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ENERGY TRANSDUCTION IN PHOTOSYNTHETIC BACTERIA

XI. FURTHER RESOLUTION OF CYTOCHROMES OF *b* TYPE AND THE NATURE OF THE CO-SENSITIVE OXIDASE PRESENT IN THE RESPIRATORY CHAIN OF *RHODOPSEUDOMONAS CAPSULATA*

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SUMMARY

1. In membranes prepared from dark grown cells of *Rhodopseudomonas capsulata*, five cytochromes of *b* type (E'_0 at pH 7.0 $+413\pm5$, $+270\pm5$, $+148\pm5$, $+56\pm5$ and -32 ± 5 mV) can be detected by redox titrations at different pH values. The midpoint potentials of only three of these cytochromes (b_{148}, b_{56}, and b_{-32}) vary as a function of pH with a slope of 30 mV per pH unit.

2. In the presence of a CO/N_2 mixture, the apparent E'_0 of cytochrome b_{270} shifts markedly towards higher potentials (+355 mV); a similar but less pronounced shift is apparent also for cytochrome b_{150}. The effect of CO on the midpoint potential of cytochrome b_{270} is absent in the respiration deficient mutant M6 which possesses a specific lesion in the CO-sensitive segment of the branched respiratory chain present in the wild type strain.

3. Preparations of spheroplasts with lysozyme digestion lead to the release of a large amount of cytochrome c_2 and of virtually all cytochrome *cc'*. These preparations show a respiratory chain impaired in the electron pathway sensitive to low KCN concentration, in agreement with the proposed role of cytochrome c_2 in this branch; on the contrary, the activity of the CO-sensitive branch remains unaffected, indicating that neither cytochrome c_2 nor the CO-binding cytochrome *cc'* are involved in this pathway.

4. Membranes prepared from spheroplasts still possess a CO-binding pigment characterized by maxima at 420.5, 543 and 574 nm and minima at 431, 560 nm in CO-difference spectra and with an α band at 562.5 nm in reduced minus oxidized difference spectra. This membrane-bound cytochrome, which is coincident with cytochrome b_{270}, can be classified as a typical cytochrome "*o*" and considered the alternative CO-sensitive oxidase.

INTRODUCTION

The branched respiratory chain of the facultative photosynthetic bacterium *Rhodopseudomonas capsulata* [1, 2] contains only cytochromes of *b* and *c* type [3, 4],

the function of which is difficult to elucidate only on the basis of their spectral and thermodynamic characterization and from studies of the respiratory activities. However these techniques, combined with the availability of respiration-deficient mutants [5] have recently allowed the partial elucidation of the function and sequence of electron carriers and the localization of the branching site of the respiratory chain [2].

One of these mutants, M7, which lacks cytochrome *c* oxidase activity [5], has been particularly useful for relating the presence or the absence of a high potential *b* type cytochrome (cytochrome b_{413}) to that of cytochrome *c* oxidase activity. Since this activity is totally unaffected by CO, we have proposed that this oxidase could not be considered as a classical cytochrome *o*, but rather as a new oxidase of *b* type [4].

In contrast, CO was found to be a specific inhibitor of a second type of oxidase, as clearly demonstrated by the complete inhibition of NADH oxidase by CO in the M7 strain [2]. A mutant, M6, with a specific lesion in this alternative oxidase, is also available; however, the absence of activity of the alternative oxidase in this strain could not be related with the obvious absence of any cytochrome of *b* type, detectable by spectral or potentiometric analysis.

In this paper, studies on the effect of CO on the apparent redox potential of cytochromes of *b* type are presented, which indicate clearly that a *b* cytochrome (cytochrome b_{270}) is able to bind CO and is the locus of M6 mutation. This pigment is different from cytochrome *cc'*, a CO-binding protein commonly found in many photosynthetic bacteria.

A detailed study on the response to the pH of the medium of the apparent midpoint potentials of the oxidases and of the other membrane bound cytochromes is also reported here.

MATERIALS AND METHODS

Cells of *Rhodopseudomonas capsulata*, strain St. Louis (American Type Culture Collection No. 23782) or of the two mutant strains M6 and M7, were grown aerobically in the dark, as described previously [1, 2], and harvested at the end of the logarithmic phase (absorbance at 660 nm = 0.8–1). For the preparation of spheroplasts, the bacteria were washed once with 0.1 M phosphate buffer, pH 6.8 and suspended in 0.03 M Tris · Cl buffer, pH 8.0 containing 20 % sucrose, at a cell density corresponding to 1.0-1.5 absorbance at 660 nm. After addition of 10 mM EDTA, pH 8.0, they were incubated with lysozyme (0.5 to 0.75 mg/ml, according to the response of the cells) for variable time, ranging from 30 min to 1 h, at 30 °C, under mild stirring. After the formation of spheroplasts, which was controlled by phase contrast microscopic observations, $MgSO_4$ (50 mM) was added and the suspension was centrifuged at 10 000 × *g* for 20 min. The supernatant was saved for further analysis and the spheroplast pellet was washed once with 0.03 M Tris · Cl, pH 8.0, containing 20 % sucrose and 50 mM $MgSO_4$, suspended in the same buffer and kept in an ice bath.

The integrity of the spheroplasts was routinely controlled by measuring the activity of a marker cytoplasmic enzyme (NADH-dependent malic dehydrogenase, EC 1.1.1.37) and comparing the total units present in suspensions of intact bacteria or spheroplasts, with that measured after mechanical breaking of the same preparations.

Membranes were prepared from spheroplasts or from untreated bacteria by breaking the cells with a French pressure cell. After a low speed centrifugation, membranes were sedimented from the supernatant centrifuging at 160 000 × *g* for 90 min. The pellet, washed with 0.1 M glycylglycine buffer, pH 7.2, containing 10 mM $MgCl_2$, was finally resuspended in the same buffer.

Oxidation-reduction titrations of the cytochromes were performed using the technique described by Dutton et al. [6] under an atmosphere of pure nitrogen or of a CO/nitrogen mixture. In the latter case, the buffer was flushed previously with CO for 30 min, then transferred to the cuvette and flushed for an additional 30 min with a mixture of CO/N_2 at pCO/pN_2 3.5 at a total pressure of 1 atm (corresponding to equilibrium to 1200 μM CO dissolved). Particles were added anaerobically through a rubber septum.

Difference spectra were run with a split beam spectrophotometer as described previously [2]. Respiratory activities were measured as described in an earlier paper [1].

Protein content was estimated by the Lowry method [7] and bacteriochlorophyll was measured spectrophotometrically at 775 nm in acetone/methanol (7 : 2) extracts using an extinction coefficient of 75 $mM^{-1} \cdot cm^{-1}$ [8].

RESULTS

The dependence on pH of the apparent midpoint potential of cytochromes of b type

It has been already reported [9] that *b* type cytochromes ($E'_0 = +60$ mV and -25 mV) present in *Rps. capsulata*, carotenoid-less strain A1a *pho*$^+$, grown photosynthetically in anaerobiosis, show a marked shift of the apparent midpoint potential when the pH of the medium is varied from 6.0 to 9.0. On the other hand, we have presented evidence that a similar bacterial strain *Rps. capsulata*, strain St. Louis, when grown aerobically in the dark, also possesses a pool of medium -low potential cytochromes *b* (centered at +47 mV at pH 7.0, ref. 4), in addition to two high potential cytochromes *b* (b_{270} and b_{413}), which seemingly are induced only in these conditions of growth.

As noted previously [2] this pool of medium low potential cytochromes appears to be composed of more than one component, although it cannot be easily resolved at pH 7.0. These components can however be distinguished more easily from each other at more alkaline pH values, since in these conditions a better resolution of the redox pattern of the cytochromes and a better agreement between the experimental points and the Nernst's theoretical behaviour (for $n = 1$) can be observed. In Fig. 1, a redox titration of *b* type cytochromes performed at pH 9.0 is presented: at least three components, with apparent midpoint potentials at 85, -10 and -90 mV respectively, are readily observed. The logarithmic interpolation of the redox response of the three components is reasonably linear, indicating minimum overlapping during the redox titration. When redox titrations at lower pH are analyzed on the basis of the results obtained at pH 9.0, i.e. on the basis of three components, less satisfactory results are obtained and apparent $n > 1$ observed. The reason for a more ideal behaviour of the redox titrations at alkaline pH values is not clear; a better equilibration of the membrane components with the redox dyes (some of which become more reducing at increasing pH) can be suggested.

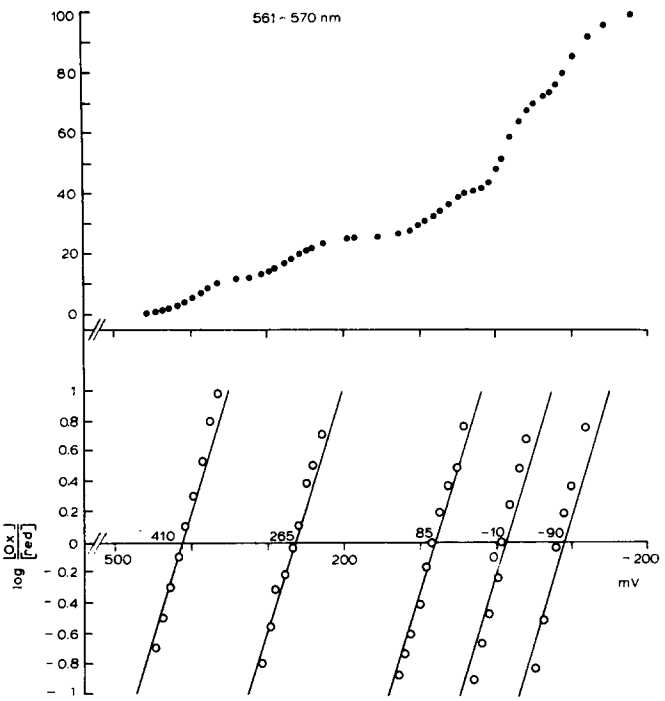

Fig. 1. Oxido-reduction titrations of cytochromes *b* (561–570 nm) in membranes from *Rhodopseudomonas capsulata*, St. Louis. The measurement was performed under nitrogen, using a buffer system containing HEPES, MES, TRICINE (30 mM each) and KCl (50 mM) at pH 9.0. The protein concentration in the assay was 2.5 mg/ml.

A complete picture of the dependency on pH of the midpoint potential of cytochromes *b* is shown in Fig. 2. The three medium low potential components present a linear decrease of their potentials from pH 7.0 to pH 9.0, with an average slope of about 30 mV per pH unit. The two high potential components, on the other hand, do not show any appreciable shift in this pH range.

Similar results were also obtained in the two respiratory mutants M6 and M7.

The identification of CO binding cytochromes of the respiratory chain

Spectra of CO-binding cytochromes associated with the membranes can be observed in both the wild type strain and in the respiration deficient mutants M6 and M7 [2]. The nature of these pigments has been elucidated examining the effect of CO on the apparent midpoint potential of cytochromes. For the sake of clarity, we shall summarize here the main biochemical characteristics of the respiratory chains of M6 and M7 mutants; the reader should refer to these properties in following the experiments described below.

M7 strain: lack of cytochrome *c* oxidase activity and absence of cytochrome b_{413}; NADH and succinate-dependent respiration inhibited only by high concentrations of KCN and hardly inhibited by antimycin; respiration completely inhibited by CO.

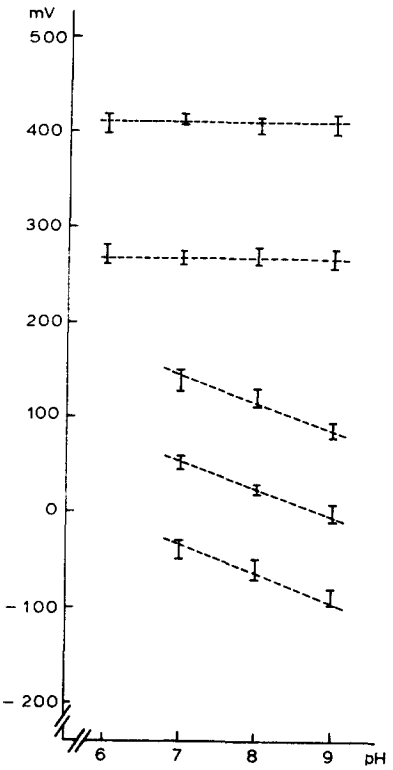

Fig. 2. Dependency of the mid-point potential of cytochromes *b* on pH. Conditions as in Fig. 1, except that the pH of the buffer system varied in the range from 6.0 to 9.0.

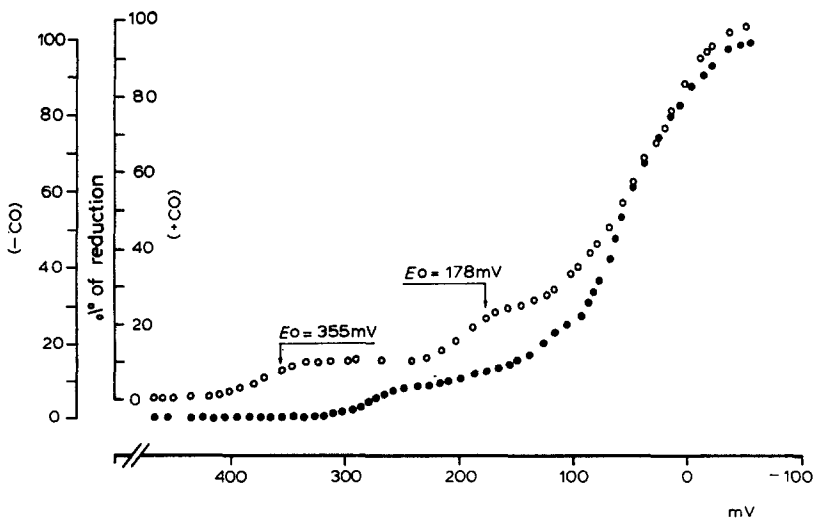

Fig. 3. Potentiometric titration of cytochromes *b* (561–570 nm) in membranes from M7, in the presence or absence of carbon monoxide. The measurements were performed at pH 7.0 under nitrogen (●) or under CO/nitrogen (○) atm, as described under Material and Methods. The protein concentration in the assay was 2.1 mg/ml.

M6 strain: active in cytochrome *c* oxidase and endowed with all *b* type cytochromes detectable in the wild type strain; respiration highly sensitive to KCN and antimycin, but insensitive to CO.

As presented in Fig. 3, when redox titrations of membranes from the M7 mutant are performed at pH 7.0 in an atmosphere of nitrogen/carbon monoxide, a very marked shift in the apparent midpoint potential of cytochrome b_{270} toward higher potentials (+355 mV) is observed. A small shift in potential (30 mV more positive under the same conditions) is also apparent for another *b* type cytochrome with a midpoint potential of about 150 mV at pH 7.0. However, this last observation is rather uncertain, due to the overlapping of the other cytochromes of the "b_{47}" pool.

Similar CO-induced shifts are also present in the wild type strain; however in the wild type, the data are not as clear-cut since in presence of CO, cytochrome b_{413} and cytochrome b_{270} (shifted to +350 mV under this condition) overlap during redox titrations.

Thus, a high potential *b* type cytochrome, present only in aerobic cells, is able to interact strongly with CO; this cytochrome could represent the alternative oxidase operating in parallel with cytochrome *c* oxidase (b_{413}) in the wild type strain. The conclusive proof of this hypothesis was obtained by comparing the properties of the high-potential cytochrome *b* in M7 and M6, mutants lacking respectively cytochrome *c* oxidase and the alternative oxidase. As previously mentioned [2], a cytochrome with E'_0 at pH 7.0 at about +285 mV and therefore analogous to cytochrome b_{270} is present in M6. However, as shown in Fig. 4, the potential of this cytochrome does not shift in the presence of CO, although the formation of a complex with this ligand seems to be demonstrated by dithionite-reduced plus CO minus dithionite-reduced differential spectra (see below). Thus the phenotypic character of M6, i.e. the

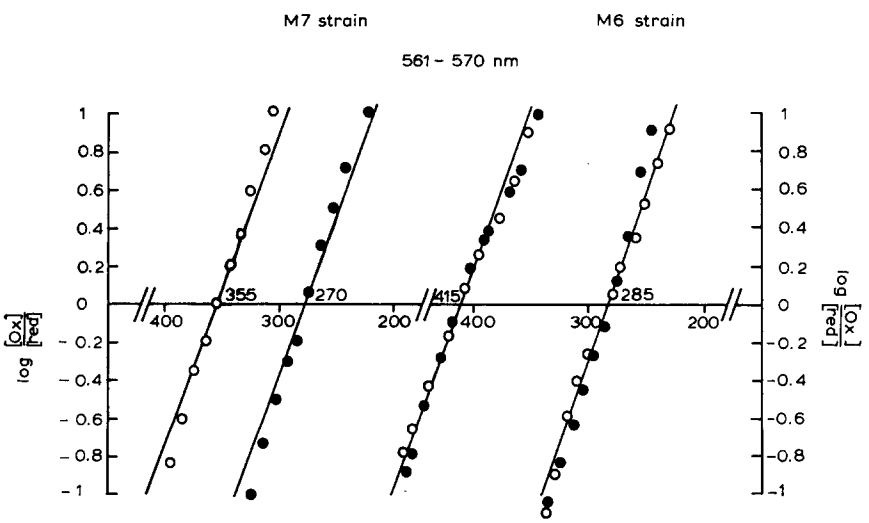

Fig. 4. Resolution of potentiometric titrations of high potential cytochromes *b* (561–570 nm) in membranes from M7 and M6 mutants in the presence (○) or absence (●) of carbon monoxide. The conditions were as in Fig. 3.

TABLE I

NADH OXIDASE ACTIVITIES IN MEMBRANES FROM *RPS. CAPSULATA*, STRAIN A1a *pho*$^+$ AND M7 MUTANT

The NADH activity was 32 μequiv/h · mg protein in membranes from the A1a *pho*$^+$ strain and 18 μequiv/h · mg protein membranes from the M7 strain. NADH oxidase was measured using an O_2 electrode (Yellow Springs Mod. 5400) at 25 °C. High intensity illumination of the reaction chamber was provided by two 500 W spot lamps; two round flasks filled with water were used as a heat filter and a focusing device between the lamps and the electrode. Green light was obtained using a Wratten filter number 58 (470–610 nm).

	Activity (%)	
	A1a pho$^+$	M7
Control	100	100
+KCN ($5 \cdot 10^{-5}$ M)	69	100
+KCN ($5 \cdot 10^{-5}$ M)+CO (1.200 μM)	27	30
Green light	45	50
White light	65	95

absence of an alternative oxidase, is paralleled by the lack of any effect of CO on the thermodynamic characteristics of cytochrome b_{270}.

On the other hand, the apparent redox potential of cytochrome b_{150} is shifted, although the same experimental uncertainty described above for M7 is present, also with membranes preparations from M6.

The complex between CO and cytochrome b_{270} appears to be photodissociable as indicated by the relief of the CO inhibition of NADH oxidase in M7 membranes by light. A stimulation of the CO-inhibited activity can be obtained either with white light or with green light (Table I), suggesting the cytochrome nature of the functional alternative oxidase. Confirmative results on this point were also obtained using the the carotenoid-less mutant A1a *pho*$^+$, which contains a normal branched respiratory pathway: membranes of this strain can be brought into a condition of the respiratory chain, analogous to that of M7 mutant by addition of 5.10^{-5} M KCN, which completely blocks the cytochrome *c* oxidase-dependent branch of the chain and consequently inhibits about 30 % of the overall NADH oxidase activity [1, 2]. As also shown in Table I, the residual respiratory activity in KCN-inhibited A1a *pho*$^+$ membranes behaves essentially as that of M7, in that it is strongly inhibited by CO and the inhibition is totally reversed by white light and also partially by green light. These results suggest that in the A1a *pho*$^+$ strain as well, a CO-binding photodissociable cytochrome is involved in a respiratory pathway alternative to that including cytochrome *c* oxidase activity.

The intracellular localization of cytochromes c_2 (c_{342}) *and cc'*

It has been known since a long time that some photosynthetic bacteria, among which *Rps. capsulata* [10] contain a soluble (or easily solubilizable) CO-binding cytochrome, called *cc'*. This pigment contains two covalently bound protohemes and presents an unusual reduced minus oxidized spectrum characterized by a broad absorption band in the 550–560 region [11]. Its CO-binding capacity, at least when

free in solution, and its autooxidability have lead to speculations that this cytochrome could function as a terminal oxidase in the aerobic metabolism of some facultative photo-synthetic bacteria [12]. On the other hand, very detailed studies by Taniguchi and Kamen [13] have excluded the presence of *cc'* in aerobically grown cells of *Rhodospirillum rubrum* which possesses a CO-inhibited *b* type oxidase; in addition, the rate of autooxidation of cytochrome *cc'* was shown to be too slow to account for a role of a terminal oxidase [14].

However, more recent studies [15] suggest the possibility that a form of membrane-bound *cc'*, characterized by different spectral properties and, perhaps, by thermodynamic and kinetic properties as well, could be present in the aerobic membranes.

Large amounts of *cc'* are found in *Rps. capsulata* cell-free extracts, either when grown photosynthetically or aerobically in air-saturated media. It is possible therefore that the CO-inhibited oxidase, which we have identified as cytochrome b_{270}, is related to *cc'*. It would be possible to clarify this possibility only by studying the chemical characteristics of the prosthetic group of the isolated oxidase, an approach which is at present unfeasible. However, the studies on the intracellular localization of cytochrome *cc'* described below, seem to exclude the possibility that *cc'* be related to the alternative oxidase.

Spheroplasts from aerobically grown *Rps. capsulata* were prepared by digestion of the cell wall with EDTA and lysozyme and the release of cytochromes during cell

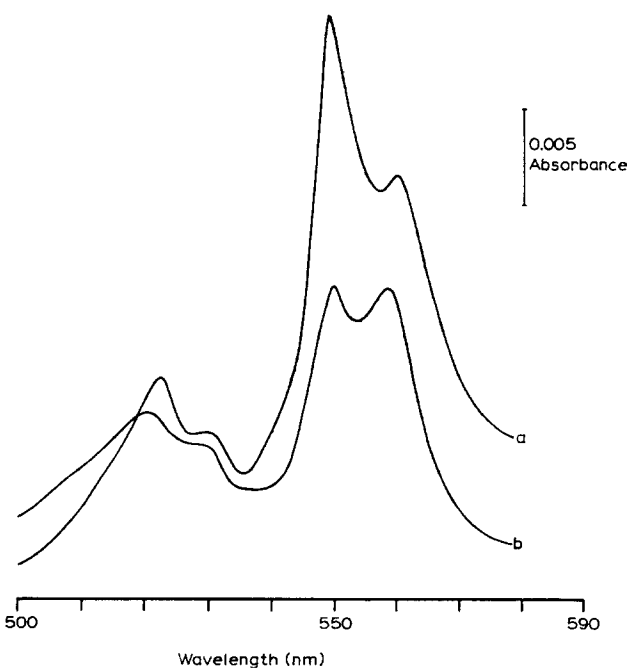

Fig. 5. Dithionite minus ferrycianide difference spectra of bacteria (a) and spheroplasts (b) of *Rhodopseudomonas capsulata*, St. Louis. Bacteria and spheroplasts were broken with a French pressure cell and the spectra were recorded using the whole extracts. The bacteriochlorophyll concentration in both assays was 3 μg/ml.

TABLE II

AMOUNTS OF DIFFERENT CYTOCHROMES PRESENT IN TOTAL EXTRACTS OF WHOLE CELLS AND SPHEROPLASTS

	Whole cells		Spheroplasts	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Cytochrome c_{342}	1930	1840	395	950
Cytochrome *cc'*	308	453	52	39
Cytochromes *b*	620	802	442	810
CO-binding cytochrome *b*	116*	191*	116*	191*

* The data indicated were assumed to be identical to those measured in membranes prepared from spheroplasts, on the basis that in these membranes, the only CO-binding pigment was a *b* type cytochrome. (For details about these calculations, see text). The data are expressed as nmol/mg bacteriochlorophyll.

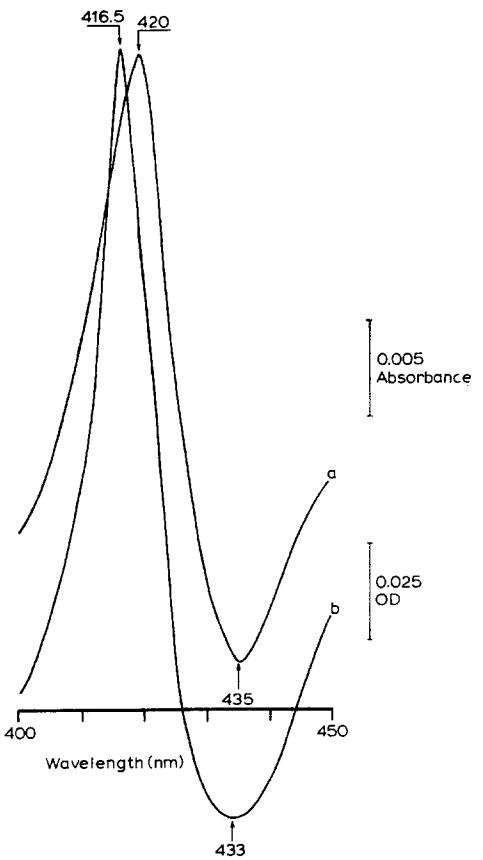

Fig. 6. Dithionite reduced minus reduced plus CO difference spectra of bacteria (b) and spheroplasts (a) of *Rhodopseudomonas capsulata*, St. Louis.

wall digestion was followed by spectral analysis of the total cell-free extracts and of sedimented membranes. In agreement with previously reported data on photosynthetically grown cells [16], a large percentage of cytochrome c_2 (c_{342}) is lost in the supernatant during cell wall digestion, as shown in Fig. 5; the decrease in cytochrome c_2 in spheroplasts was variable in different preparations, ranging from 50 to 80% (Table II).

That this solubilization is due to the presence of this cytochrome in the periplasmic space and not to the accidental damage of the plasma membrane, was firmly established by the concurrent measurement of the activity of a cytoplasmic marker enzyme (NADH-dependent malic dehydrogenase) which was recovered nearly completely (85–90 %) in sedimented spheroplasts.

The effect of cell wall digestion was still more pronounced on the CO-binding cytochromes: a large decrease of absorbance was observed in the dithionite-reduced plus CO minus dithionite-reduced difference spectra of French press broken spheroplasts, as compared to that of broken untreated bacteria. It should be noted that the maximum of the difference spectrum in the Soret region changes from 416.5 in whole bacteria to 419.5 nm in M7 spheroplasts (Fig. 6). The CO-binding protein released could be totally recovered in the supernatant of unbroken spheroplasts and its spectrum was coincident with that of purified cytochrome *cc'* isolated from photosynthetically grown *Rps. capsulata* (Fig. 7).

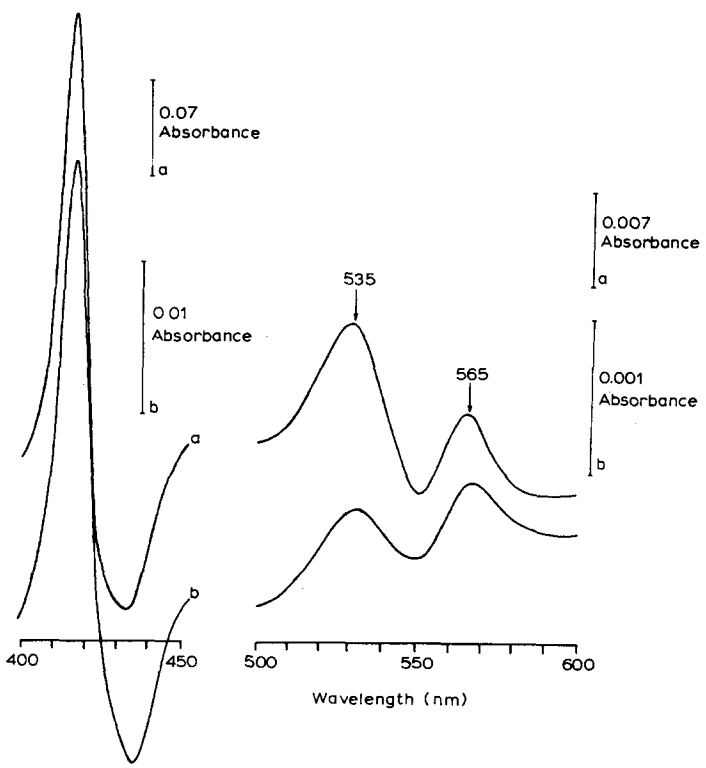

Fig. 7. CO difference spectra of purified *cc'* (a) and the first supernatant (b) obtained after centrifugation of unbroken spheroplasts.

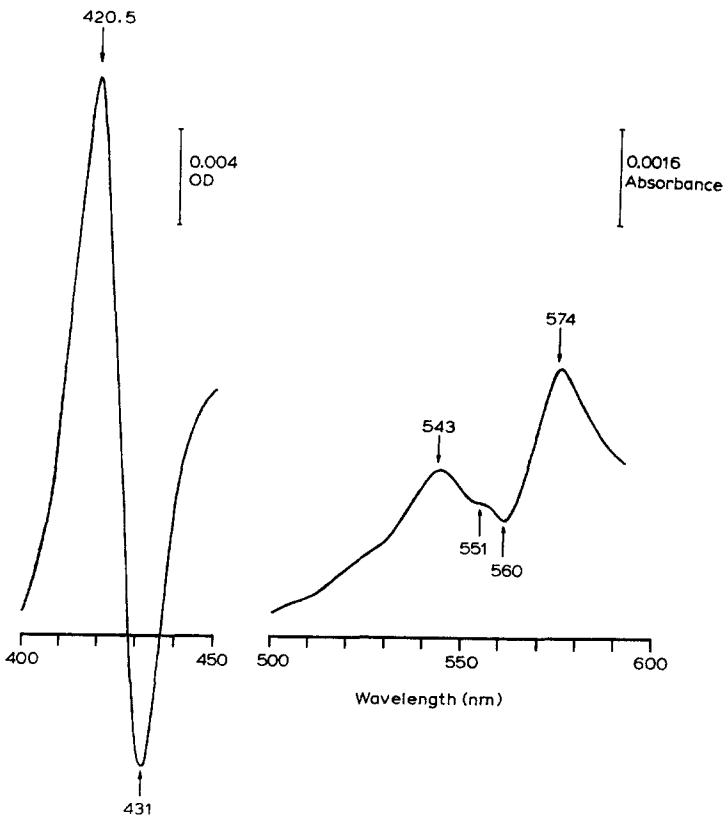

Fig. 8. CO difference spectrum of washed membranes from spheroplasts of *Rhodopseudomonas capsulata*, St. Louis. The bacteriochlorophyll concentration was 2 μg/ml and that of the protein was 6.2 mg/ml.

When membranes were sedimented and washed from broken spheroplasts, the presence of a CO-binding pigment which could not be washed out could be observed. The amount of this cytochrome, on a bacteriochlorophyll basis, was the same as that present in unbroken spheroplasts and was also identical with that present in extensively washed membranes prepared from untreated bacteria. The CO spectrum of this membrane-bound cytochrome presented maxima at 420.5, 543 and 574 nm, and minima at 431 and 560 nm (Fig. 8) and was therefore significantly different from that of purified *cc'* (especially in the Soret region which presents a maximum at 416 nm for *cc'*). The spectrum in the Soret region was also different from that observed in the whole extract of broken bacteria (Fig. 6) which presented maxima at 416.5 and minima at 434 nm. This latter spectrum probably represents that of a mixture of cytochrome *cc'*, which is predominant, and of another membrane-associated CO-binding pigment. A similar situation could be observed in all the three strains used, St. Louis, M6 and M7.

Redox titrations at pH 7.0 performed in the absence or in the presence of CO in membranes prepared from spheroplasts indicated that the digestion of the cell

wall did not affect the amount of CO-binding cytochrome b_{270} present in the preparation; a marked decrease in the amount of the other CO-binding pigment (b_{150}) was apparent. This indicates that most of the membrane bound *b* cytochromes interacting with CO in these preparations is represented by b_{270}. Since the same situation is observable in M6 one can tentatively conclude rather paradoxically that mutated b_{270}, present in M6, can still bind CO, although this ligand does not affect its redox characteristics. However, the experimental difficulties in establishing unequivocally this correlation should be underlined although it should be emphasized that a disproof of these experiments would not have any bearing on the basic conclusions that cytochrome b_{270} is involved in the alternative oxidase and that the mutation in M6 affects cytochrome b_{270}.

On the basis of these results, an approximate evaluation could be made of the contributions of cytochromes of *b* and *cc'* type to the overall spectrum obtained in whole membranes (reduced minus oxidized and reduced plus CO minus reduced, respectively). For these calculations, it was assumed that the amount of membrane-associated CO-binding cytochrome *b* could be measured from the CO spectrum of washed membranes prepared from spheroplasts and that the amount of *cc'* could be calculated from the CO spectrum of broken bacteria corrected for the contribution of the CO-cytochrome *b* complex. The contribution of the other possible CO-binding cytochromes $b(b_{150})$ was neglected in these calculations, due to the experimental uncertainty of its presence (cf. above), and to the very reduced amount of this pigment possibly present in membranes prepared from spheroplasts.

The results of such calculations are shown in Table II; in this table, the amount of *b* and *c* type cytochromes present in whole extracts from intact bacteria or spheroplasts and corrected for the contribution of *cc'* to the reduced minus oxidized spectra are also reported. The extinction coefficients used are as follows: for reduced plus CO minus reduced spectra of cytochromes *cc'* and *b* measured between the maximum of the Soret band and the minimum of the trough, 340 $mM^{-1} \cdot cm^{-1}$ [17] and 170 $mM^{-1} \cdot cm^{-1}$ (reported by Daniel [18] for cytochrome *o* of *Acetobacter suboxidans*) respectively; for the reduced minus oxidized difference spectrum of *cc'* at 552 and 561 mm, an extinction coefficient of 15.6 $mM^{-1} \cdot cm^{-1}$ was assumed [17]. The data of Table II indicate clearly that the preparation of spheroplasts results in the release of virtually all cytochrome *cc'* and a large amount of cytochrome c_2 and only in a minor and sometimes negligible decrease in the content of cytochromes *b* associated with the membranes.

The respiratory activities of preparations from spheroplasts and bacteria were also compared. The results, reported in Table III, were measured directly in the whole extract of broken bacteria or spheroplasts, in order to maximize the differences due only to the release of periplasmic proteins, and since it was observed that no important artefact was caused by the presence in the assay of the cytoplasmic fraction, except for the NADH-cytochrome *c* reductase activity. Results in the same direction, although less clear cut, could be also obtained, however, with sedimented membranes derived from bacteria and spheroplasts. The data obtained demonstrate that the loss of cytochrome *cc'* and the partial loss of cytochrome c_2, result in a marked decrease of NADH- and cytochrome *c* oxidase activities, and in a less marked inhibition of the antimycin sensitive NADH-cytochrome *c* reductase; NADH-DCIP reductase (presumably NADH dehydrogenase) and NADH-oxidase in the presence of $5 \cdot 10^{-5}$M

TABLE III

RESPIRATORY ACTIVITIES IN THE TOTAL EXTRACT FROM BROKEN WHOLE CELLS AND SPHEROPLASTS

The activities are expressed as nmol of substrate oxidized/h per mg of bacteriochlorophyll.

	Whole cells	Spheroplasts
NADH oxidase	81	55
+KCN ($2 \cdot 10^{-5}$ M)	27	28
NADH-cytochrome *c* reductase	14.5	11
+Antimycin	7.2	5.6
NADH-DCIP reductase	8.6	7.6
Cytochrome *c* oxidase	46.2	28.5

KCN (i.e. NADH-oxidase through the alternative branch of the respiratory chain) are unaffected, suggesting that neither cytochrome c_2 nor cytochrome *cc'*, are involved in this CO-sensitive electron transport pathway.

DISCUSSION

The data presented in this paper confirm and extend previous results on the composition and function of the respiratory chain of the facultative photosynthetic bacterium *Rps. capsulata*, grown in the dark in an air-saturated medium. In particular, the essential role of cytochrome b_{270} in the activity of the alternative respiratory branch has been elucitated, which can only be inhibited by high concentrations of KCN or by CO. Since it has been possible to demonstrate a direct interaction of this protein with CO and a drastic effect of this ligand on its thermodynamic characteristics, it is quite likely that cytochrome b_{270} acts as the terminal oxidase of the alternative branch, interacting directly with oxygen. All the data collected so far are in agreement with the notion that cytochrome b_{270} can be classified as a typical cytochrome *o* [19]: its CO-reduced minus reduced spectrum is quite similar to that of other cytochromes *o* previously described in the literature [20] and its reduced minus oxidized spectrum presents an α band at about 563 nm (cf. spectrum in Fig. 9). This cytochrome is distinctly different from the other high potential cytochrome *b* (cytochrome b_{413}) present in the same membranes and described extensively in previous papers [4]. Basic is the difference in role of these two carriers in the electron transport dependent energization of the membrane, since cytochrome b_{413}, but not cytochrome b_{270}, seems to be involved in oxidative phosphorylation [1]; in this connection, it is noteworthy that the midpoint potential of both oxidases is independent of the pH.

The characteristics of the mutation of cytochrome b_{270} present in the M6 strain, if substantiated by further experiments, could be of considerable theoretical interest since neither the redox properties nor the possibility of binding CO seem to be drastically affected, while the effect of CO on the apparent midpoint potential is lacking. This property would indicate that the mutation involves changes in the binding affinity for CO of the oxidized versus the reduced forms of this cytochrome. This thermodynamic property however is not necessarily linked directly to the lack of functionality in the respiratory chain.

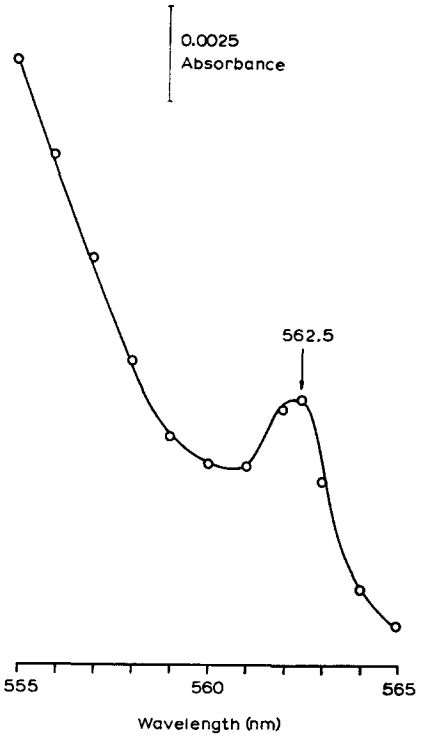

Fig. 9. Spectrum obtained plotting the differences between the oxidation reduction levels observed varying the potential of the medium (pH 7.0) from 340 to 100 mV at different wavelengths, using as reference a wavelength of 575 nm. The protein concentration in respiratory membranes of *Rps. capsulata*, St. Louis, was 2.6 mg/ml. The assay medium contained HEPES, MES, TRICINE (30 mM each), and KCl (50 mM) at pH 7.0.

The study of the pH dependence of the apparent midpoint potentials of *b*-type cytochromes has allowed to resolve the cytochromes of the *b* pool in the medium-low potential range into three components, which were not easily distinguishable at pH 7.0. The number of cytochromes *b* associated with the membrane amounts therefore to at least five; among these, two are coincident with the two oxidases and not all of the other three appear to be involved in respiration, since maximally only 60 % of the total dithionite reducible cytochrome *b* pool seems to be reduced by physiological respiratory substrates [2]. The most likely candidate for this role is cytochrome b_{60}, for which a role in photosynthetic electron flow was also suggested [21].

A similar composition in cytochromes of *b* type in both conditions of growth, has also been reported for the facultative photosynthetic bacterium *Rps. spheroides* [22].

The considerable release of cytochrome c_2 during the preparation of spheroplasts from aerobic cells confirms previous results obtained with photosynthetic cells [16] and also demonstrates the asymmetric structure of the bacterial membrane involved in the respiratory energy conservation. In addition, the release of c_2 is accompanied by a marked decrease of some respiratory activities all related to the phos-

phorylating branch of the chain [1]; this observation offers a strong support to the previous suggestion [2] for an involvement of cytochrome c_2 in respiration. This experimental approach has also demonstrated the periplasmic location of cytochrome *cc'* and offered the technical possibility of removing the whole of this CO-binding protein from the membrane with a relatively mild procedure. On the basis of the respiratory activities of these preparations, any role of this soluble cytochrome in the respiration of isolated membranes can be excluded, a conclusion which confirms previous data on *R. rubrum* [13]. Moreover, by this technique it has been possible to differentiate clearly between cytochrome b_{270} and cytochrome *cc'*, on the basis of their association to the membrane and of their absorption spectra. The most conclusive demonstration of this point, however, derives again from the availability of the M6 respiration-deficient strain in which cytochrome b_{270} appears to be mutated, but which possesses cytochrome *cc'* in the same amount and with properties identical to those of the wild type strain.

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