BBA 46113

# IRON-CONTAINING PROTEINS IN *CHROMATIUM*

## I. SOLUBILIZATION OF MEMBRANE-BOUND CYTOCHROME

S. J. KENNEL AND M. D. KAMEN

*Department of Chemistry, University of California, San Diego, La Jolla, Calif. 92037 (U.S.A.)*

(Received January 11th, 1971)

SUMMARY

Variations in the nature of the iron-containing proteins in *Chromatium* sp. strain D, grown under varying conditions of substrate and as a function of growth phase, are investigated. The results obtained show no significant changes induced by manipulation of these parameters. At least 80% of the heme protein present is membrane-bound and can be released only by detergent treatment. All but a small fraction is of *c* type, a minor protoheme component (cytochrome *b'*) being present. The membrane-bound cytochrome cannot be identified with those which are freely soluble.

## INTRODUCTION

Recently, many reports have appeared about the photooxidation of cytochromes in *Chromatium* strain D[1-4]. In these researches cells cultured under different growth conditions have been used. Interpretation of data obtained has required the assumption that membrane-bound redox components were identical with those which were buffer-soluble. In this report we present results of a systematic survey of the iron-containing proteins of the bacterium as a function of growth conditions. The detergent solubilization of membrane-bound cytochrome is also described. The identification of functional membrane-bound cytochromes with previously isolated soluble heme proteins does not appear to be warranted.

## METHODS AND MATERIALS

### *Growth media*

The basic salts solution contained per l: 10.0 g NaCl, 0.5 g $K_2HPO_4$, 0.5 g $KH_2PO_4$, 1.0 g $NH_4Cl$, 0.05 g $MgCl_2 \cdot 6\,H_2O$, 5 mg $FeCl_3 \cdot 6\,H_2O$, and 2.0 g $Na_2S_2O_3 \cdot 5\,H_2O$. Additions were: sterile $Na_2S \cdot 9\,H_2O$ to 0.1 g/l and the appropriate carbon source. The different carbon sources were 4.0 g/l $NaHCO_3$ (sterilized by filtration), 4.0 g/l sodium acetate, 4.0 g/l sodium malate, or 4.0 g/l sodium succinate. Cells for inocula were always cultured autotrophically and then diluted 10-fold in the appropriate medium. The cells were grown at 30° in 1-l prescription bottles with 40-W showcase lamp illumination (approx. 50 ft candles) for 48 h heterotrophically, and

60 h autotrophically. Determination of the growth curve was done in an 18-l glass carboy. Samples were removed by displacement with $N_2$. Cell concentration was monitored by cell count employing a calibrated microscope slide grid. Turbidity was assayed with a Klett-Summerson Photoelectric Colorimeter equipped with a No. 66 red filter.

*Cell breakage*

Cells were harvested and broken immediately after suspension in 3 vol. of 50 mM Tris–HCl (pH 8.0) in a Servall Ribi Cell Fractionator operated at 20000 lb/inch$^2$. The broken-cell suspension was readjusted to pH 8.0 with 4 M NaOH and centrifuged 10 min at 30000 $\times$ *g*. The supernatant fluid was decanted and centrifuged 2 h at 100000 $\times$ *g*. The resulting pellet ("crude chromatophores") was resuspended in Tris buffer. The supernatant fraction of this centrifugation was designated "soluble protein".

*Protein fractionation*

The crude soluble protein was passed through a small DEAE–cellulose column to remove ferredoxin. The cytochrome left on this column was eluted with 0.02 M Tris–HCl, 0.25 M NaCl, pH 7.3. The column bypass and the salt wash were pooled and desalted on Sephadex G-25. The desalted protein was chromatographed either on DEAE–cellulose or on Sephadex G-100 after concentration by precipitation with saturated ammonium sulfate.

*Sodium dodecyl sulfate gel electrophoresis*

0.8 cm $\times$ 5 cm 10% acrylamide gels were poured in 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate (pH 7.2) according to the method of WEBER AND OSBORN[5]. Samples were pre-incubated overnight at 4° in 4 M urea, 1.0% sodium dodecyl sulfate and 1.0% mercaptoethanol in a final volume of 40 $\mu$l (ref. 6). Prior to loading, the samples were heated to 60° for 30 min to ensure complete dissociation. Crude chromatophores were treated as described; occasionally, they were subjected to a prior extraction with acetone–methanol (7:2, v/v) to remove lipids. In each gel, a current of 10 mA was applied for 4 h at 35 V. The gels, stained with amido black, were scanned at 620 nm in a Gilford 2000 Spectrophotometer (slit width of 0.5 nm) equipped with a model 2410 linear transport.

*Membrane cytochrome preparation*

Crude chromatophores were suspended in 50 mM Tris–HCl (pH 8.0). Acetone (−10°) was added to a final concentration of 90% (v/v). The suspension was filtered through Whatman No. 1 paper by vacuum and the residue was resuspended in buffer. After a second 90% acetone extraction, the residue was washed twice with 100% acetone. The major portion of acetone was allowed to evaporate, and the resultant acetone powder was suspended in 50 mM Tris–HCl (pH 8.0), 0.5 M NaCl, and stirred magnetically for 24 h at 4°. The mixture was centrifuged for 15 min at 30000 $\times$ *g* and the pellet was suspended in the same salt solution with 2% cholate addition. After 24–48 h, the soluble cytochrome was separated by centrifugation as before. Most of the detergent could be removed by Sephadex G-25 chromatography, but if all of the detergent were removed, the protein residue became insoluble.

*Other measurements*

Protein was estimated by biuret assay[7] standardized against bovine serum albumin Fraction V (Sigma Chemical Co.). Cytochrome was measured by spectroscopy of pyridine hemochromes. The extinction coefficient used was 19.1 $mM^{-1}$ (reduced *minus* oxidized) at 550 nm (ref. 8). The contribution of protoheme (<5 %) could be ignored. Bacteriochlorophyll was determined as described by VAN NIEL AND ARNOLD[9].

The glucose-6-phosphate dehydrogenase assay mixture contained 0.025 M Tris–HCl (pH 9.0), 1.0 mM NAD, and 1 mM glucose 6-phosphate. The increase in absorbance at 340 nm was monitored. One unit was taken as $\Delta A_{340}/30$ min. Spectra were recorded on a Cary Model 14R recording spectrophotometer.

## RESULTS

*Variation of substrate*

The yields as functions of various cell parameters are shown in Table I. Although the total steady-state cell yield varies up to a factor of 4 from autotrophic to hetero-

TABLE I

YIELD OF CELL PARAMETERS AS A FUNCTION OF CARBON SOURCE (STEADY-STATE GROWTH)

| | *Carbon source* | | | |
|---|---|---|---|---|
| | *$CO_2$* | *Acetate* | *Malate* | *Succinate* |
| Cell yield (g wet wt./l) | 1.0 | 4.0 | 4.0 | 2.2 |
| Protein (g/l) | 0.22 | 0.68 | 0.76 | 0.37 |
| Bacteriochlorophyll (μmoles per g protein) | 273 | 441 | 310 | 400 |
| Cytochrome (μmoles per g protein) | 2.18 | 2.29 | 2.16 | 1.65 |

TABLE II

DISTRIBUTION OF CYTOCHROME AND PROTEIN AS A FUNCTION OF CARBON SOURCE

| | *Carbon source* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *$CO_2$* | | *Acetate* | | *Malate* | | *Succinate* | |
| | *Cytochromes per 50 g wet wt.* | | | | | | | |
| *Fraction* | *μmoles* | % | *μmoles* | % | *μmoles* | % | *μmoles* | % |
| Large cell debris | 1.3 | 6 | 0.95 | 6 | 0.82 | 5 | 0.42 | 4 |
| Crude chromatophore | 16.1 | 76 | 12.6 | 79 | 14.6 | 80 | 10.0 | 84 |
| Soluble | 3.9 | 18 | 2.3 | 15 | 2.8 | 15 | 1.5 | 12 |
| Total | 21.3 | 100 | 15.8 | 100 | 18.2 | 100 | 11.9 | 100 |
| | *Protein per 50 g wet wt.* | | | | | | | |
| | *g* | % | *g* | % | *g* | % | *g* | % |
| Large cell debris | 0.4 | 5 | 0.47 | 6 | 0.34 | 3 | 0.12 | 2 |
| Crude chromatophores | 5.3 | 62 | 3.6 | 50 | 5.3 | 54 | 5.0 | 66 |
| Soluble | 2.8 | 33 | 3.2 | 44 | 4.3 | 43 | 2.5 | 32 |
| Total | 8.5 | 100 | 7.3 | 100 | 9.9 | 100 | 7.6 | 100 |

TABLE III

CONCENTRATIONS OF SOLUBLE REDOX COMPONENTS

Values expressed as $\mu$moles per kg wet wt. of cells.

| *Component* | *Extinction coefficient* ($\varepsilon_{mM}$) | *Wave-length* | *Carbon source* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $CO_2$ | | *Acetate* | | *Malate* | | *Succinate* | |
| | | | *DEAE* | *G-100* | *DEAE* | *G-100* | *DEAE* | *G-100* | *DEAE* | *G-1* |
| Ferredoxin | 20 | 388 | 25.5 | — | 14.1 | — | 33.1 | — | 21.7 | — |
| High potential iron sulfur protein | 16 | 388 | 39 | 36 | 23 | — | 63 | 45 | 48 | 35 |
| Cytochrome *c'* | 100 | 425 | 25 | 30 | 16 | — | 22 | 26 | 14 | 13 |
| Cytochrome *c*-553 | 150 | 418 | 23 | 21 | 18 | — | 22 | 23 | 18 | — |
| Cytochrome *b'* | 100 | 403 | — | 8.4 | — | 5.2 | — | 4.4 | — | 2.: |

trophic growth, the amounts of bacteriochlorophyll per protein and the concentrations of cytochrome per total protein are less variable.

When the cells are broken and fractionated, the distribution of components shown in Table II results. It will be noted that the different substrates have relatively little effect on the percentages of cytochrome and protein in various fractions. In all cases, membrane-bound cytochrome constitutes more than 80% of the total cytochrome. This is not true of total protein, 30–45% of which is soluble.

The soluble proteins were chromatographed, using two different procedures (see METHODS), to test for systematic variations in a particular redox component possibly involved in utilization of a particular substrate. Table III presents the results of this study. In general, while gross variations were not found, minor fluctuations did occur. The yield of ferredoxin was most variable, but because of lability such variations were not regarded as significant. Lability was indicated in that yields from fresh cells were 2–3 times higher than those from frozen cells. The variation of "high potential iron sulfur protein" concentration was significant and reproducible. High potential iron sulfur protein has been shown to be an efficient electron carrier in subchromatophore particles (S. J. KENNEL, unpublished data). The concentrations of cytochrome *c*-553 and cytochrome *c'* were nearly invariant. Some significance may be attached to the fact they always occurred in equimolar amounts.

Finally, we note the presence in minor amounts of protoheme in *Chromatium* obtained as a high-spin cytochrome ("cytochrome *b*'"), first observed in this laboratory by Dr. T. E. Meyer, and spectrally similar to horseradish peroxidase. It may have such a function. The relative yields of this cytochrome *b'* are preliminary because it has not yet been obtained pure so that its spectra and extinction coefficients are not known quantitatively.

The spectra of the crude chromatophore fractions from the different cell cultures are shown in Fig. 1. No notable differences are evident. The infrared region is especially interesting in light of the published spectral differentiation of photosystems[10]. If spectrally unique systems exist, they must be present in constant amounts under all growth conditions tested, or else the bulk bacteriochlorophyll absorbance masks a small fluctuation at different wavelengths of active center photopigments.

Sodium dodecyl sulfate gel electrophoresis was performed on the crude chro-

matophore preparations which had been freed of lipid by acetone–methanol extraction. The protein patterns of the stained gels are shown in Fig. 2. The protein patterns exhibited only minor differences, regardless of carbon source. The only variation was found in the succinate cell preparation. Although the gel pattern was similar to the others, the bands had not traveled as far. This may have resulted from a small amount of salt in the sample.

*Cell growth curve*

An 18-l carboy of malate medium was inoculated with autotrophically grown cells. The cell number and turbidity were monitored as a function of time (Fig. 3). The initial peak in turbidity during the lag phase was caused by the deposition of

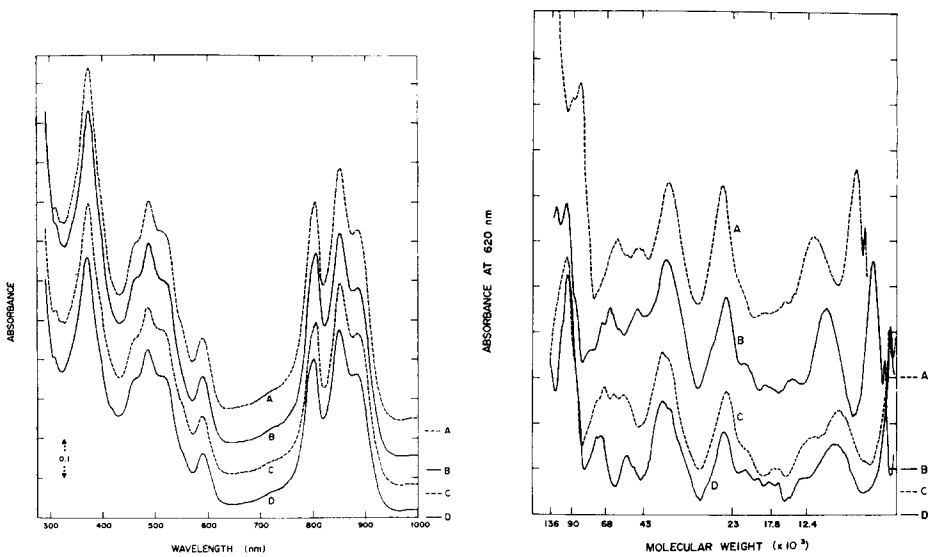


Fig. 1. Spectra of crude chromatophore fraction. Cells were grown with the following carbon sources: Curve A, malate; Curve B, succinate; Curve C, $CO_2$; Curve D, acetate.

Fig. 2. Sodium dodecyl sulfate acrylamide gel electrophoresis of lipid-depleted crude chromatophore fractions. Cells were grown with the following carbon sources: Curve A, succinate; Curve B, acetate; Curve C, malate, and Curve D, $CO_2$. The gels were scanned at 620 nm after staining with amido black.

TABLE IV

CELL YIELD FROM GROWTH-CURVE EXPERIMENT

| | *Cell type* | | | |
|---|---|---|---|---|
| | *Early log* | *Log* | *Late log* | *Stationary* |
| Cell yield (wet wt. per l) | 0.80 | 1.4 | 2.3 | 3.4 |
| Protein (g/l) | 0.080 | 0.158 | 0.23 | 0.313 |
| Bacteriochlorophyll (μmoles per g protein) | 295 | 302 | 445 | 498 |
| Cytochrome (μmoles per g protein) | 1.92 | 2.17 | 1.88 | 3.02 |
| Soluble glucose-6-phosphate dehydrogenase (units/g) | 21 | 57 | 111 | 228 |

sulfur particles[11]. Samples were taken at the times indicated in Fig. 3 and were designated "early log", "log", "late log", and "stationary phase" cells. As routine, the cells were immediately processed. The cell yield statistics are presented in Table IV.

As the culture matured, fewer and fewer cells were actively dividing. These cells must have been in a different metabolic state. As the age of the culture increased, they represented an increasing percentage of the total cell mass. An increase of glucose 6-phosphate activity in the soluble protein fraction was noted as the cultures aged. This may have been evidence of increased membrane permeability. Data in

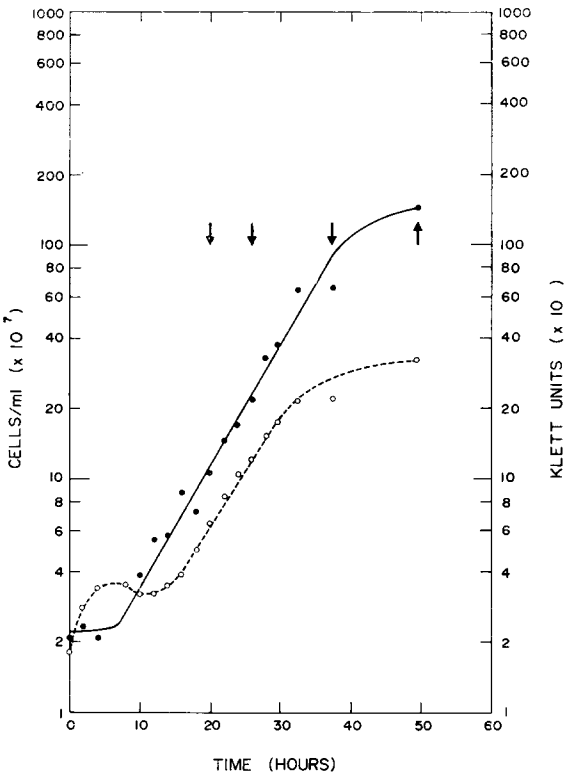


Fig. 3. Growth curve on malate medium. ○---○, Klett units; ●—●, cells/ml. The arrows designate points of harvest. From left to right: early log phase, log phase, late log phase, and stationary phase.

TABLE V

DISTRIBUTION OF CYTOCHROME AND PROTEIN AS A FUNCTION OF GROWTH CURVE

| | *Early log* | | *Log* | | *Late log* | | *Stationary* | |
|---|---|---|---|---|---|---|---|---|
| | % *protein* | % *cyto-chrome* | % *protein* | % *cyto-chrome* | % *protein* | % *cyto-chrome* | % *protein* | % *cyt chrom* |
| Large cell debris | 13 | 7 | 13 | 5 | 16 | 7 | 19 | 6 |
| Crude chromatophores | 48 | 83 | 56 | 89 | 58 | 87 | 50 | 85 |
| Soluble | 40 | 10 | 31 | 6 | 26 | 6 | 31 | 9 |

Table V show that this was not the case in terms of soluble protein and cytochrome. With the exception of the early log phase cells, no systematic increase in soluble protein or cytochrome was seen. Data for the early log phase cells were not consistent with this statement because all of the membrane fraction did not associate with the pellet in the centrifugation step.

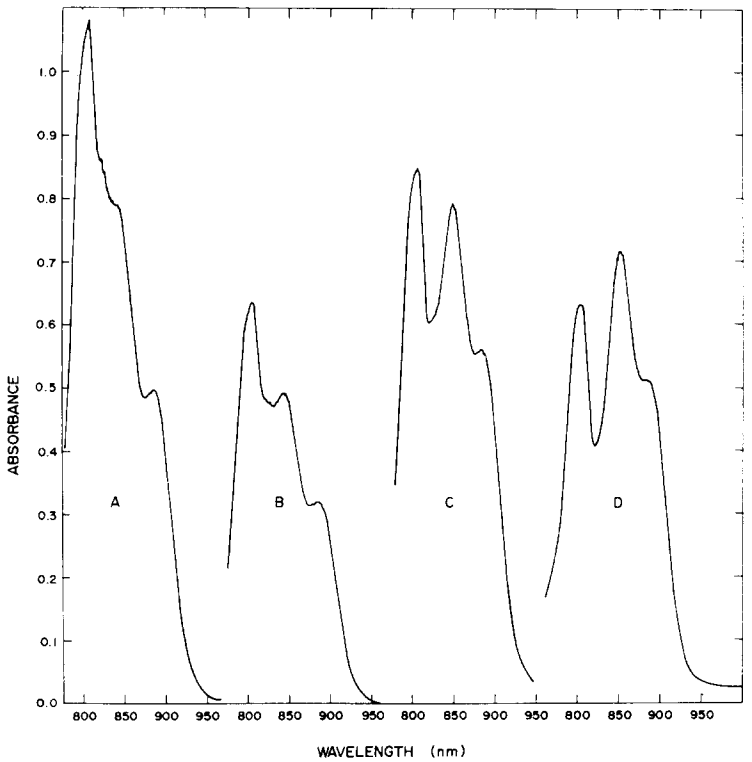


Fig. 4. Infrared spectra of crude chromatophores of cells harvested at various phases of growth. Curve A, stationary; Curve B, late log; Curve C, log, and Curve D, early log.

Table IV also shows the systematic increase of bacteriochlorophyll with cell age. The infrared spectra of the crude chromatophore fractions (Fig. 4) indicate that this increase in bacteriochlorophyll is mainly due to the components with absorption maxima at 820 nm and 800 nm (B820 and B800).

*Detergent solubilization of cytochrome*

In Tables II and V, it is seen that 80–85 % of the total cytochrome remains membrane-bound after cell disruption. Similar data are obtained when cells are broken by sonication.

The membrane-bound cytochrome, as well as all membrane proteins, can be divided into two classes: (1) Proteins which are soluble in buffer, but owing to specific association or merely membrane entrapment, appear in the membrane portion of the cell, and (2) proteins which are inherently insoluble owing to their secondary and tertiary structure or intrinsic hydrophobic character.

These two classes of proteins can be separated in *Chromatium*. The crude chro-

matophore fraction is extracted with acetone until the residue is free of pigment. The resultant acetone powder is treated with buffer to solubilize the first class of proteins. Extraction of the insoluble residue with cholate dissolves nearly all of the remaining cytochrome (Table VI). The cholate-soluble cytochrome represents at least 50 % of the total cell cytochrome.

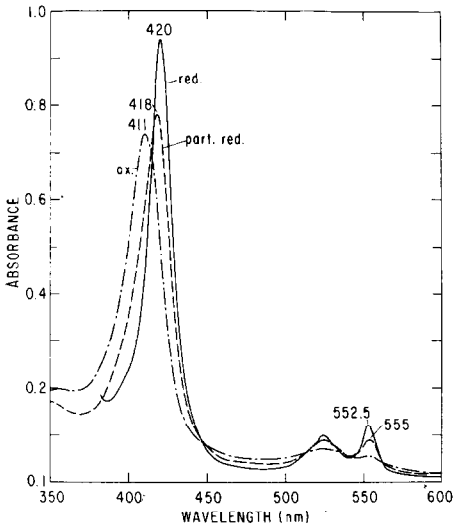


Fig. 5. Spectra of the cholate-soluble cytochrome. —·—, completely oxidized; — — —, partially reduced with 0.04 % (v/v) $\beta$-mercaptoethanol; ———, completely reduced with $Na_2S_2O_4$.

Spectra shown in Fig. 5 indicate that the crude extract is a mixture of two spectral forms differentiable on the basis of electrochemical potential. The high-potential cytochrome has an alpha peak at 555 nm ("*c*-555") and is easily reduced with 10 mM mercaptoethanol. The oxidized-*minus*-reduced spectrum is similar to those obtained in light-*minus*-dark spectra of various membrane fractions poised at high redox potential. The lower redox potential cytochrome, reduced by dithionite, has an alpha maximum at 552.5 nm ("*c*-553"), There is no evidence of flavin absorbance at 480 and 450 nm. Similar treatment, as above, of purified soluble *Chromatium* flavocytochrome *c*-553 (ref. 12) does not result in loss of flavin absorbance or any other spectral change.

TABLE VI

CHOLATE SOLUBILIZATION OF CYTOCHROME

| *Fraction* | *Carbon source* | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $CO_2$ | | *Acetate* | | *Malate* | | *Succinate* | |
| | *μmoles* | % | *μmoles* | % | *μmoles* | % | *μmoles* | % |
| Acetone powder of crude chromatophore | 15.0 | 100 | 12.6 | 100 | 14.2 | 100 | 9.6 | 100 |
| Buffer soluble | 2.9 | 19 | 1.6 | 13 | 2.2 | 15 | 2.1 | 22 |
| Cholate soluble | 10.8 | 72 | 7.4 | 59 | 10.4 | 73 | 6.5 | 68 |
| Residue | 0.9 | 6 | 1.4 | 11 | 0.7 | 5 | 0.8 | 8 |
| Recovery | 14.6 | 97 | 10.4 | 83 | 13.3 | 93 | 9.4 | 98 |

## DISCUSSION

Recent experiments in this laboratory have indicated differences in growth pattern and metabolism of *Chromatium* grown autrophically and heterotrophically[12]. Other work has appeared recently concerning the cytochrome content of *Chromatium*[13]. The present work was undertaken to ascertain the effects of different growth conditions on the presence of redox components in the cell. It was postulated that any systematic variation found with a particular mode of growth might be the basis for assignment of a particular function. However, our metabolism studies revealed only minor changes in the concentrations of the iron-containing proteins chosen for assay, possibly indicating either no specialized involvement of these proteins in carbon metabolism during heterotrophic growth or, more likely, that synthesis of these proteins was under constitutive control.

The ratio of various bacteriochlorophyll spectral froms (B890, B850, and B800) seems to be independent of carbon metabolism. By challenging the cell with carbon sources of various oxidation states one would expect that the photosynthetic apparatus would be required to produce varying amounts of ATP and reducing power. Thus the substrate oxidation level could affect the presence and/or activity of the available photosystem(s). If the differentiated forms of bacteriochlorophyll do represent two (or more) photosystems, substrate variation should cause fluctuations in the infrared absorption spectra. Within the limits of detection in our experiments, bacteriochlorophyll forms do not vary with substrate provided for growth. The similarity of protein patterns on polyacrylamide gel electrophoresis is added evidence that no major substrate-controlled changes occur in the photosynthetic apparatus.

It is well established that the amount of bacteriochlorophyll per cell increases with decreasing culture illumination intensity[14]. This fact is shown again in the data of Table IV. As the cell density increases, light scattering increases, thus lowering the effective illumination intensity per cell. Further, Fig. 4 shows that the increase in bacteriochlorophyll arises mainly from increases in the B820 and B800 forms. This is consistent with the proposed light-harvesting function of the "820" subchromatophore particle isolated by THORNBER[3].

Finally, our studies show that more than 80% of the cytochrome of *Chromatium* is membrane-bound. This is consistent with several proposed models for *Chromatium* photosynthesis[1–4]. A large portion of this membrane-bound cytochrome can be solubilized with cholate. The crude cholate extract is a mixture of two spectral forms. The oxidized-*minus*-reduced difference spectra obtained are similar to the light-induced difference spectra. Furthermore, these cytochromes do not appear identical to any of the known buffer-soluble forms of *Chromatium* cytochrome *c*. Thus, while virtually all the soluble iron-containing proteins appear accountable as cytochrome *c'* and flavocytochrome *c* with inclusion of a small protoheme component (cytochrome *b'*)[13], the major fraction of the cell cytochrome is membrane-bound and not to be identified tacitly as bound forms of the soluble cytochromes. This may be significant in terms of extinction coefficients as well as detailed morphological models of the photosynthetic unit. Investigation of the structure, function, and identity of the detergent-soluble cytochromes is now proceeding.

ACKNOWLEDGMENTS

These researches were supported by grants-in-aid from the National Science Foundation (GB-7033X) and the National Institutes of Health (HD-01262). S. J. K. is the recipient of a U. S. Public Health Service Predoctoral Fellowship (USPHS 2-T01-GM-01045-08).

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