Biochimica et Biophysica Acta, 423 (1976) 413–430

BBA 47026

ENERGY TRANSDUCTION IN PHOTOSYNTHETIC BACTERIA

X. COMPOSITION AND FUNCTION OF THE BRANCHED OXIDASE SYSTEM IN WILD TYPE AND RESPIRATION DEFICIENT MUTANTS OF *RHODOPSEUDOMONAS CAPSULATA*

DAVIDE ZANNONI, BRUNO ANDREA MELANDRI and ASSUNTA BACCARINI-MELANDRI

Institute of Botany, University of Bologna, Bologna (Italy)

(Received July 28, 1975)

SUMMARY

The respiratory chain of *Rhodopseudomonas capsulata*, strain St. Louis and of two respiration deficient mutants (M6 and M7) has been investigated by examining the redox and spectral characteristics of the cytochromes and their response to substrates and to specific respiratory inhibitors. Since the specific lesions of M6 and M7 have been localized on two different branches of the multiple oxidase system of the wild type strain, the capability for aerobic growth of these mutants can be considered as a proof of the physiological significance of both branched systems "in vivo".

Using M6 and M7 mutants the response of the branched chain to respiratory inhibitors could be established. Cytochrome oxidase activity, a specific function of an high potential cytochrome *b* ($E'_0 = +413$ mV) is sensitive to low concentrations of KCN ($5 \cdot 10^{-5}$ M); CO is a specific inhibitor of an alternative oxidase, which is also inhibited by high concentrations of KCN (10^{-3} M). Antimycin A inhibits preferentially the branch of the chain affected by low concentrations of cyanide.

Redox titrations and spectral data indicate the presence in the membrane of three cytochromes of *b* type ($E'_0 = +413, +260, +47$ mV) and two cytochromes of *c* type ($E'_0 = +342, +94$ mV). A clear indication of the involvement in respiration of cytochrome b_{413}, cytochrome c_{342} and cytochrome b_{47} has been obtained. Only 50 % of the dithionite reducible cytochrome *b* can be reduced by respiratory substrates also in the presence of high concentrations of KCN or in anaerobiosis.

The presence and function of quinones in the respiratory electron transport system has been clearly demonstrated. Quinones, which are reducible by NADH and succinate to about the same extent can be reoxidized through both branches of the respiratory chain, as shown by the response of their redox state to KCN.

The possible site of the branching of the electron transport chain has been investigated comparing the per cent level of reduction of quinones and of cytochromes *b* and *c* as a function of KCN concentrations in membranes from wild type and M6 mutants cells. The site of the branching has been localized at the level of quinones-cytochrome b_{47}.

A tentative scheme of the respiratory chains operating in *Rhodopseudomonas*

capsulata, St. Louis and in the two respiration deficient mutants, M6 and M7 is presented.

INTRODUCTION

Previous studies [1] have indicated that the respiratory chain of *Rhodopseudomonas capsulata*, strain St. Louis, a facultative photosynthetic bacterium of the Rhodospirillaceae family, transfers electrons from respiratory substrates to O_2 through a branched pathway. This possibility was suggested mainly on the basis of the different sensitivity of NADH and cytochrome *c* oxidases to KCN and of the differential inhibition by antimycin A of NADH and succinate dependent oxidative phosphorylation. These observations made "in vitro" on isolated aerobic membranes were substantiated by the capability for aerobic growth, on a malate containing medium, of a respiratory mutant of *Rps. capsulata* (isolated by Marrs and Gest and denominated M7 [2]), which lacks the terminal cytochrome *c* oxidase.

Oxido-reduction titrations of the *b* type cytochromes present in membranes prepared from St. Louis and M7 strains have given a direct demonstration that cytochrome *c* oxidase activity is associated with the presence of a *b*-type cytochrome having a midpoint potential of about +410 mV at pH 7.0 [3].

A detailed study of the nature and the interactions of the different electron carriers operating in aerobically grown cells of *Rps. capsulata*, is reported in this paper. Additional evidence on the existence and function of two terminal oxidases showing a different behaviour in respect to inhibitors is also reported. Moreover on the basis of the level of reduction of quinones and *b*- and *c*-type cytochromes, as a function of KCN concentrations, the localization of the branching point of the respiratory chain has been elucidated. This work integrates studies on the light dependent electron transport system of the same organism [4, 5] and offers the possibility of a better understanding of the interrelationships existing between photosynthesis and respiration in the dual function membrane of *Rps. capsulata*.

MATERIALS AND METHODS

Rps. capsulata (American Type Culture Collection No. 23782) and the mutant strains M6, M7 and A1a pho$^+$ were grown for many generations in the dark in Feunbach bottles, under mechanical shaking and strong aeration. Before the transferring to aerobic dark conditions [1] the bacteria were grown anaerobically in the light. The medium described by Ormerod et al. [6] was used for both aerobic and anaerobic growth conditions; when M6 and M7 were grown photosynthetically, this medium was supplemented with Na-ascorbate at 0.5 mg/ml [2].

Membranes were prepared in 0.1 M glycylglycine buffer pH 7.2 containing 10 mM Mg^{2+}, as described in a previous paper [1], except that they were washed once with the same buffer; the preparations were stored in ice under nitrogen. Respiratory activities were measured as outlined in detail elsewhere [1].

The technique used for determination of the oxido-reduction potentials of the cytochromes was that originally reported by Dutton et al. [7]; the conditions for these assays have been already described [3].

Difference spectra during respiration or under controlled ambient redox conditions were obtained with a dual wavelength spectrophotometer by varying the scanning wavelength between 515 and 575 nm and maintaining the reference monochromator fixed at 540 nm.

Quinones were extracted from membranes following essentially the method introduced by Kröger et al. [8] and modified by Redfearn et al. [9]: membranes, corresponding to 5 mg of protein in a volume of 2 ml of buffer, were treated at room temperature with 10 ml of a mixture containing methanol+petroleum ether (6 : 4). After a low speed centrifugation the upper phase was saved and the lower phase was again extracted with petroleum ether and centrifuged. To the combined petroleum ether extracts, methanol (95 %) was added, followed by vigorous stirring. After separation of the two phases, the petroleum ether phase was dried under flushing nitrogen.

Spectra were then recorded on the ethanol dissolved extract in the range of 340-230 nm with a Cary model 15 spectrophotometer. An extinction coefficient of 12.5 $cm^{-1} \cdot mM^{-1}$ was used.

Protein content was estimated by the Lowry method [10].

RESULTS

(1) *Respiratory activities in membranes prepared from Rps. capsulata, St. Louis, M6 and M7 mutant strains.*

Our earlier suggestion [1] of a branched respiratory electron transport chain in membranes from *Rps. capsulata*, grown aerobically in the dark, was based mainly on the different degree of sensitivity to KCN of NADH oxidase and of cytochrome *c* oxidase. Fig. 1 compares the effect of cyanide on NADH oxidase of M6 and M7 membranes with that of the wild type. It is evident that there is a marked difference in response among the three kinds of respiratory membranes; in fact in M6 this activity is markedly inhibited by low concentrations of KCN (K_i about $5 \cdot 10^{-6}$ M), which are of the same order of magnitude as those inhibiting cytochrome *c* oxidase in wild type [1]. On the contrary, in M7, NADH oxidase is affected only by very high concentrations of this inhibitor (K_i about $5 \cdot 10^{-4}$ M). The two concentrations at which 50 % inhibition of activity is observed in M7 and M6, respectively, compares well with the values previously reported for the byphasic pattern of inhibition of the same activity in the wild type membranes [1]. From these data it appears that cytochrome *c* oxidase represents the only pathway for NADH oxidation in M6 while in M7 the transfer of electrons appears to be channeled to oxygen only through the branch of the chain independent from cytochrome *c* oxidase. Further support to the idea that only one or the other of the two branches of the chain is operating in the two mutant strains is presented in Table I, where a complete picture of the various respiratory activities is compared in the three bacterial strains.

The absolute values of the respiratory activities can vary markedly from one preparation to the other, expecially as NADH oxidase is concerned. However, the response to inhibitors always gives reproducible results. The possibility that the rate of NADH oxidation can be related to the growth phase of the culture (and also controlled by the O_2 concentration in the medium) cannot be ruled out and will need careful investigations. As previously reported [2, 3] cytochrome *c* oxidase is nearly

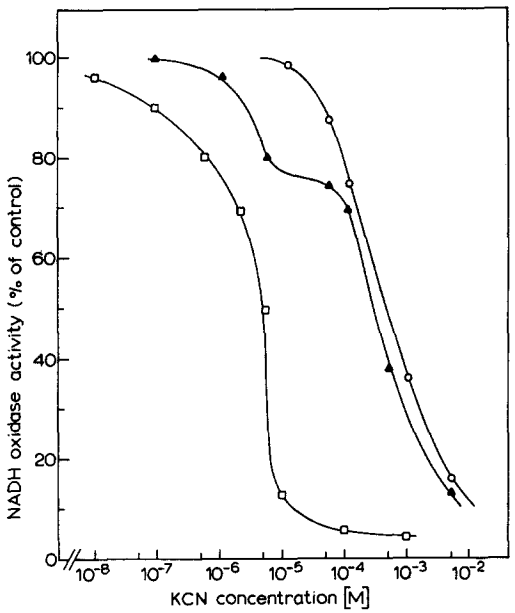


Fig. 1. Inhibition of NADH oxidase in membrane fragments prepared from *Rhodopseudomonas capsulata*, St. Louis, M6 and M7. The membranes were preincubated with KCN for 5 min, and the reaction was started by addition of NADH (0.15 mM); the rates reported were taken after 1 min of reaction and following a second addition of the substrate. 100 % of activity was 55, 18, 26.4 μequiv · h^{-1} · mg $protein^{-1}$ for *Rps. capsulata* (St. Louis), M6 and M7, respectively. ▲—▲, *Rps. capsulata* (St. Louis); □—□, M6; ○—○, M7.

TABLE I

RESPIRATORY ACTIVITIES IN MEMBRANES PREPARED FROM *RPS. CAPSULATA*, ST. LOUIS, M6 AND M7

The activities are expressed as microequivalents of substrate oxidized h^{-1} · mg $protein^{-1}$.

Electron donor	μequiv · h^{-1} · mg $protein^{-1}$		
	St. Louis	M6	M7
NADH	68	20.8	31.6
+Antimycin A (10 μM)	31.5	1.7	22.1
+CO (1 200 μM)	36.5	20.6	4.4
Succinate	6.4	4.6	4.1
+Antimycin A (10 μM)	3.2	0.5	2.5
Reduced cytochrome *c*	13.2	13.4	0.8

absent in the M7 mutant whereas this activity is again present in M6 where its rate appears to be of the same order of magnitude as that observed in the wild type. In agreement with previous data [11, 1] antimycin A inhibits only 50–60 % of NADH and succinate oxidase activities in the wild type strain; on the other hand, a nearly complete inhibition of these two activities is observed in M6 which is unable to oxidize NADH and succinate through an alternative pathway. A partial inhibition

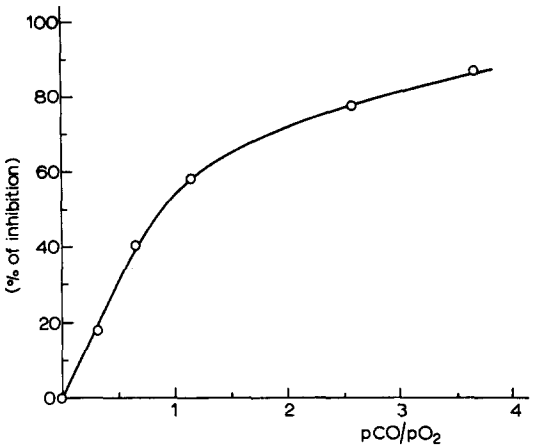


Fig. 2. NADH oxidase activity in membranes from M7, as a function of pCO/pO_2. The reaction mixture was flushed for several minutes with CO and O_2 previously mixed together at fixed ratios. The reaction was started by addition of NADH. The activity in absence of CO was 25.2 μequiv · h^{-1} · mg $protein^{-1}$.

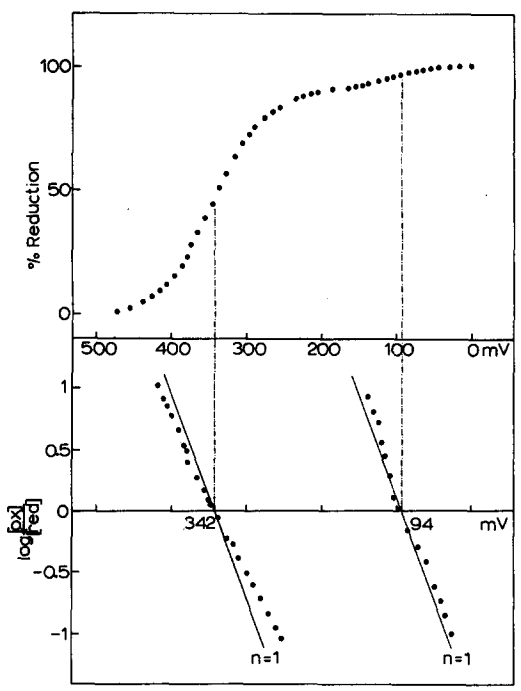


Fig. 3. Potentiometric titrations performed at 551-540 nm in membranes from *Rps. capsulata*, St. Louis. The assay contained 2.26 mg protein per ml.

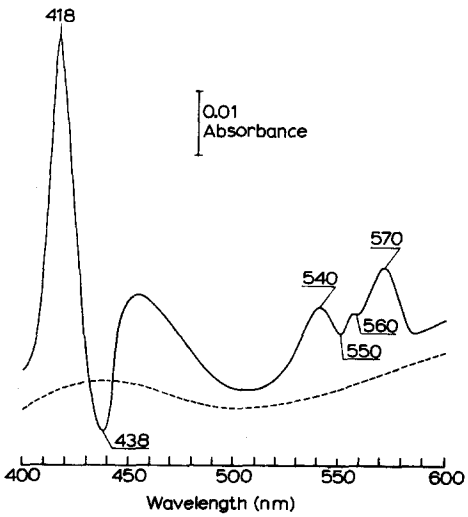


Fig. 4. Dithionite reduced plus CO minus dithionite reduced difference spectrum in membranes from *Rps. capsulata*, St. Louis. The assay contained 2.15 mg protein/ml. ---, baseline before CO addition.

(30–40 %) of these activities is observed also in M7 in the presence of antimycin A; this could suggest a possible role of a *b*-type cytochrome in the pathway independent of cytochrome *c* oxidase.

Clear cut evidence of the difference in nature of the two terminal oxidases is represented by their markedly different response to CO. This ligand inhibits completely oxidation of NADH in M7, whereas no effect on the same activity can be detected in M6. Intermediate response to the inhibitor is observed in the wild type membranes, which possess both oxidases.

These data are also in agreement with the observed lack of effect of CO (unpublished data) on cytochrome *c* oxidase activity in membranes from wild type cells.

In Fig. 2 the % inhibition of the rate of NADH oxidase in M7 is reported as a function of pCO/pO_2 ratio. An increase in pCO/pO_2 ratios, at a constant pressure of 1 atmos., produces a gradual inhibition of the activity; 90 % inhibition of NADH oxidase is reached at pCO/pO_2 value of 3.5, corresponding to 1200 μM CO dissolved.

These data open the possibility of further studies on the nature of the alternative oxidase, which must interact with CO; preliminary data show the presence in aerobic membranes of CO binding pigments; the nature of these pigments is now under active investigation. Identical dithionite-reduced plus CO minus dithionite-reduced difference spectra can be observed in aerobic membranes from M6, M7 and wild type (Fig. 4); this observation, per se, cannot therefore shed any light on the functional role of this pigment.

(2) *Characterisation of some electron carriers of the respiratory chain of Rps. capsulata.*

In a previous paper [3] we have shown that the cytochromes detectable by

differential spectra of membranes prepared from *Rps. capsulata*, St. Louis belong only to the *b* and *c* class. Oxido-reduction titrations, performed at 561–570 nm, at pH 7.0, have resolved the *b* cytochromes in at least three components with midpoint potentials equal to $+413\pm5$, $+259\pm5$, $+47\pm5$ mV, respectively. The unequivocal role of terminal cytochrome *c* oxidase was given to the high potential *b* (b_{413}) on the basis of the observed correlation existing between the disappearance of this component and the lack of cytochrome *c* oxidase activity in membranes from the M7 strain. Moreover, the presence in membranes from M6 strain of this cytochrome is associated with the parallel presence of cytochrome *c* oxidase activity. In fact this mutant presents the same composition in *b* type cytochromes as the wild type strain. Therefore, the absence of the alternative oxidase does not correlate with the absence of anyone of these three *b*-type components. In Fig. 3 a potentiometric titration at 551–540 nm of membranes prepared from *Rps. capsulata* is shown. Two *c* type cytochromes with midpoint potentials at $+342\pm5$ and $+94\pm5$ mV at pH 7.0 can be detected; they correspond to 85 % and 15 % of the total *c* type cytochrome. Membranes from M6 and M7 show approximately the same percentage composition in cytochrome *c*.

Although the absolute quantities of dithionite reducible cytochrome *b* (0.6–0.8 nmol/mg protein) and *c* (1.0–1.2 nmol/mg protein) vary slightly in different preparations and are always higher in the two mutant strains, the ratio of total cyto-

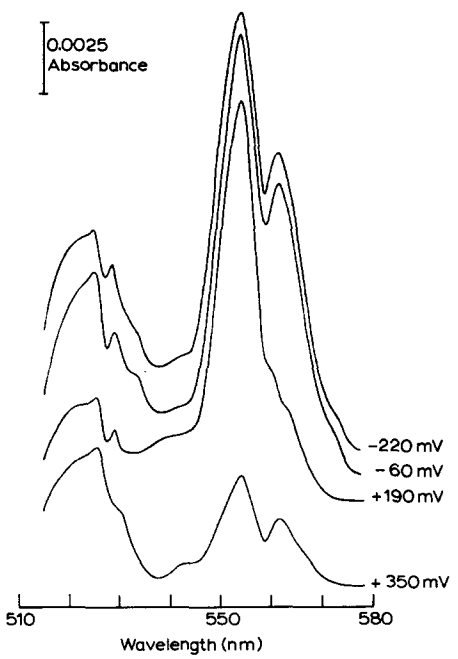


Fig. 5. Absorption spectra at controlled redox potentials of membranes from *Rps. capsulata*, St. Louis. The assay contained 0.34 mg protein per ml. The spectrum recorded at −220 mV was corrected for the contribution given by dyes at this potential. The spectra correspond to differential spectra calculated against a baseline recorded on the same sample completely oxidized with ferricyanide ($E_h = +490$ mV).

chrome *b* to total cytochrome *c* remains virtually constant (ratio *b*/*c* about 0.6). This value differs from the data of Klemme et al. [11] in *Rps. capsulata* KBl, who reported a *b*/*c* ratio of approx. 2; different *b*/*c* ratios can be measured in different strains of *Rps. capsulata*, as observed for example in the mutant A1a pho$^+$ from *Rps. capsulata* 37 b4, grown photosynthetically [4] or heterotrophically (D. Zannoni, unpublished observations). The agreement between the reported differential spectra [3] and the potentiometric titrations of the cytochromes *b* and *c* has been confirmed by spectra performed at different ambient redox potentials. Typical experimental results are shown in Fig. 5. In this figure spectra recorded at potentials of +350, +190, +60, −220 mV are depicted; the redox potentials chosen correspond to plateau regions of typical redox titrations of *b* type cytochromes measured at 561–570 nm. The data are presented as differential spectra referred to a completely oxidized respiratory chain (E_h = +490 mV). A clear cut spectrum of a cytochrome of *b* type is already apparent at +350 mV; this component corresponds to cytochrome b_{413}, the functional cytochrome *c* oxidase in these membranes. A second *b* type component, which becomes evident as a shoulder at 564 nm in the 190–350 potential span should represent the cytochrome b_{260} [3] observed in redox titration experiments. The major contribution to the absorbance peak at 561 nm appears at more negative poten-

TABLE II

THE EFFECT OF RESPIRATORY SUBSTRATES ON THE LEVEL OF REDUCTION OF *b* AND *c* CYTOCHROMES IN MEMBRANES FROM *RPS. CAPSULATA*, ST. LOUIS, M6 AND M7

The assay contained 1.2 mg of protein per ml. The data are expressed as per cent of the total dithionite reducible cytochromes.

Experimental conditions	% of reduction; electron donor					
	NADH			Succinate		
	St. Louis	M6	M7	St. Louis	M6	M7
(a) Cytochromes of *b* type (561–570 nm)						
Steady state	18	20	30	10	20	30
Anaerobiosis	39	45	57	41	45	46
Steady state +Antimycin A (10 μM)	26	32	38	25	38	41
Anaerobiosis +Antimycin A 10 μM)	40	46	58	43	47	48
(b) Cytochromes of *c* type (551–540 nm)						
Steady state	40	30	85	45	35	78
Anaerobiosis	85	76	85	88	80	78
Steady state +Antimycin A (10 μM)	25	13	85	25	12	78
Anaerobiosis (10 μM)	88	80	85	88	80	78

tials, in agreement with the presence of a predominant pool of *b* cytochrome with an apparent $E'_0 = +47$ mV. No further reduction of cytochrome *b* could be detected at potentials more negative than −60 mV.

Most of the *c*-type cytochrome is reduced at +190 mV in agreement with the quantitative predominancy of cytochrome c_{342} (compare Fig. 4). A minor component corresponding to cytochrome c_{94} and representing 15 % of total cytochrome *c* is also evident; no other *c*-type cytochrome is present below −60 mV.

Table II (a) summarizes and compares the effectiveness of NADH and succinate on the reduction of cytochromes $b_{561-570}$ in *Rps. capsulata* w.t. and in the two mutant strains, M6 and M7. In all the three strains the percentage reduction of cytochromes reached in steady state respiration and anaerobiosis is virtually the same, when either NADH or succinate is added.

However, it must be noted that in M7 a significantly higher level of reduction, compared with that characteristic of the other two strains, is usually observed, especially in the steady state, both with NADH and succinate. This last response could be related to the lack in M7 of the terminal cytochrome *c* oxidase [2] which results in a complete and stable reduced state of cytochrome c_{342} (see Table IIb). Additional experiments on the level of reduction of cytochrome *b* in the presence of $5 \cdot 10^{-5}$ M KCN, which blocks cytochrome *c* oxidase, give support to this suggestion (see below Fig. 8a).

Addition of antimycin A to membranes from w.t. and M6 causes an increase in the steady state level of reduction of *b* cytochromes which is more marked in M6, in agreement with the greater effectiveness of this antibiotic on the NADH and succinate oxidase activities of these membranes.

Little enhancement of the steady state level of reduction of *b* cytochromes in the presence of antimycin A can be observed in M7, indicating that the effect of this antibiotic is predominant in the branch of the chain, not functional in this mutant. It is evident that the whole pool of *b* cytochromes cannot be reduced by the respiratory substrates used in these experiments. A different situation (Table IIb) is observed when measuring the effect of NADH and succinate on the reduction of cytochrome *c* (551–540 nm). In fact in anaerobiosis the level of reduction of cytochrome *c* reaches values of about 80–90 % indicating that practically all c_{342} (cytochrome c_2) is substrate reducible in the respiration of *Rps. capsulata*. Looking at the steady state level of reduction of cytochrome *c*, induced either by NADH or succinate, a different behaviour can be observed in the three bacterial strains. While in the wild type cytochrome *c* is reduced to 40–50 %, in M6 a lower steady state level (30 %) is reached; in contrast in M7 the level of reduction of cytochrome *c*, obtainable only in anaerobiosis in the other strains, is already reached in aerobiosis. All these observations clearly prove that cytochrome c_{342} is part of the respiratory electron transport chain and is the physiological electron donor for cytochrome *c* oxidase (cytochrome b_{413}); consequently the lack of a functional cytochrome *c* oxidase in M7 results in a dead end branch in the respiratory pathway.

Reduced cytochrome c_{342} is promptly reoxidized in w.t. or M6 strains during anaerobic-aerobic transitions or following exhaustion of respiratory substrates, or inhibition by antimycin A; these phenomena are however completely absent in M7, in which under all conditions a stable reduction of cytochrome c_{342} is observed (unpublished results). Oxido-reduction spectra induced by addition of NADH and

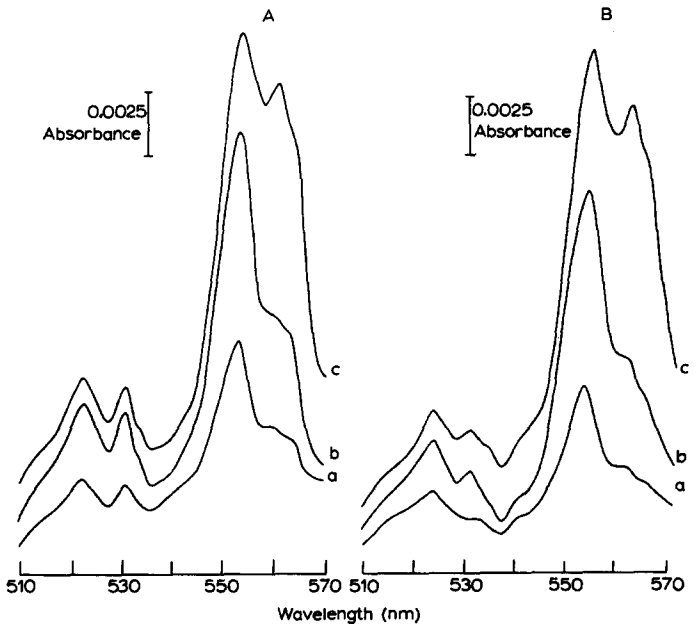


Fig. 6. The effect of respiratory substrates on the redox state of cytochromes. (A), in presence of NADH; (B), in presence of sodium succinate; (a), steady state condition (this condition was achieved by addition of the respiratory substrates together with a mixture of H_2O_2+Catalase to allow the recording of the spectrum), (b), anaerobiosis, (c), sodium dithionite. The respiratory activities were 62 and 5.8 μequiv $\cdot$ h^{-1} $\cdot$ mg $protein^{-1}$ for NADH and succinate oxidase activities, respectively.

succinate to respiratory membranes from *Rps. capsulata*, St. Louis, are shown in Fig. 6: these spectra, recorded at different oxido-reductive levels of the cytochromes, i.e. during steady state respiration, anaerobiosis and after addition of dithionite, are in good quantitative agreement with the data reported in Table IIa and b.

Quinones

The presence of quinones in photosynthetically and aerobically grown cells of Rhodospirillaceae has been widely documented [12, 13]. Although the direct participation of these carriers in the light dependent electron transport chain has been demonstrated [13–15] their specific role in bacterial photosynthesis is still a matter of debate [16]. The possibility of the existence of different pools of quinones, reducible by light and by respiratory substrates, has also been suggested [17]. A detailed study on the function and localization of quinones in the oxidative electron transport system has been described only for *Rhodopseudomonas palustris*, grown aerobically in the dark [18].

Previous data indicated Q_{10} as the only quinone present in photosynthetically and aerobically grown cells of *Rps. capsulata* [12]. We have investigated the role of quinone in the respiratory chain of *Rps. capsulata* by means of two different experimental approaches, namely by irradiation of the membranes with ultraviolet light and by the rapid solvent extraction technique [8, 9]. Ultraviolet irradiation causes a severe inhibition of NADH and succinate oxidase activities which is more drastic

TABLE III

EFFECT OF ULTRAVIOLET IRRADIATION ON VARIOUS RESPIRATORY ACTIVITIES OF MEMBRANES FROM *RPS. CAPSULATA*, ST. LOUIS

Membranes (3 mg/ml) were irradiated for 2 h at +2 °C with an ultraviolet lamp (Hanau, model S 81-PL 327). The activities are expressed as microequivalents of substrate oxidized · h^{-1} · mg protein^{-1}.

	Control activity	Ultraviolet irradiated	
		Activity	% of control
NADH oxidase	66	5.2	8
NADH-DCIP reductase	14.6	11.7	80
NADH-cytochrome *c* reductase	39.8	9.5	24
Succinate oxidase	6.4	2.7	43
Succinate-DCIP reductase	4.5	2.9	65
Succinate-cytochrome *c* reductase	4	2	50
Cytochrome *c* oxidase	15.2	8.3	55

for NADH- than for succinate-oxidase (Table III). The same behaviour is also shown by NADH- cytochrome *c* and succinate-cytochrome *c* reductase activities. The inhibitions observed are not due to the damage of the specific dehydrogenases involved as demonstrated by the much lower decrease in these activities, which is however higher for succinic than for NADH dehydrogenase. The difference in the degree of inhibition of these two activities can be explained by a different lability of the two systems, or by the existence of different rate limiting steps.

The residual activities show approximately the same degree of sensitivity to Antimycin A as observed in untreated membranes suggesting the involvement of quinones in both branches of the chain (unpublished observation).

Although irradiation of membranes by ultraviolet light causes an irreversible destruction of the quinones [18], as demonstrated by the spectrum shown in Fig. 7, aspecific damage to respiratory activities caused by prolonged ultraviolet irradiation has previously been reported [18] and is also evident from the data of Table III. In fact also cytochrome *c* oxidase activity appears to be affected by this treatment.

Therefore, although on the whole, the results, obtained by ultraviolet irradiation of the membranes, indicate an involvement of quinone in the transferring of electrons from NADH and succinate to oxygen, this technique does not allow a clear cut demonstration of the function of this carrier.

Localization of the site of branching of the respiratory chain

Measurements of the redox level of ubiquinone obtained by the rapid solvent extraction technique substantiate the conclusions drawn by the ultraviolet irradiation experiments and offer some indication about the localization of the branching of the respiratory chain.

Since interference in the redox spectra of carotenoids and quinones extracted

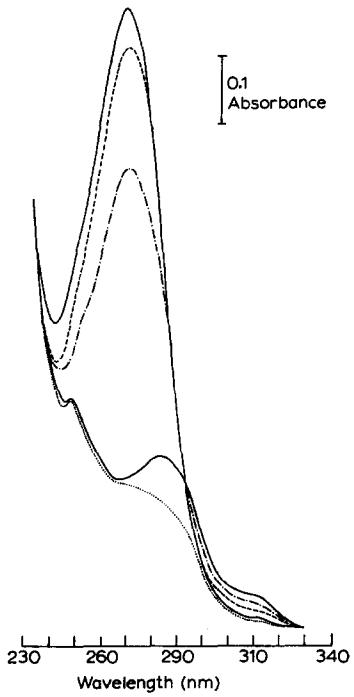


Fig. 7. Absorption spectra of chemically extracted quinones from membranes of Ala pho$^+$. — (upper), oxidized by addition of 3 μl of 5 % KOH in 95 % ethanol; ---, ·····, oxido-reductive state of quinones extracted from membranes preincubated with O_2 in absence of substrates and with NADH (12 mM) or succinate (40 mM) and oxygen, respectively. — (lower), reduced by addition of KBH_4; ····, quinones extracted from ultraviolet irradiated membranes (conditions as in Table III).

from *Rps. capsulata*, St. Louis was observed, membranes from the carotenoid less mutant Ala pho$^+$ from *Rps. capsulata* 37 b4, grown aerobically in the dark, were used in this study. The respiratory chain of this mutant shows a comparable behaviour in respiratory activities and sensitivity to inhibitors with that of *Rps. capsulata*, strain St. Louis (unpublished results).

Spectra of extracted quinones at different degree of reduction are shown in Fig. 7. The absorption maximum of all the spectra of completely or partially oxidized quinones is centered at 275 nm. This is in agreement with previous data which showed that Q_{10} was the only quinone present in *Rps. capsulata* [12]. In the same figure the spectrum of chemically reduced quinone is also shown. For sake of completeness a spectrum of quinones, extracted from ultraviolet irradiated membranes, is also reported; alteration of spectral properties is apparent and no changes can be induced by oxidizing or reducing agents, indicating that the quinones have been structurally damaged by the ultraviolet treatment.

The extent of reduction of quinones induced by respiratory substrates during steady state respiration, anaerobiosis and in the presence of inhibitors is reported in Table IV. It is evident that NADH and succinate cause approximatively the same state of reduction of quinone in all the conditions tested, suggesting the accessibility of this carrier to both substrates. The level of reduction reached in steady state (37 %)

increases in anaerobiosis to about 80 % in the presence of NADH and 65 % in presence of succinate.

We have shown previously [1] that in membranes from *Rps. capsulata*, St. Louis a great difference in sensitivity to CN^- between NADH oxidase and cytochrome *c* oxidase activities exists: these data hold true also for Ala pho^+ mutant. This phenomenon has been interpreted as a difference of CN^- sensitivity of the two oxidase systems and is also supported by the different behaviour of NADH oxidase in M6 and M7 (see Fig. 1). We have therefore compared the state of reduction of quinones in the presence of two different concentrations of cyanide.

$5 \cdot 10^{-5}$ M cyanide, which blocks completely cytochrome *c* oxidase activity in w.t. membranes and NADH oxidase in M6, increases the substrate induced level of reduction of quinones to only 47 %, indicating that reduced quinone can be reoxidized through the alternative oxidase; in fact, higher concentrations of KCN ($5 \cdot 10^{-3}$ M), which inhibits completely NADH oxidase in w.t. and M7, brings this level of reduction to a value comparable to those obtained in the anaerobic state.

The possibility of an alternative pathway of oxidation of quinones is also confirmed by the steady state level of reduction of this carrier in presence of antimycin A which only partially blocks NADH and succinate activities; and does not influence at all the redox state of quinone in steady state respiration.

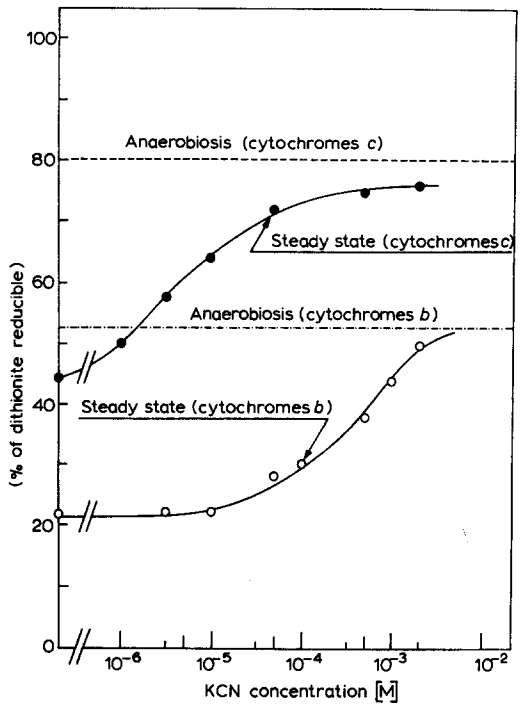
As has been shown in relation to quinones, studies on the percentage reduction of the electron carriers, as a function of KCN concentrations could give some hint on the possible site of branching of the respiratory chain. We have therefore examined the degree of reduction of cytochromes *b* and *c*, at increasing concentrations of KCN, in membranes from *Rps. capsulata*, St. Louis (Fig. 8a). In agreement with the data reported in Table II, during NADH respiration in absence of CN^-, only 20 % of the total dithionite reducible *b* cytochromes appears reduced, while *c* cytochromes are reduced at 45 %. Addition of KCN at millimolar concentration

TABLE IV

EFFECT OF KCN AND ANTIMYCIN A ON THE REDOX STATE OF QUINONES DURING RESPIRATION OF NADH AND SUCCINATE BY MEMBRANES, FROM THE Ala pho^+ STRAIN

Membranes corresponding to 5 mg of protein in 2 ml were incubated for 10 min at 25 °C with continuous shaking in the presence of NADH (12 mM) or succinate (40 mM). The redox state of quinones was then determined by the rapid extraction technique (see Methods). Aerobic conditions were achieved by adding H_2O_2 in the presence of catalase a few seconds before quenching the reaction; anaerobiosis was maintained by flushing the system continuously with nitrogen. The average amount of quinones extractable was about 10 nmol per mg protein. When experiments were performed in the presence of KCN or antimycin A, the membranes were preincubated 5 min with the inhibitor before addition of the substrates.

Exogenous electron donor	Steady state respiration (% of reduction)				Anaerobiosis
	No addition	+Antimycin A (10 μM)	+KCN (5.10^{-5} M)	+KCN (5.10^{-3} M)	
None	10	–	–	–	20
NADH	37	38	47	70	78
Succinate	37	37	48	62	64



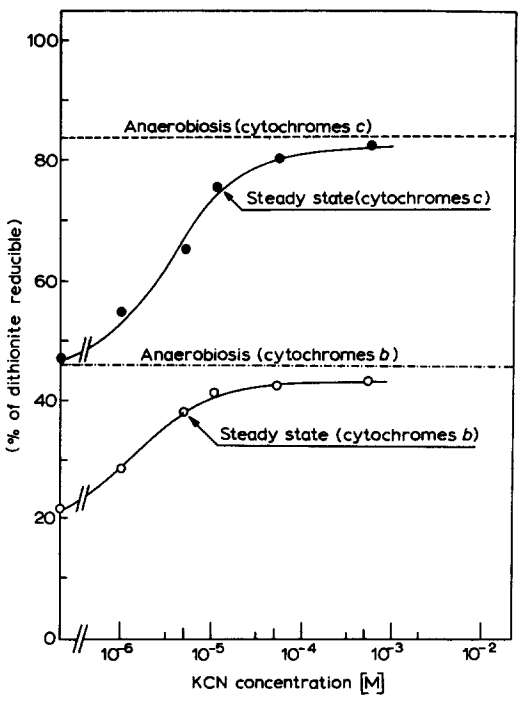


Fig. 8. (a) Redox state of cytochromes *b* and *c* as a function of KCN concentrations in membranes from *Rps. capsulata*, St. Louis. (b) Redox state of cytochromes *b* and *c* as a function of KCN concentrations in membranes from M6. The data are expressed as per cent of the total dithionite reducible cytochromes.

causes an increase of the levels of reduction of *b* and *c* cytochromes to 53 % and 76 %, close to the levels observed in anaerobic conditions. However, this transition takes place at quite different concentrations of KCN for cytochromes *b* and *c*. In fact, while for cytochromes *c*, 50 % of the maximal increase in the state of reduction is observed at about $2 \cdot 10^{-5}$ M, the same effect is observed for cytochromes *b* only at $5 \cdot 10^{-4}$ M. These values compare well with the concentrations of KCN which inhibit 50 % NADH oxidase through the two alternative pathways of respiration.

That the different response of the cytochromes in the St. Louis strain indicate a reoxidation of these carriers through pathways varying in sensitivity to CN^-, is confirmed by similar experiments performed in M6, a strain possessing only one oxidase highly sensitive to KCN (compare Fig. 1). In this mutant the state of reduction of both cytochromes *b* and *c* is affected at comparable degree by the inhibitor (Fig. 8b).

DISCUSSION

The results presented in this paper, together with previously published data obtained "in vivo" and "in vitro" [1–3] demonstrate conclusively that a branched respiratory chain is operating in *Rps. capsulata*, grown aerobically in the dark. A decisive contribution for these conclusions has been made by the availability of respiration deficient mutants with specific lesions in the two terminal oxidases [2]. These mutants have been particularly useful for the identification of the cytochrome (b_{413}) involved in cytochrome *c* oxidase activity [3], for the localization of the branching point of the chain and for a clear-cut characterization of the sensitivity to some specific inhibitors of the different oxidase systems.

Several electron carriers present in aerobic membranes have been characterized in this study; at the present state of knowledge however a direct involvement in respiration has been clearly demonstrated only for some of them. Besides cytochrome *c* oxidase, cytochrome c_{342} (c_2) also clearly participates in respiration since it is largely and very rapidly reduced by respiratory substrates and oxidized by O_2 only when b_{413} is present. Therefore cytochrome c_2 can be tentatively considered as the physiological electron donor to b_{413}. In this connection it is also important to underline that cytochrome c_{342} is present also in photosynthetically grown cells [4, 5] and that in these cells it is localized, in an easily solubilizable form, on the side of the membrane facing the periplasmic space [19].

Ubiquinone (probably only Q_{10}) is also clearly involved in respiration since most of the quinone pool, which on a molar basis exceeds cytochromes by a factor varying from five to ten, is substrate reducible in anaerobiosis and since oxidation of quinone through both branches of the respiratory chain has been demonstrated. It is not clear at present if the part of quinone not reducible by substrates (20–30 %) is really unaccessible to reducing equivalents from the respiratory chain.

A more complex situation exists as far as cytochromes of *b* type are concerned; no more than 60 % of the total dithionite reducible *b* cytochromes are reduced by substrates in anaerobiosis. It appears, therefore, that about 40 % of the *b* pool is either damaged during the preparation of the membranes [20] or not involved in the respiratory chain. Redox titration experiments [3] have demonstrated the

presence of a large pool of cytochrome *b* centered at about +47 mV at pH 7.0, representing nearly 70 % of the total *b* cytochromes in w.t. strain. This pool appears surely formed of more than one component as is evident from the large potential span (more than 240 mV) needed for its complete reduction. Preliminary redox experiments at pH's different from 7.0 have indeed indicated that several *b* type components can be resolved. A positive conclusion reached in this study is that certainly one or more *b* type cytochromes are involved in both branches of the respiratory chain in strict connection with the quinone pool and located at the oxygen side of the rotenone sensitive site and before or coincident with the antimycin A binding site.

A tentative scheme of the respiratory chains of *Rps. capsulata*, St. Louis and of the respiration deficient mutants, M6 and M7, is shown in Fig. 9; this scheme does not include cytochrome b_{260} and c_{94} since no evidence for their role in respiration could be obtained.

The participation also in photosynthetic cyclic electron flow of *Rps. capsulata*, Ala pho$^+$ of a cytochrome *b* and of a cytochrome *c* with midpoint potentials equal to +60 and +340 mV at pH 7.0, respectively, i.e. with redox characteristics similar to those two respiratory cytochromes, has been recently demonstrated [4, 5]. The possibility that in Rhodospirillaceae photosynthetic and respiratory electron flows share common electron transport components is therefore very strong and has also received support by the studies of Connelly et al. [21] and by the recent reconstitutions of cyclic electron flow obtained by incubating reaction center preparations with membranes prepared from bacteriochlorophyll less mutants grown heterotrophically [22, 23].

In a previous paper [1] we have demonstrated that respirations through the two alternative pathways of the branched chain differ in their efficiency of oxidative phosphorylation since one or two coupling sites are located after the branching point and only in the segment of the chain containing cytochrome b_{413}. The presence of

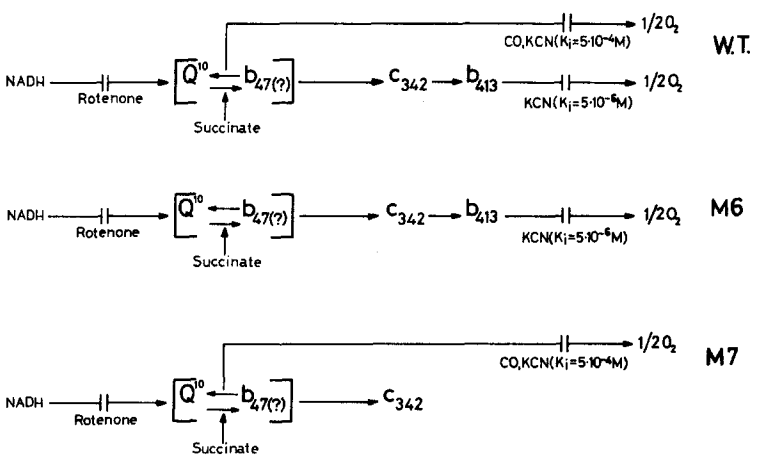


Fig. 9. Tentative scheme of the respiratory chains of *Rps. capsulata*, St. Louis and of the respiration deficient mutants M6 and M7.

multiple oxidase systems at different degrees of coupling in bacteria has been widely documented [24–26] and it is conceivable that the development of alternative ways of oxidation is related to the need for an independent regulation of the energy charge and of the reducing power under extremely variable conditions of growth. This need is particularly evident for facultative photosynthetic bacteria, since their natural environment, characterized by the availability of light in semiaerobic conditions is consistent with the simultaneous presence in the cell of respiratory and photosynthetic metabolism. This condition would need the presence of a rather active mechanism of dissipating reducing equivalents in the presence of the high energy charge maintained by cyclic photophosphorylation. As a reasonable working hypothesis, this function can be possibly performed by the alternative oxidase system which is partially uncoupled.

ADDENDUM

Data presented in an accompanying paper by La Monica and Marrs [27] are in good agreement with the operativity of a branched respiratory system also in cells of the same organism when grown photosynthetically in anaerobiosis i.e. under conditions extreme and opposite to those utilized in the present study. Moreover by means of inhibitor studies further support for a branching of the chain at the site demonstrated by our data is given.

ACKNOWLEDGEMENTS

We wish to thank Dr. B. Marrs and H. Gest for their generous gift of the two mutant strains M6 and M7 and Dr. G. Drews for the A1a pho$^+$ mutant. We are also indebted to Dr. O. T. G. Jones for critically reading the manuscript. This work was supported partially by Consiglio Nazionale delle Ricerche (C.N.R.) of Italy (grant No. C.T. 73.00660.04).

REFERENCES

1 Baccarini-Melandri, A., Zannoni, D. and Melandri, B. A. (1973) Biochim. Biophys. Acta 314, 298–311
2 Marrs, B. and Gest, H. (1973) J. Bacteriol. 114, 1045–1051
3 Zannoni, D., Baccarini-Melandri, A., Melandri, B. A., Evans, E. H., Prince, R. C. and Crofts, A. R. (1974) FEBS Lett. 48, 152–155
4 Evans, E. H. and Crofts, A. R. (1974) Biochim. Biophys. Acta 357, 78–88
5 Evans, E. H. and Crofts, A. R. (1974) Biochim. Biophys. Acta 357, 89–102
6 Ormerod, J. G., Ormerod, K. S. and Gest, H. (1961) Arch. Biochem. Biophys. 94, 449–463
7 Dutton, P. L., Wilson, D. F. and Lee, C. P. (1970) Biochemistry 9, 5077–5082
8 Kröger, A. and Klingenberg, M. (1966) Biochem. Z. 344, 317–336
9 Redfearn, E. R. and Pumphrey, A. M. (1966) Biochim. Biophys. Acta 118, 413–418
10 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
11 Klemme, J. H. and Schlegel, H. G. (1968) Arch. Mikrobiol. 63, 154–169
12 Carr, N. G. and Exell, G. (1965) Biochem. J. 96, 688–692
13 Clayton, R. K. (1962) Biochem. Biophys. Res. Commun. 9, 49–53
14 Clayton, R. K. and Yan, H. F. (1972) Biophys. J. 12, 867–881
15 Slooten, L. (1972) Biochim. Biophys. Acta 275, 208–218
16 Codgell, R. J., Brune, D. C. and Clayton, R. K. (1974) FEBS Lett. 45, 344–347

17 Parson, W. W. (1967) Biochim. Biophys. Acta 143, 263–265
18 King, M. T. and Drews, G. (1973) Biochim. Biophys. Acta 305, 230–248
19 Prince, R. C., Baccarini-Melandri, A., Hauska, G. A., Melandri, B. A. and Crofts, A. R. (1975) Biochim. Biophys. Acta 387, 212–227
20 Lambowitz, A. M. and Bonner, W. D. Jr. (1974) J. Biol. Chem. 249, 2428–2440
21 Connelly, J. L., Jones, O. T. G., Saunders, V. A. and Yates, D. W. (1973) Biochim. Biophys. Acta 292, 644–652
22 Jones, O. T. G. and Plewis, K. M. (1974) Biochim. Biophys. Acta 357, 204–214
23 Garcia, A. F., Drews, G. and Kamen, M. D. (1974) Proc. Natl. Acad. Sci. U.S. 71, 4213–4216
24 White, D. C. and Sinclair, P. R. (1970) Adv. Microbiol. Physiol. 5, 173–211
25 Meyer, D. J. and Jones, C. A. (1973) Eur. J. Biochem. 36, 144–151
26 King, M. T. and Drews, G. (1975) Arch. Microbiol., 102, 219–231
27 La Monica, R. F. and Marrs, B. L. (1976) Biochim. Biophys. Acta 423, 431–439