JOURNAL OF BACTERIOLOGY, June 1985, p. 897–901
0021-9193/85/060897-05$02.00/0

Vol. 162, No. 3

Kinetic Studies on Formation of Cytochrome Oxidase of *Rhodopseudomonas capsulata* after a Shift from Phototrophic to Chemotrophic Growth†

HENDRIK HÜDIG AND GERHART DREWS*

Institute of Biology II, Microbiology, Albert-Ludwigs-University, 7800 Freiburg, Federal Republic of Germany

Received 4 December 1984/Accepted 25 February 1985

Rhodopseudomonas capsulata **cells were shifted from phototrophic (anaerobic, light) to chemotrophic (semiaerobic, dark, 10% air saturation) growth conditions. During the adaptation period of 4 h, the bacteriochlorophyll content of cells and membranes decreased, and a newly synthesized 65-kilodalton polypeptide of the cytochrome oxidase was incorporated into the membrane fraction. The enzymatic activity of the cytochrome oxidase increased strongly after a lag time of 2 h. The amount of cytochrome oxidase protein does not follow the same kinetics. The relative amount of a membrane-bound cytochrome *c* of low molecular weight, which has been proposed to be a donor for the cytochrome oxidase, increased during adaptation.**

Rhodopseudomonas capsulata is a facultative phototrophic bacterium, growing either aerobically in the dark (chemotrophic) or anaerobically in the light (phototrophic). Studies on the differentiation of the membrane system of *R. capsulata* after transition of the cells from chemotrophic to phototrophic growth conditions revealed the synthesis of new photosynthetic units, the formation of vesicular intracytoplasmic membrane, and the lowering of respiratory activities, especially of succinate-cytochrome *c*-oxidoreductase and cytochrome *c* oxidase (4, 5, 8, 16, 17, 23, 24).

Chemotrophically grown *R. capsulata* produces ATP by oxidative phosphorylation (1, 19). Only *b*-type cytochromes function in the two terminal oxidases of the branched respiratory chain, which differ in their midpoint potential and sensitivity to KCN and CO (30, 31). Under phototrophic conditions ATP is formed by photophosphorylation driven by a cyclic electron transport.

An active preparation of the membrane-bound terminal high-potential cytochrome *c* oxidase of *R. capsulata* contained a *b*-type cytochrome and a polypeptide with an M_r of 65,000 (12). The active form of the cytochrome oxidase appeared as a dimer with an M_r of 130,000 containing one molecule of protoheme (13). The oxidase was incorporated into phospholipid vesicles to measure proton extrusion after pulses of ferrocytochrome *c* per oxidase turnover. In accordance with the pH shift of its midpoint potential, the purified oxidase showed a proton extrusion of 0.24 H^+/e^- with uptake of 1 H^+/e^- from the liposomes during the reduction of oxygen to water (15).

In this communication we describe the kinetics of cytochrome oxidase formation in *R. capsulata* cells transferred from phototrophic to chemotrophic semiaerobic growth conditions. The appearance of new oxidase protein in the membrane fraction and the specific cytochrome *c* oxidase activity were measured. The results support the idea that the induction of respiratory activity of the cells involves regulation of synthesis and activity of the terminal *b*-type oxidase and also changes of the *c*-type cytochromes bound to the membrane of *R. capsulata*.

MATERIALS AND METHODS

Organisms and culture conditions. *R. capsulata* wild-type strain 37b4 (DSM 938; German Collection of Microorganisms, Göttingen, Federal Republic of Germany) was cultivated anaerobically in a 12-liter fermentor (Biostat S; B. Braun-Melsungen, Melsungen, Federal Republic of Germany) equipped with a device for the measurement and control of PO_2 at 30°C in a malate medium (3) with illumination by incandescent bulbs (3,000 lx behind the fermentor). Formation of cytochrome oxidase was measured in phototrophic cultures shifted during the exponential-growth phase (A_{660} = 0.17; 0.5-cm light path) to chemotrophic conditions by bubbling air through the medium with shaking (400 to 500 rpm) in the dark. The oxygen concentration was measured with an autoclavable Clark-type electrode (Ingold, Frankfurt, Federal Republic of Germany) fitted with a recorder unit. A constant dissolved oxygen concentration was maintained with an air stream regulated by a proportional-integral operating valve. Oxygen concentrations were given as the percent saturation of culture medium with air. The Clark electrode was calibrated with air-saturated culture medium. Samples taken from the fermentor at different times of incubation were immediately poured on ice. Chloramphenicol (100 μg/ml), NaN_3 (1 mM), and phenylmethylsulfonyl fluoride (1 mM, final concentration) were added to stop biosynthetic processes and protein degradation.

Membrane isolation. The cells were disrupted at 112 mPa in a French pressure cell (200 mg [wet weight] of packed cells per ml of 50 mM Tris-hydrochloride buffer [pH 8.0] plus 1 mM phenylmethylsulfonyl fluoride and 0.1 mM EDTA). DNAse I was added, and the suspension was centrifuged at 27,000 × *g* for 30 min. The sediment was discarded, and the supernatant was centrifuged at 160,000 × *g* for 90 min. The sediment was washed twice in the buffer given above and used immediately for enzyme measurement and Triton X-100 extraction.

Immunological procedures. Crossed immunoelectrophoresis (CIE) was performed by a modified procedure that has been previously described (27). The membrane fraction was

* Corresponding author.

† Dedicated to Norbert Pfenning on the occasion of his 60th birthday.

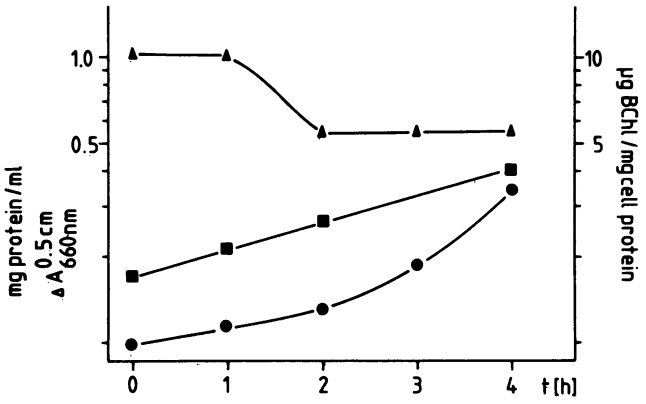

FIG. 1. Cell density, cell protein, and Bchl concentration in cultures of *R. capsulata* transferred from phototrophic (anaerobic, light) to chemotrophic growth conditions (dark, 10% air saturation). The time scale indicates the hours after a shift in culture conditions. See the text for details. Symbols: ▲, micrograms of BChl per milligram of cell protein; ■, A_{660} of the cell suspension (light path, 0.5 cm); ●, milligrams of cell protein per milliliter.

suspended in 80 mM Tris (TRIZMA; Sigma Chemical Co., Muenchen, Federal Republic of Germany)–40 mM sodium-acetate–2.5 mM EDTA (pH 8.8) (CIE buffer) to a protein concentration of 10 mg/ml. Triton X-100 (E. Merck AG, Darmstadt, Federal Republic of Germany) was added to a final concentration of 5% (wt/vol). Samples were then incubated for 30 min at 30°C with stirring. Insoluble material was sedimented by centrifugation at 100,000 × *g* for 1 h, and 5 to 20 μl of the supernatant (100 μg of protein) was used for CIE. More than 90% of the cytochrome oxidase (based on enzymatic activity) and of other membrane-bound proteins (measured by protein determination after centrifugation) was solubilized. Electrophoresis in the first dimension was performed in CIE buffer with 0.5% agarose (Serva, Heidelberg, Federal Republic of Germany) and 0.5% Triton X-100 (flat gel; 2.7 ml; 7.6 by 2.6 cm) at 0.5 mA/cm for 3 h at 4°C. The agarose gel slice was then quickly transferred to a second gel (2.7 ml; 5 by 5 cm) containing 2 to 5 mg of antibodies. Electrophoresis in the second dimension ran for 16 h at 0.5 mA/cm at 4°C.

After electrophoresis the agarose gels were washed for 12 h in 0.5 liter of 0.5% NaCl solution containing 0.025% Triton X-100 and stained with 0.3% Coomassie brilliant blue in 50% ethanol and 10% acetic acid for 3 h. Gels were destained in the same solution without Coomassie brilliant blue. Immunoprecipitation rockets were quantified by using a standard curve prepared with purified oxidase.

Immunoblotting of the proteins after sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described (26). Proteins blotted on nitrocellulose filters were incubated overnight at room temperature with rabbit antibodies (50 μg/ml) against the purified polypeptides. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Sigma) were diluted 1:1,000 with saline containing 3% bovine serum albumin and 0.7% goat serum. The preparations were incubated with the second antibody for 3 h at room temperature and afterwards stained with 4-chloro-1-naphthol (E. Merck) as described (11). Antibodies against the different membrane constituents were purified from rabbit serum as described (10).

Analytical procedures. Cytochrome *c* oxidase was assayed at 30°C by using reduced horse heart ferrocytochrome *c* (type II-A, Sigma) as the electron donor (12, 18). Bacteriochlorophyll (Bchl) was extracted from cells and membranes in an acetone-methanol mixture (7:2 [vol/vol]), and the concentrations were calculated by using an extinction coefficient of $\varepsilon = 76\ mM^{-1} \times cm^{-1}$ at 770 nm (2). Membranes and membrane constituents were solubilized in sodium dodecyl sulfate buffer at 60°C and separated by slab gel polyacrylamide gel electrophoresis according to the method of Laemmli (21). Heme staining of polyacrylamide gels was performed as described (9). Protein concentration was determined by the method of Lowry et al. (22) with serum albumin as the standard.

RESULTS

Growth and pigment content in shift experiments. Cells of *R. capsulata* contained 10.3 μg of Bchl per mg of cell protein when growing under phototrophic conditions, with a doubling time for cell protein of 2 h. When these cells were shifted to chemotrophic conditions at 10% air saturation in the medium, the doubling time for cell protein increased after a lag time of 3 h to nearly 6 h; the specific Bchl content was reduced to ca. 6 μg of Bchl per mg of cell protein after 2 h (Fig. 1). After 4 h of chemotrophic growth the cells were growing exponentially at a doubling time of 1.5 h. The carotenoid content decreased from 6.4 nmol/mg of cell

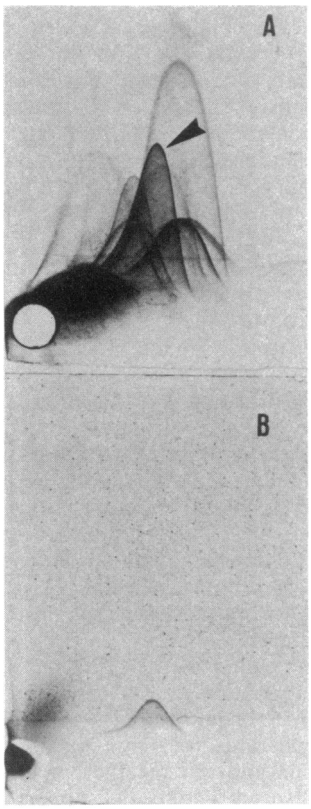

FIG. 2. CIE of Triton X-100 (5% [wt/vol]) extracts from membranes of chemotrophically grown cells. The experimental conditions are described in the text. Membrane protein (100 μg) was separated in the first dimension. The second dimension contained 5 mg of antibodies against the membranes of the wild-type strain 37b4 of *R. capsulata* (A) or 2 mg of antibodies against the purified cytochrome oxidase (B). The arrow indicates the precipitation line of the *b*-type oxidase.

protein under phototrophic conditions to 4.4 nmol/mg of cell protein after 4 h of chemotrophic growth.

Oxidase activity and protein content in the membrane fraction. During the shift experiment the high-potential *b*-type cytochrome *c* oxidase was determined by measuring the specific enzyme activity and the amount of oxidase protein in the membrane (calculated from quantitative CIE; Fig. 2). The specificity of the antibodies against the purified cytochrome *c* oxidase was verified by immunoblotting of the 65,000-molecular-weight polypeptide and by CIE (see Fig. 5a).

When phototrophic cultures were shifted to chemotrophic growth with 10% air saturation, the oxidase protein content per total amount of membrane protein increased more than threefold during the first hour of chemotrophic growth and then steadily decreased to nearly the former level (Fig. 3; see Fig. 5a). The specific oxidase activity, however, decreased during the first hour nearly 10-fold (Fig. 3). After 4 h of chemotrophic growth the enzyme activity had increased 3.5-fold compared with that of phototrophically adapted cells. These differences in kinetics of enzyme activity and protein formation may be due to the unbalanced growth during the first hours of chemotrophic growth.

Oxidase activity and protein content of whole cells. Since the membrane content of cells changed during a shift experiment, we wished to determine the oxidase concentration and activity not only on a membrane basis, as described above, but also on the basis of cell protein. We supposed that the observed Bchl content of whole cells (Fig. 1) reflected the membrane-bound Bchl content and that during membrane isolation Bchl was maintained or at least reduced in the same degree in all membrane fractions.

Therefore, we calculated the ratio of oxidase protein to total cell protein on the basis of (micrograms of oxidase protein/micromoles of Bchl) × (micromoles of Bchl/milligram of cell protein) as described before (25). The ratio of cytochrome oxidase protein to total cell protein remained nearly constant during the 4 h of chemotrophic growth (Fig. 4). The activity of oxidase per milligram of cell protein, however, increased strongly after 2 h of chemotrophic growth.

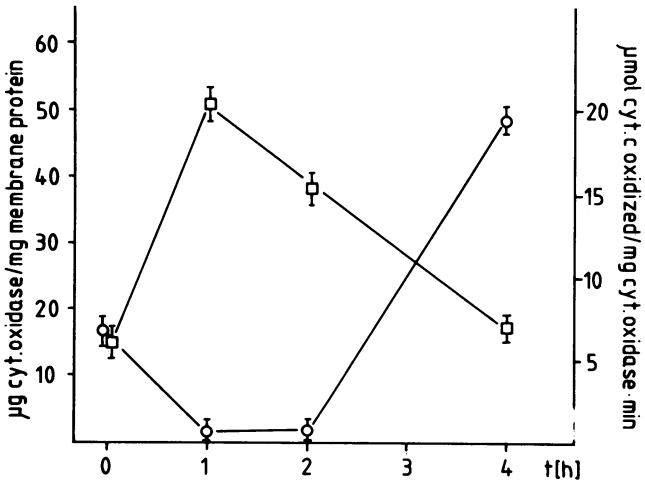

FIG. 3. Cytochrome *c* oxidase after transition from phototrophic to chemotrophic growth conditions (10% air saturation). Values and bars indicate the means and standard deviations of three different experiments, respectively. Symbols: □, micrograms of cytochrome oxidase protein per milligram of membrane protein; ○, cytochrome oxidase activity per milligram of oxidase protein.

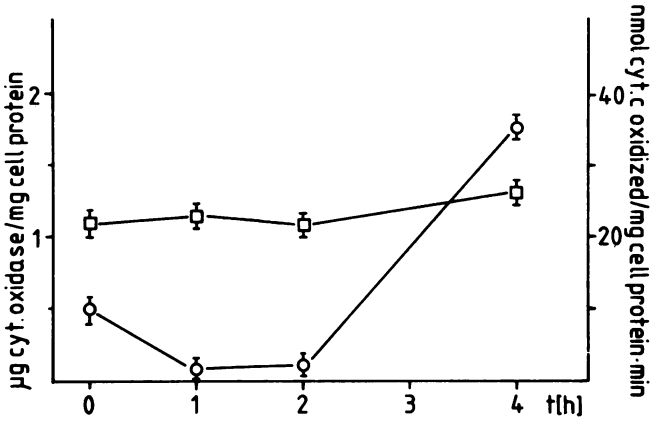

FIG. 4. Cytochrome *c* oxidase after transition from phototrophic to chemotrophic growth conditions (10% air saturation). Shown are the oxidase activity and oxidase protein content per total amount of cell protein. Values and bars indicate the means and standard deviations of three different experiments, respectively. Symbols: □, micrograms of cytochrome oxidase protein per milligram of cell protein; ○, cytochrome oxidase activity per milligram of cell protein.

Heme protein content of the membrane. Mesoheme-binding polypeptides with apparent M_rs of 35,000, 30,000, and 25,000 were present in membranes of phototrophically, as well as of chemotrophically, grown cells, whereas a *c*-type cytochrome with an apparent M_r of 32,000 was present only in membranes of phototrophically grown cells. It disappeared under chemotrophic conditions, and a membrane-bound *c*-type cytochrome with an apparent M_r of 12,000 appeared (Fig. 5b). The cytochrome oxidase could not be visualized by heme staining because the cytochrome *b* diffused away during electrophoresis.

There was a polypeptide of 40 kilodaltons which disappeared completely after adaptation to chemotrophic conditions but was present in low concentrations under phototrophic conditions (Fig. 5a). This polypeptide showed immunological cross-reactivity with the soluble cytochrome c_2 of *R. capsulata* (Fig. 5a). It was not seen by heme staining, when membranes of phototrophically or chemotrophically grown cells were used (Fig. 5b), but bound heme when cells were grown under microaerobical conditions in the dark in the presence of dimethyl sulfoxide (data not shown).

DISCUSSION

During adaptation of phototrophically grown cells to chemotrophic conditions, growth was retarded because the membrane to be changed from a photosynthetic to a respiratory system of energy transduction. The Bchl concentration decreased and the cytochrome oxidase concentration increased. However, since other biosynthetic processes were initiated after the shift in growth conditions, the ratio of oxidase protein to cell protein remained constant (Fig. 4). After 4 h of chemotrophic growth the specific oxidase activity had reached a stable level, which was characteristic of chemotrophically grown cells under full aeration (17). Our results and published data indicate that the high-potential *b*-type cytochrome oxidase is that enzyme of the respiratory chain which is regulated by changes in oxygen partial pressure. Similar results have been obtained by studies on the a/a_3-type oxidase of *R. sphaeroides* (28). In *Escherichia coli* the synthesis of the cytochrome *o*-type oxidase was

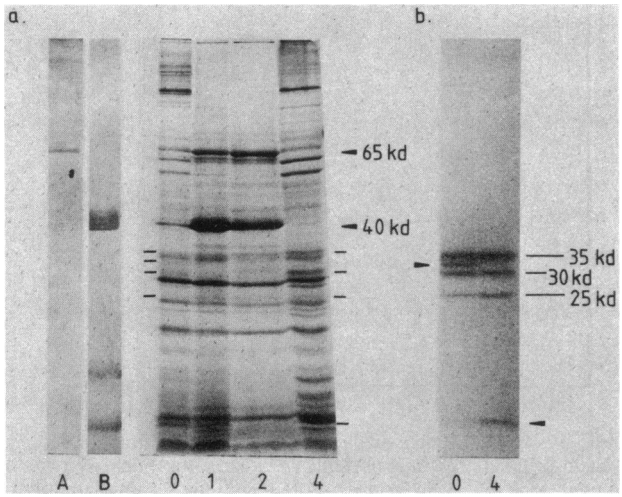

FIG. 5. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (12% acrylamide). (a) Lane numbers refer to 0 to 4 h of chemotrophic growth. Membrane protein (30 μg) was applied to each slot. Staining was performed with Coomassie brilliant blue. Lanes A and B show immunoblots stained with 4-chloro-1-naphthol as described in the text. Lane A shows the 65-kilodalton polypeptide of the cytochrome oxidase as detected by immunoblotting of lane 1 with antibodies against the purified oxidase. Lane B shows the immunoblotting of lane 1 with antibodies against the purified cytochrome c_2 of *R. capsulata*. Bars indicate proteins with positive heme stain as shown in (b). (b) Heme staining of polypeptide separated by sodium dodecyl sulfate-polyacrylamide slab gel (12% acrylamide) electrophoresis. Lanes 0 and 4 refer to 0 h (phototrophic) and 4 h (chemotrophic) growth conditions. Only membranes of phototrophic (0 h) and chemotrophic (4 h) cells were stained. See the change in cytochrome *c* composition (arrows). BChl-depleted membranes showed the same results.

suppressed by lowering of the aeration rate, whereas the synthesis of a cytochrome *d* was induced (20). The activity of the high-potential *b*-type oxidase in *R. capsulata* seems to be regulated not only by incorporation of new oxidase protein into the membrane but also by the enzyme activity, probably owing to a separate incorporation of the protoheme.

Preliminary results indicate that during adaptation to chemotrophic growth the synthesis of other components of respiratory chain of *R. capsulata*, mainly *c*-type cytochromes, are regulated. Thus, the relative concentration of a membrane-bound cytochrome *c* of low molecular weight, which has been proposed to be a donor for the cytochrome oxidase (14), increased during adaptation. The function of the 32,000-molecular-weight cytochrome *c* (6, 7, 29), which disappeared during adaptation, as well as of the other heme staining components, has to be determined.

ACKNOWLEDGMENTS

We thank Martin D. Kamen for discussion and for reading the manuscript and Robert G. Bartsch for a gift of the purified cytochrome c_2. H.H. thanks Helwig Reidl for valuable information, Dieter K. Hammer for preparation of antisera, and Roland Dierstein for helpful advice to establish the CIE. We thank Johanna Nährig for preparing the figures and Heide Alexander for typing the manuscript.

The work was supported by a grant of the Deutsche Forschungsgemeinschaft to G.D.

LITERATURE CITED

1. **Baccarini-Melandri, A., D. Zannoni, and B. A. Melandri.** 1973. Respiratory sites of energy conservation in membranes from dark grown cells of *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **314**:298–311.
2. **Clayton, R. K.** 1966. Spectroscopic analysis of bacteriochlorophylls in vitro and in vivo. Photochem. Photobiol. **5**:669–688.
3. **Drews, G.** 1965. Die Isolierung schwefelfreier Purpurbakterien. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Suppl. Heft. **1**:170–178.
4. **Drews, G.** 1978. Structure and development of the membrane system of photosynthetic bacteria. Curr. Top. Bioenerg. **8**:160–207.
5. **Drews, G., and J. Oelze.** 1981. Organisation and differentiation of membranes of phototrophic bacteria. Adv. Microb. Physiol. **22**:1–92.
6. **Gabellini, N., J. R. Boyer, E. Hurt, B. A. Melandri, and G. Hauska.** 1982. A cytochrome b/c_1-complex with ubiquinol-cytochrome c_2 oxidoreductase activity from *Rhodopseudomonas sphaeroides* GA. Eur. J. Biochem. **126**:105–111.
7. **Gabellini, N., and G. Hauska.** 1983. Characterization of cytochrome *b* in the isolated ubiquinol-cytochrome c_2 oxidoreductase from *Rhodopseudomonas sphaeroides* GA. FEBS Lett. **153**:146–150.
8. **Garcia, A. F., G. Drews, and H. H. Reidl.** 1981. Comparative studies of two membrane fractions isolated from chemotrophically and phototrophically grown cells of *Rhodopseudomonas capsulata*. J. Bacteriol. **145**:1121–1128.
9. **Guikema, J. A., and L. A. Shermann.** 1981. Electrophoretic profile of cyanobacterial membrane polypeptides showing heme-dependent peroxidase activity. Biochim. Biophys. Acta **637**:189–201.
10. **Harboe, N., and A. Ingild.** 1975. Immunization, isolation of immunoglobulin, estimation of antibody titre, p. 161–164. *In* N. H. Axelson, J. Kroll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis, vol. 4. Universitetsvorlaget, Oslo, Norway.
11. **Hawkes, R., E. Niday, and J. Gordon.** 1982. A dot-immunobinding assay for monoclonal and other antibodies. Anal. Biochem. **119**:142–147.
12. **Hüdig, H., and G. Drews.** 1982. Isolation of a *b*-type cytochrome oxidase from membranes of the phototrophic bacterium *Rhodopseudomonas capsulata*. Z. Naturforsch. **37c**:193–198.
13. **Hüdig, H., and G. Drews.** 1982. Characterization of a *b*-type cytochrome *c* oxidase of *Rhodopseudomonas capsulata*. FEBS Lett. **146**:389–392.
14. **Hüdig, H., and G. Drews.** 1983. Characterization of a new membrane-bound cytochrome *c* of *Rhodopseudomonas capsulata*. FEBS Lett. **152**:251–255.
15. **Hüdig, H., and G. Drews.** 1984. Reconstitution of *b*-type cytochrome oxidase from *Rhodopseudomonas capsulata* in liposomes and turnover studies of proton translocation. Biochim. Biophys. Acta **765**:171–177.
16. **Kaplan, S.** 1978. Control and kinetics of photosynthetic membrane development, p. 809–839. *In* R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
17. **Kaufmann, N., H. H. Reidl, J. Golecki, A. F. Garcia, and G. Drews.** 1982. Differentiation of membrane system in cells of *Rhodopseudomonas capsulata* after transition from chemotrophic to phototrophic growth conditions. Arch. Microbiol. **131**:313–322.
18. **King, M. T., and G. Drews.** 1976. Isolation and partial characterization of the cytochrome oxidase from *Rhodopseudomonas palustris*. Eur. J. Biochem. **68**:5–12.
19. **Klemme, J. H., and H. G. Schlegel.** 1969. Untersuchungen zum Cytochrom-Oxidase-System aus anaerob im Licht und aerob im Dunkeln gewachsenen Zellen von *Rhodopseudomonas capsulata*. Arch. Mikrobiol. **68**:326–354.
20. **Kranz, P. G., and R. B. Gennis.** 1983. Immunological characterization of the cytochrome *o* terminal oxidase from *Escherichia coli*. J. Biol. Chem. **258**:10614–10621.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

22. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. **Oelze, J., and G. Drews.** 1972. Membranes of photosynthetic bacteria. Biochim. Biophys. Acta **265**:209–239.
24. **Oelze, J., and G. Drews.** 1981. Membranes of phototrophic bacteria, p. 131–195. *In* B. K. Gosh (ed.), Organization of prokaryotic cell membranes, vol. 2. CRC Press, Boca Raton, Fla.
25. **Reidl, H. H., J. R. Golecki, and G. Drews.** 1983. Energetic aspects of photophosphorylation capacity and reaction center content of *Rhodopseudomonas capsulata*, grown in a turbidostat under different irradiances. Biochim. Biophys. Acta **725**:455–463.
26. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. **76**:4350–4354.
27. **Weeke, B.** 1975. Rocket immunoelectrophoresis, crossed immunoelectrophoresis, p. 37–56. *In* N. H. Axelson, J. Kroll, and B. Weeke (ed.), A manual of quantitative immunoelectrophoresis, vol. 4. Universitetsvorlaget, Oslo, Norway.
28. **Whale, F. R., and O. T. G. Jones.** 1970. The cytochrome system of heterotrophically grown *Rhodopseudomonas sphaeroides*. Biochim. Biophys. Acta **233**:146–157.
29. **Wood, P. M.** 1980. Do photosynthetic bacteria contain cytochrome c_1? Biochem. J. **189**:384–391.
30. **Zannoni, D., B. A. Melandri, and A. Baccarini-Melandri.** 1976. Composition and function of the branched oxidase system in wild type and respiratory deficient mutants of *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **423**:413–430.
31. **Zannoni, D., B. A. Melandri, and A. Baccarini-Melandri.** 1976. Further resolution of cytochromes of *b*-type and the nature of CO-sensitive oxidase present in the respiratory chain of *Rhodopseudomonas capsulata*. Biochim. Biophys. Acta **449**:386–400.