-3}$)	*Molecular size* ($\times 10^{-3}$)	*Ref.*
Chromatium flavocytochrome *c*-552	1.30	72		34
Myoglobin (sperm whale)	2.10	17.4		35
Mitochondrial cytochrome *c* (horse heart)	2.30	12.4		36
Chlorobium thiosulfatophilum cytochrome *c*-555	2.42	10		37
C. ethylicum cytochrome *c*-551.5	2.37		11	
Rps. spheroides cytochrome *c*-551.5	2.01		21	
Rps. palustris cytochrome *c*-551.5	2.15		16	
A. nidulans cytochrome *c*-549	2.08		19	

* V/V_0 = ratio of elution volume/void volume observed in Sephadex G-75 chromatography.
** Calculated from amino acid composition.

Heme content, absorptivity values, and tentative amino acid compositions of low-potential photosynthetic cytochromes c

Absorptivity values were obtained by comparing absorption spectra in 0.1 M phosphate buffer (pH 7.0) and in 0.1 M NaOH *plus* 25% (v/v) pyridine. The alkaline pyridine ferrohemochrome absorptivity value of homogeneous beef heart cytochrome *c*, $\varepsilon_{mM,\ 550\ nm} = 31.1$ (T. FLATMARK, personal communication), was used for all the cytochromes reported here. These results are given in Table II. The adsorption spectra of the cytochromes at 80° K were measured with the Dewar cuvette assembly described elsewhere[16].

Amino acid analyses were performed on aliquots of solutions of known heme content. The samples were hydrolyzed anaerobically for 48 h in constant boiling HCl at 110° and analyzed according to the procedure of DUS *et al.*[17] The amino acid compositions as determined relative to heme content are given in Table III. The serine and threonine values were not corrected for partial destruction during hydrolysis. Tryptophan was not determined. The cysteine values obtained were corrected for the 40 %

TABLE II

WAVELENGTH MAXIMA AND ABSORPTIVITIES OF LOW-POTENTIAL CYTOCHROMES *c*

$A_{\gamma,r}/A_{\gamma,o}$ is the ratio of reduced Soret absorbance to oxidized Soret absorbance. $A_{\gamma,r}/A_{\alpha,r}$ is the ratio of reduced Soret absorbance to reduced α peak absorbance.

		Oxidized			*Reduced*			$A_{\gamma,r}/A_{\gamma,o}$	$A_{\gamma,r}/A_{\alpha,r}$
		α	γ	δ	α	β	γ		
A. nidulans	λ_m	525	407.3	352	548.8	521	417.0		
	ε_{mM}*	10.8	131		27.5	16.4	198	1.51	7.2
Rps. spheroides	λ_m	527	409.2	352	551.5	523	419.1		
	ε_{mM}*	10.3	133		29.5	16.7	216	1.62	7.32
C. ethylicum	λ_m	526	407.8	352	551.5	522.5	418.0		
	ε_{mM}*	9.6	118		30.8	16.0	196	1.66	6.35
Rps. palustris	λ_m		407.7	350	551.5	523	418.5	1.56	6.3

* The absorptivity values are calculated per heme.

TABLE III

AMINO ACID COMPOSITIONS OF LOW-POTENTIAL CYTOCHROMES *c*

Amino acid	*D. vulgaris**	*D. gigas***	*C. ethylicum*	*Rps. spheroides****,†	*A. nidulans*†
Asp	12	18	9	11	12
Thr	5	5	(5)††	(5)††	(10)††
Ser	6	6	(2)††	(6)††	(5)††
Glu	5	4	3–4	9	15
Pro	4	4	2	5	8
Gly	9	11	6–7	7	10
Ala	10	9	9	14	8
Cys	8	8	(6)†††	(2)†††	(2)†††
Val	8	8	3	4	8
Met	3	0	0	2	1
Ile	0	4	3	2	7
Leu	2	4	1	10	13
Tyr	3	2	1	1	3
Phe	2	2	1	2	2
His	9	8	6	3	3
Lys	20	17	12	2	7
Arg	1	0	0	9	3
Trp	0	1	—§	—§	—§
Total	107	111	70–72	94	117
Formula weight	14 135 (if 4 hemes	14 356 (if 4 hemes)	9 266 (if 3 hemes)	10 617 (if 1 heme)	14 315 (if 1 heme)

* From AMBLER[4] (sequence determination).
** From AMBLER *et al.*[25] (sequence determination).
*** From MEYER *et al.*[8].
† The composition was calculated per single heme.
†† The values for serine and threonine were not corrected for loss during hydrolysis.
††† Cysteine values reported were corrected for the average 40% loss observed with various cytochromes *c*, especially horse heart cytochrome *c*.
§ Tryptophan was not determined.

loss routinely observed with horse heart cytochrome *c* treated in the manner followed here. No evidence for methionine (nor its sulfoxide or sulfone) was detected in the analyses of *C. ethylicum* cytochrome *c*-551.5, and consequently it is concluded that none was present in the protein. For the *Rps. spheroides* and *A. nidulans* cytochromes, only methionine, but not its oxidative products methionine sulfoxide or sulfone, was observed. By analogy to results with horse heart cytochrome *c*, it appears unnecessary to consider any loss of methionine.

Cytochrome purity criteria

The following criteria of purity of the cytochromes were observed. As a first approximation, a protein was judged to be homogeneous if the $A_{280\ nm,\ ox.}/A_{\gamma,\ ox.}$ ratio was constant throughout a zone eluted from a DEAE-cellulose or Sephadex G-75 chromatogram at the last step of purification. As a second approximation, the formula weight per heme, calculated from the amino acid analysis results, was compared with the molecular size determined by the gel-permeation technique. If the tentative formula weight was greater than the molecular size, the protein was assumed to be impure. By these criteria the *Rps. spheroides* and *A. nidulans* cytochromes were essentially homogeneous. The ultimate criterion of purity is accepted to be the determination of the amino acid sequence, which has recently been completed for *C. ethylicum* cytochrome *c*-551.5 by R.P. AMBLER (private communication) (see DISCUSSION). For *C. ethylicum* cytochrome *c*-551.5, the tentative formula weight per heme multiplied by three or four would approximate the molecular size. Therefore, if this protein has three hemes, the sample analyzed was pure.

Oxidation–reduction potentials

Oxidation–reduction potentials were estimated by titrating anaerobically a mixture of cytochrome (10–20 μM) and flavin mononucleotide (50 μM) in 0.1 M potassium phosphate buffer (pH 7.0) with a sodium dithionite solution (8.7 mg/ml in the same buffer). The ratio of oxidized to reduced cytochrome was calculated from the height of the α-peak absorption after each addition of reductant. The ratios of oxidized to reduced FMN were calculated from the difference in absorbance at 450 nm after the contribution of the cytochrome was substracted. Although FMN bleaches in the presence of high concentrations of sulfite[18], the small quantities produced in the titration were not sufficient to constitute a source of error. The midpoint oxidation–

TABLE IV

PROPERTIES OF LOW-POTENTIAL CYTOCHROMES *c*

The protein size is expressed in molecular weight units.

Source	*α peak* (λ_m)	*Molecular size* ($\times 10^{-3}$)	$E_{m,7}$	*pI*	*Complex with CO*	$A_{280}/A_{\gamma,r}$	*Concn. of c_3 heme* (*μmoles/kg cells*)	$(NH_4)_2SO$ *satn. to precipitat* (%)
A. nidulans	548.8	19	−260[5]	4.4	Yes[5]	0.10	15	20–60
Rps. spheroides	551.5	21	−254[8]	4.3	Yes[8]	0.08	4	60–90[8]
C. ethylicum	551.5	11	−194	4.1		0.04	80	80–100
Rps. palustris	551.5	16	−150[9]	6.1		0.18	0.5	50–90

reduction potentials, $E_{m,7}$, were calculated, assuming that, for FMN, $E_{m,7} = -219$ mV (ref. 19). The oxidation–reduction potentials of the purified cytochromes are given in Table IV.

Isoelectric points

Isoelectric points were measured according to VESTERBERG AND SVENSSON[20] using 1 % (v/v) carrier ampholytes (LKB Producter AB, Stockholm) and about 1 mg of each cytochrome. The temperature of the electrolysis cell was maintained at 4° during the run and the pH of 1-ml fractions was determined at 24° following the run. The cytochrome content of the fractions was measured with a Cary 14R spectrophotometer. See Table IV for results.

DISCUSSION

Our results are in agreement with earlier observations[5–7] which suggested that certain photosynthetic bacteria have cytochromes *c* similar to Desulfovibrio cytochromes c_3 in redox potential and absorption spectra. The oxidation–reduction potentials of the photosynthetic bacterial cytochromes range between — 150 and — 260 mV at pH 7.0, values which are comparable to that of *D. vulgaris* cytochrome c_3 (for which $E_{m,7} = -205$ mV) as well as other cytochromes c_3 reported[21]. The absorption spectra of this group of cytochromes are similar in band shapes and absorptivities, but there are small variations in band maxima. The absorption spectra differ from those of mitochondrial cytochrome *c* in several details. Most notable is the very large ratio of reduced to oxidized Soret absorbance in cytochrome c_3 and the presence of a shoulder on the short wavelength side of the reduced Soret peak. For the four cytochromes *c* described here $A_{\gamma red.}/A_{\gamma ox.} = 1.5–1.6$ as compared with the ratio 1.3 for horse heart cytochrome *c*. At liquid nitrogen temperature, there is no splitting of the α-peak of cytochrome c_3, whereas the α peak of horse heart cytochrome *c* is split into three components 2–3 nm apart[21,22].

The differences in oxidation–reduction potentials and absorption spectra between mitochondrial *c* and cytochromes c_3 indicate these two groups of cytochromes probably do not share the same heme ligands. Studies with horse heart cytochrome *c* (ref. 23) and with *Pseudomonas fluorescens* cytochrome *c*-551 (ref. 24) indicate that the fifth and sixth ligands are most likely histidine and methionine. From the amino acid sequences of *D. vulgaris*[4] and *D. gigas*[25] cytochromes c_3, it is evident that the fifth ligand is probably the histidine located immediately adjacent to each of the four cysteine clusters, as is true for the single cysteine cluster of mitochondrial cytochromes *c*. Because methionine is absent from *D. gigas* cytochrome c_3 and from *C. ethylicum* cytochrome *c*-551.5, it seems unlikely that methionine serves as the sixth ligand for any cytochrome c_3, even if present in the peptide chain. In all the cytochromes c_3 as well as in the analogous low potential photosynthetic bacterial cytochromes there are sufficient histidines present to provide both the fifth and sixth ligands. However, it should be noted that only a relatively low degree of homology appears among the available amino acid sequences of cytochromes c_3 (refs. 3, 25 and 26; R. P. AMBLER, private communication) and frequent gaps must be inserted to align the hemes when comparing the several cytochromes. Therefore it is difficult to conceive how similar

three-dimensional structures might arise from the different peptide chains if the structures require that two histidines be liganded per heme.

It may be relevant to the nature of ligands responsible for the characteristic cytochrome c_3 absorption spectra that the alkaline hemochromogen spectra of all cytochromes *c* tested with 0.1 to approx. 1 M NaOH as solvent, depending on the cytochrome, closely approximated the c_3 spectra. The cytochromes tested to date include mitochondrial (horse heart) cytochrome *c*, *R. rubrum*[27] and *Rps. palustris*[9] cytochromes c_2 and *c'*, and *Chromatium* cytochromes *c*-552 and *c'*. Only at high pH do the spectra of these cytochromes *c* assume what seems to be the limiting hemochrome character which prevails in the cytochromes c_3 at neutral pH. Indeed the spectra of *C. ethylicum* cytochrome *c*-551.5 is unchanged between pH 7 and pH approx. 13. If a unique ligand is responsible for the cytochrome c_3 spectra, that ligand may gain access to the hemes of other cytochromes *c* at high pH.

The heme content of purified *D. vulgaris* cytochrome c_3 was originally thought to be two[2], but more recently has been reported to be closer to three[3] per protein of $13 \cdot 10^3$ molecular size. Examination of the amino acid sequence of Desulfovibrio cytochrome c_3 (refs. 4 and 25) indicates four possible heme binding sites.

Recent measurements on *D. vulgaris* NCIB 8303 cytochrome c_3 (T. E. MEYER, unpublished observations performed in the laboratory of Dr. R. P. AMBLER) indicate 3.96 ± 0.36 hemes per molecule based on alkaline pyridine ferrohemochrome determinations on a sample for which the molar protein concentration was determined by normalizing the results of amino acid analysis to the number of amino acids determined in the sequence. The heme content is therefore identical to the number of heme binding sites determined through sequence studies. The earlier lower estimates of the heme content of *D. vulgaris* cytochrome c_3 may have been due to presence of impurities and also to the fact that protein determinations were based on dry weight, a procedure which may be insufficiently reliable.

The amino acid sequence of *C. ethylicum* cytochrome *c*-551.5 has recently been determined by R. P. AMBLER (personal communination[26]). The cytochrome peptide chain consists of 68 amino acid residues and contains three possible heme binding sites. Close, although not identical, comparison between the amino acid composition reported here and that derived from the sequence indicates that the protein as reported here is very close to purity and has three hemes. That the calculated formula weight of $9.3 \cdot 10^3$ for *C. ethylicum* cytochrome *c*-551.5 is low relative to the molecular size of $11 \cdot 10^3$ as estimated by gel-permeation analysis may indicate that this small tri-heme cytochrome does not behave as an ideal globular protein in a molecular sieve, and indeed the formation of multiple bands in DEAE-cellulose and Sephadex G-100 chromatography *plus* the tendency to precipitate over a wide range of $(NH_4)_2SO_4$ concentrations indicate that the protein tends to polymerize in solution.

A comparison between calculated formula weights per heme and the molecular sizes of *A. nidulans* cytochrome *c*-549 and *Rps. spheroides* cytochrome *c*-551.5 indicates that for both cytochromes the molecular sizes are roughly twice the formula weight per heme. If these two cytochromes prove to be monoheme and dimeric, or diheme and monomeric, they may be essentially pure. However, if the cytochromes are triheme like *C. ethylicum* cytochrome *c*-551.5, the samples analyzed were not pure.

In the light of the observations recorded here, it seems appropriate to extend the usage of the designation "cytochrome c_3" (ref. 28). We suggest that for the present

the name "cytochrome c_3" be used to distinguish from the mitochondrial and analogous cytochromes c a class of distinctive cytochromes c with characteristic absorption spectra and low oxidation–reduction potentials. The cytochrome c_3 class could include all cytochromes which resemble the Desulfovibrio cytochromes c_3, regardless of protein size or heme content.

Cytochrome c_3 of Desulfovibrio species has been implicated in various low redox potential electron transport systems[29–32]. In contrast, no clear idea exists regarding the role played by analogous cytochromes in photosynthetic systems. However, SYBESMA[34] has observed the slow light-induced oxidation and rapid dark reduction of a cytochrome "*c*-551" in intact. *C. ethylicum* cells and suggested that this cytochrome might be the major soluble cytochrome *c*-551 isolated by OLSON AND SHAW[7]. If this suggestion is valid, it will be necessary to more closely investigate this bacterial photosystem which operates at a redox potential about 200 mV more reducing than any so far characterized.

If the cytochrome c_3 is a part of a photosynthetic electron transfer pathway, its occurrence is greatly restricted. To date, cytochrome c_3 as a major soluble cytochrome has been found only in *C. ethylicum* and *A. nidulans* and is apparently absent from a second green-sulfur photosynthetic bacterium, *Chlorobium thiosulfatophilum*. The cytochrome occurs at only low concentrations in but two of some six purple photosynthetic bacteria examined. None was detected in *R. rubrum*, *Chromatium*, *Rps. gelatinosa* or *R. molischianum*.

The distribution of cytochrome c_3, while not providing any obvious clue to function, might be valuable in the study of the natural relationships among bacteria, as it may be much more general than previously assumed. Present results encourage further exploration of classes of bacteria and microorganisms other than those described in this report.

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