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THE HEMOPROTEIN CONTENT OF *CHROMATIUM* sp. STRAIN D

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SUMMARY

1. The hemoprotein content of autotrophic *Chromatium* strain D has been measured from difference spectra, using solvent-extracted and intact cells.

2. The quantities of the individual cytochromes were estimated by absorbance differences at single wavelengths and by peak–trough differences in spectra obtained from reduced *versus* oxidized, and CO-treated reduced *versus* reduced preparations. The concentrations found in intact cells were 1.15, 0.49 and 0.56 nmoles heme per mg protein for cytochromes c_{553}, *cc'* and c_{555}**, respectively. In intact cells, the corresponding values were 1.33, 0.47 and 0.67 nmoles heme per mg protein.

3. The mesoheme content of the cells, estimated as pyridine hemochromogen, was 2.08 nmoles heme per mg protein, while the amount of protoheme was negligible.

INTRODUCTION

A number of hemoproteins have been isolated from, or detected in, cells of photosynthetic bacteria[2–15]. These include the *b*-type cytochrome, cytochrome c_2 and cytochrome *cc'* isolated from Athiorhodaceae such as *Rhodospirillum rubrum*, and the low-potential cytochrome *c* (ref. 16) detected in *Rhodopseudomonas spheroides* and cytochrome *o* (ref. 12) detected in *R. rubrum*, although not yet isolated from them.

A *c*-type cytochrome c_{553} and cytochrome *cc'* have been isolated from the obligatorily photosynthetic sulfur bacterium *Chromatium*[10], and in addition the presence of a high-potential *c*-type pigment has been inferred by BARTSCH[17] and by the present authors[18].

There is, however, relatively little information about the quantities of these pigments that are found in photosynthetic cells. The total hemoprotein of *Chromatium* chromatophores has been determined as pyridine hemochromogen[19]. CUSANOVICH *et al.*[15] have estimated the content of individual hemoproteins in chromatophores. It is probable that the values for intact cells may differ considerably from those for chromatophores, since some of the hemoproteins are known to be more soluble than

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** The components of the cytochrome system in *Chromatium* were named according to the recommendations of the International Union of Biochemistry on Nomenclature and Classification of Enzymes[1].

others. Methods by which the cytochromes can be determined are described in this paper, and used to arrive at values for the concentration of the different components in whole cells of *Chromatium*, strain D.

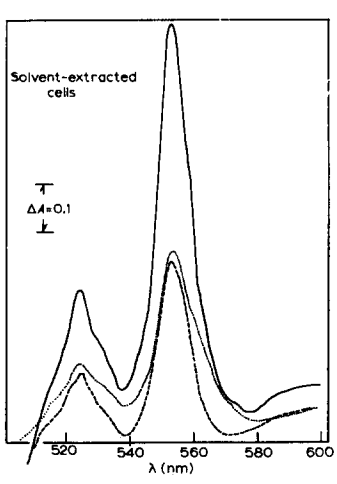
MATERIALS AND METHODS

Growth of the organism and preparation of starved cultures have been described previously[18]. Such starved cells were used as starting material in all preparations and experiments. Solvent-treated cells were prepared by extracting a concentrated suspension with about 10 vol. ethanol–acetone (1:9 mixture, by vol.) at 0°, and collecting the cells by centrifugation. The extraction with the ethanol–acetone mixture was repeated once, and the cells then dried by washing twice with diethyl ether at 0°, and left at room temperature under a gentle air stream until the diethyl ether had evaporated.

Difference spectra were measured using a Cary 14, or Shimadzu MPS50 recording spectrophotometer. All test materials were suspended in 0.1 M phosphate buffer (pH 7) containing 5% sucrose to reduce settling. Compensation for the light absorption by ferricyanide, when used in the test suspension, was achieved by adding an identical amount to a third cuvette placed in the reference beam.

Estimation of quantities of cytochromes

The quantitative estimation of absorbing materials in scattering suspensions presents considerable difficulties. In order to compensate as far as possible for such



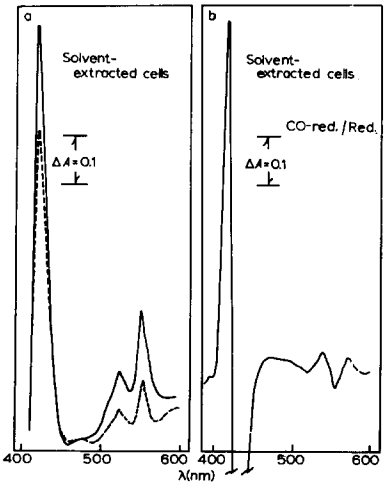


Fig. 1. Difference spectra (reduced−oxidized) of solvent-extracted cells (20 mg protein/ml) suspended in 0.1 M phosphate buffer containing 5% sucrose (pH 7.0). Cary Model 14. ———, dithionite reduced *vs.* ferricyanide oxidized; – – – –, ascorbate reduced *vs.* ferricyanide oxidized; ·····, dithionite reduced *vs.* ascorbate reduced.

Fig. 2. a. Difference spectra (reduced−oxidized) of solvent-extracted cells (6.7 mg protein/ml) suspended in 0.1 M phosphate buffer containing 5% sucrose (pH 7.0). Shimadzu MPS 50. ———, dithionite reduced *vs.* ferricyanide oxidized; – – – –, dithionite reduced *vs.* ascorbate reduced. b. Difference spectrum (CO-reduced *vs.* reduced) of solvent-extracted cells (6.7 mg protein/ml) suspended in phosphate buffer containing 5% sucrose (pH 7.0). Shimadzu MPS 50. ———, dithionite reduced and CO bubbled *vs.* dithionite reduced.

effects, several kinds of difference spectrum measurements were used, including changes in peak–trough extinction differences. Published values for the corresponding spectral differences in purified preparations were then used to calculate intracellular contents. Most measurements were made with solvent-extracted cells.

Cytochrome c_{555}. Measurements were based on the absorbance differences between preparations treated with ascorbate and ferricyanide (Figs. 1 and 2a). Only this cytochrome component is reduced by ascorbate, while ferricyanide effectively oxidizes the pigment in solvent-extracted preparations. Absorbances at troughs on both short- (536 nm) and long- (565 nm) wavelength sides of the α-band, which is centered at 555 nm, were used in calculations. Absorbance at the trough at 451 nm on the longer wavelength side of the γ-peak (422 nm) was used.

TABLE I

QUANTITIES OF CYTOCHROMES IN SOLVENT-EXTRACTED CELLS

Difference spectra	*Cytochrome (nmoles heme per mg protein)*		
	c_{553}	cc'	c_{555}
Ascorbate reduced *vs.* ferricyanide oxidized (α-band) *			0.55
Ascorbate reduced *vs.* ferricyanide oxidized (γ-band) *			0.57
Dithionite reduced *vs.* ascorbate reduced *	1.24	0.55	
CO–dithionite reduced *vs.* dithionite reduced **	1.15	0.37	
CO–dithionite reduced *vs.* dithionite reduced ***	1.06	0.55	
Average	1.15	0.49	0.56

*, **, *** Different preparations of cells were used in these determinations.

Using the values for ΔA (reduced − oxidized) = 47 $mM^{-1} \cdot cm^{-1}$ at 422 nm given by BARTSCH[5] for the pigment from *R. rubrum*, the changes in peak to trough absorption can be shown to have the following values: $\Delta A_{555\,nm} - 1/2\ (\Delta A_{565\,nm} + \Delta A_{536\,nm}) = 22.7\ mM^{-1} \cdot cm^{-1}$ for the α-band and $\Delta A_{422\,nm} - \Delta A_{451\,nm} = 60.8\ mM^{-1} \cdot cm^{-1}$ for the γ-band.

From measurements with a number of solvent-extracted preparations, the cytochrome c_{555} content of the cells was estimated to be about 0.56 nmole (Table I). PARSON[20] has suggested the value ΔA (reduced − oxidized) =, for cytochrome c_{555} in *Chromatium* to be 90 $mM^{-1} \cdot cm^{-1}$. Using this value the cytochrome c_{555} content will be estimated about a half of the value listed in Table I.

Cytochrome c_{553} *and cytochrome* cc'. In isolated preparations, these components have similar redox potentials, and both form compounds with CO when in the reduced form. Measurements of their intracellular concentrations therefore depend on the use of several types of difference spectra, and application of known molar extinction coefficients to the solution of the resultant differential equations. Measurements of the following kinds were made:

(1) Reduced *versus* oxidized cytochrome c_{553} *plus* cytochrome cc', using ascorbate- and dithionite-treated preparations (Fig. 2a). Cytochrome c_{555} does not contribute to the difference spectra observed, since it is reduced in the ascorbate-treated

sample. Absorbance differences at a single wavelength and peak–trough differences were used to calculate the amounts of the two components present, again making use of the values of BARTSCH[16] for the molar difference extinction coefficients, and of the following peak–trough values computed from published difference spectra.

Reduced *versus* oxidized cytochrome c_{553}: $\Delta A_{553\text{ nm}} - 1/2\ (\Delta A_{580\text{ nm}} + \Delta A_{538\text{ nm}}) = 20.5\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ at the α-band and $\Delta A_{424\text{ nm}} - \Delta A_{460\text{ nm}} = 78\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ at the γ-band.

Reduced *versus* oxidized cytochrome *cc'*: $\Delta A_{553\text{ nm}} - 1/2\ (\Delta A_{580\text{ nm}} + \Delta A_{538\text{ nm}}) = 3.54\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ at the α-band and $\Delta A_{424\text{ nm}} - \Delta A_{460\text{ nm}} = 69\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ at the γ-band.

(2) Reduced–CO *versus* reduced cytochrome c_{553} and cytochrome *cc'*, obtained by bubbling CO through a portion of sample reduced with dithionite (Fig. 2b). Calculation of the amounts of the two pigments present were then made using the following constants[16].

Reduced–CO *versus* reduced cytochrome c_{553}: $\Delta A_{418\text{ nm}} - 1/2\ (\Delta A_{400\text{ nm}} + \Delta A_{470\text{ nm}}) = 19.6\ \text{mM}^{-1}\cdot\text{cm}^{-1}$ for the γ-bands.

Reduced–CO *versus* reduced cytochrome *cc'*: $\Delta A_{418\text{ nm}} - 1/2\ (\Delta A_{400\text{ nm}} + \Delta A_{470\text{ nm}}) = 260.5\ \text{mM}^{-1}\cdot\text{cm}^{-1}$.

The large discrepancy in the difference spectra of the two pigments in the γ-band region made these wavelengths more suitable for calculations than the α-band region. However, calculations based on the difference spectra of two pigments in the α-band region were also made.

Values for the quantities of the cytochrome pigments, calculated in these ways, are given in Table I.

Experiments with intact cells

Although the use of solvent-extracted cells allows more accurate spectra to be obtained, it is possible that such treatment causes denaturation of part of the cyto-

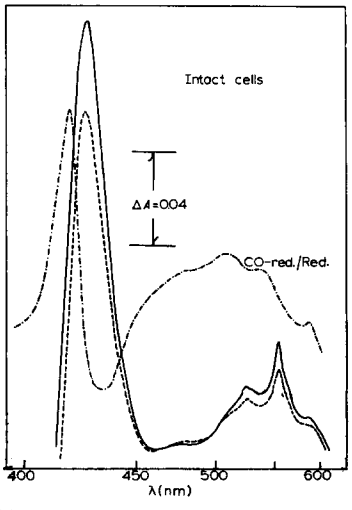


Fig. 3. Difference spectra of intact cells of *Chromatium*, suspended (1.02 mg protein/ml) in 0.1 M phosphate buffer containing 5% sucrose (pH 7.0). Shimadzu MPS 50. ———, dithionite reduced *vs.* ferricyanide oxidized; – – – –, dithionite reduced *vs.* washed starved cells; —·—, dithionite reduced and CO bubbled *vs.* dithionite reduced.

TABLE II

QUANTITIES OF CYTOCHROMES IN INTACT STARVED CELLS

Difference spectra	*Cytochrome (nmoles heme per mg protein)*		
	c_{553}	cc'	c_{555}
Aerobic cell suspension *vs.* ferricyanide oxidized *			0.67
Dithionite reduced *vs.* aerobic cell suspension **	1.13	0.83	
CO–dithionite reduced *vs.* dithionite reduced and dithionite reduced *vs.* aerobic cell suspension **	1.61	0.25	
CO–dithionite reduced *vs.* dithionite reduced and dithionite reduced *vs.* aerobic cell suspension ***	1.22	0.32	
Average	1.33	0.47	0.67

*, **, *** Different preparations of starved cells were used in these determinations.

TABLE III

SUMMARIZED QUANTITIES OF CYTOCHROMES IN *Chromatium* D

Material	*Cytochrome (nmoles heme per mg protein)*			
	c_{553}	cc'	c_{555}	*Total*
Solvent-extracted cells	1.15	0.49	0.56	2.21
Intact cells	1.33	0.47	0.67	2.47
Light-induced absorbance changes	1.37	0.42	0.65	2.44
Pyridine hemochromogen	Mesoheme			2.08
	Protoheme			0.009

chrome pigments. This could lead, for example, to an increase in the portion of the pigments able to react with CO. In order to test for this possibility, some experiments were also run using intact staved cells (Fig. 3). Ascorbate was not added to such suspensions, since cytochrome c_{555} remains reduced after starvation[18], and since the addition of ascorbate to starved suspensions brings about the gradual reduction of more cytochromes, presumably by enzymatic action. The curves shown in Fig. 3 illustrate that it is possible to calculate the amounts of pigments present by some of the means used in the depigmented cells, in spite of some loss of precision in the difference spectra. The quantities so calculated are given in Table II.

Pyridine hemochromogen derivatives of intracellular hemoproteins

Pyridine hemochromogen derivatives were prepared from solvent-extracted cells by the procedure described by TANIGUCHI AND KAMEN[18]. The quantities of protoheme and mesoheme[21] were estimated from the absorbance of the methyl ethyl ketone and aqueous phases, and are summarized in Table III. Very little, if any, pyridine protohemochromogen was found.

Oxidation–reduction potential of the cytochrome reduced by ascorbate

Titration with ferrocyanide–ferricyanide mixtures was used to determine the redox potential of the cytochrome material reduced by treatment with ascorbate, following the procedure of DAVENPORT AND HILL[22].

As shown in Fig. 4, the points obtained fall on a curve typical of a one-electron transfer, and the mid-point potential (E_0') is 340 mV. Therefore this pigment resembles cytochrome c_2 from *R. rubrum* in its oxidation–reduction potential as well as in the position of its absorption maxima. The major difference between the pigments from the two sources would seem to be that while the Rhodospirillum cytochrome is easily extracted from the cells with phosphate buffer, the *Chromatium* pigment has so far resisted solubilization.

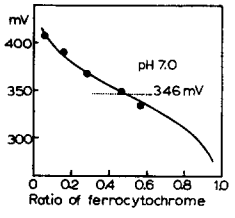


Fig. 4. Relation between reduction–oxidation potential of medium and ratio of reduced cytochrome c_{555} to total cytochrome c_{555}. The reduction–oxidation potentials of the medium were obtained by mixing ferricyanide and ferrocyanide[12].

DISCUSSION

Although *b*-type cytochromes are prominent in several Athiorhodaceae, the virtual absence of protoheme in preparations of pyridine hemochromogen derivatives from *Chromatium* indicates that such pigments are at best very minor components of the cytochrome system in these organisms. Instead, a series of *c*-type cytochromes appear to function in electron transport, with redox potentials covering much the same range as is found in cells which contain pigments of both *b*- and *c*-types. Thus BARTSCH AND KAMEN[10] give values of about 0 and 100 mV for the mid-point potentials of cytochrome *cc'* and cytochrome c_{553} from *Chromatium* at pH 7, and the present work indicates that the mid-point potential of cytochrome c_{555} is close to that of cytochrome c_2 from *R. rubrum*[23].

The methods used to calculate the concentrations of the components of the cytochrome system of *Chromatium* involve a number of assumptions. In practice, however, the consistency of the results obtained using different methods of calculation indicate that the assumptions appear to be valid ones. Thus cytochrome c_{555} does indeed appear to be the only pigment reduced on adding ascorbate to suspensions of solvent-extracted cells, and the fact that similar values for the amounts of cytochrome c_{553} and cytochrome *cc'* were arrived at from a consideration of the reduced *versus* oxidized difference spectra, and the reduced–CO *versus* reduced spectra indicates that these two pigments are both reduced by dithionite but not by ascorbate, and that both form CO compounds. Moreover, the close correspondence between the concentrations of the hemoproteins found in solvent-extracted cells and in intact cells (Table III) indicates that removal of bacteriochlorophyll and carotenoids by the

method used does little damage to the cytochromes. Denaturation would result in an increased binding of CO.

Besides these assumptions, ΔA (reduced − oxidized) values of *R. rubrum* cytochrome c_2 were used for the calculation, since the cytochrome c_{555} in *Chromatium* has not been isolated and its extinction coefficient has therefore not been measured. In fact, A values of the cytochrome c_2 differ from species to species and range from 47 to 50 $mM^{-1} \cdot cm^{-1}$ (A values are 129, 117 and 115 $mM^{-1} \cdot cm^{-1}$ for peaks of γ-band). The value suggested by PARSON[20], 90 $mM^{-1} \cdot cm^{-1}$ for cytochrome c_{555} in *Chromatium*, based on the argument that the quantum yield of photoinduced cytochrome oxidation should be close to one, indicates that this assumption may be invalid.

The quantity of functional cytochrome components present in *Chromatium* can also be estimated from the light-induced difference spectra, and Table III shows the values obtained using the difference spectra previously published[18]. The cytochrome c_{555} and cytochrome *cc'* contents agree quite well with the amounts calculated from experiments in which the pigments were oxidized and reduced chemically, and suggest that the total amount of these pigments present can be oxidized by saturating light. However, the addition of a trace of dithionite to dark, anaerobic cell suspension in these experiments was always found to result in a greater optical change at the appropriate low-light intensity suggesting that even when the cells were supplied with $Na_2S_2O_3$ and kept anaerobic, a portion of the total cytochrome c_{553} remained oxidized. There is close correspondence, therefore, between the total and functional concentrations of cytochrome c_{555} and of cytochrome *cc'*, but there may be a greater quantity of cytochrome c_{553} than is immediately accessible to the cells' reductive mechanisms.

The total amounts of the pigment estimated either from the chemical experiments or from the light-induced changes, after correction for the quantity of cytochrome c_{553} which is not reduced in the presence of $Na_2S_2O_3$, agree within close limits with the total hemochromogen measurements. It is therefore valid to conclude that the three pigments, cytochrome c_{555} and c_{553} and cytochrome *cc'*, account for by far the greater part of the hemoprotein content of these organisms. Other pigments[15], whether of *c*- or *b*-types, appear to be quantitatively of little significance.

ACKNOWLEDGEMENTS

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