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PHOTOOXIDASE SYSTEM OF *RHODOSPIRILLUM RUBRUM*

I. PHOTOOXIDATIONS CATALYZED BY CHROMATOPHORES ISOLATED FROM A MUTANT DEFICIENT IN PHOTOOXIDASE ACTIVITY

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SUMMARY

The aerobic photooxidations of reduced 2,6-dichlorophenolindophenol and of reaction-center bacteriochlorophyll (*P*-870) have been investigated in membrane vesicles (chromatophores) isolated from a non-phototrophic *Rhodospirillum rubrum* strain. In aerobic suspensions of wild-type chromatophores, continuous light elicits an increase of the levels of 2,6-dichlorophenolindophenol and of oxidized *P*-870, which reach steady-state values shortly after the onset of illumination. In contrast, light induces in mutant suspensions a transient increase of the levels of 2,6-dichlorophenolindophenol and of oxidized *P*-870, which fall to low steady-state values within a few seconds. These observations suggest that the mutation has altered a redox constituent located on the low-potential side of the photochemical reaction center, between a pool of acceptors and oxygen.

Since endogenous cyclic photophosphorylation is catalyzed by mutant chromatophores at normal rates, it appears that the constituent altered by the mutation does not belong to the cyclic electron-transfer chain responsible for photophosphorylation. However, the system which mediates the aerobic photooxidations and the cyclic system are not completely independent: endogenous photophosphorylation is inhibited by oxygen in wild-type chromatophores but not in mutant chromatophores; in addition, the inhibitor of cyclic electron flow, 2-heptyl-4-hydroxyquinoline-*N*-oxide, enhances the aerobic photooxidation of reduced 2,6-dichlorophenolindophenol by chromatophores from both strains.

These results support a tentative branched model for light-driven electron transfer. In that model, the constituent altered in the mutant strain is located in a side electron-transfer chain which connects the cyclic acceptors to oxygen.

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine.

INTRODUCTION

Chromatophores isolated from the facultative phototrophic bacterium, *Rhodospirillum rubrum*, contain a photooxidase system which mediates the aerobic photooxidation of exogenous electron donors [1]. Previous work from several laboratories has suggested that this system is a part of the cyclic chain of redox carriers responsible for endogenous photophosphorylation [2–4]. However, the phenotypic properties of strain F11, a mutant derivative of *Rsp. rubrum* selected for its inability to grow anaerobically in the light [5], suggest that the photooxidase system contains at least a specific constituent which does not belong to the cyclic redox chain. The mutant grows at normal rates in dark aerated cultures and, as the parent strain, synthesizes unaltered photopigments when the oxygen tension is kept at a low level during growth [5]. Chromatophores isolated from F11 pigmented cells support normal rates of cyclic photophosphorylation in the absence of exogenous redox compounds, but show low photooxidase activity measured by following the light-dependent uptake of oxygen which takes place in the presence of added reductants [5]. On the other hand, the restoration of photooxidase activity in a spontaneous revertant of the mutant, selected only for recovery of anaerobic phototrophic growth [5], indicates that the photooxidase system performs a physiological function which is essential for the normal photosynthetic metabolism of *Rsp. rubrum*.

In this report we describe the photooxidations of exogenous electron donors and of reaction-center bacteriochlorophyll in suspensions of mutant chromatophores. The results presented here and previously published data obtained in vivo and in vitro [4–6] favour a tentative branched model for light-driven electron transfer in which the specific photooxidase constituents are arranged in a side chain which connects a pool of secondary cyclic acceptors and oxygen.

METHODS

The wild-type strain of *Rsp. rubrum* (S1) and its non-phototrophic, photooxidase-defective derivative (F11) have been described previously [4, 5]. Cultures were performed in the dark under low oxygen tension [5], using the growth medium of Lascelles [7] supplemented with 2 g/l yeast extract. Cells were collected by centrifugation at 0–2 °C when the cultures reached 0.6 mg dry weight of cells per ml (about 3 to 4 nmol of bacteriochlorophyll per mg dry weight), and chromatophores were prepared according to a previously published procedure [4]. The bacteriochlorophyll content of chromatophores was estimated from the absorbance at 878 nm, using the extinction coefficient of 153 $mM^{-1} \cdot cm^{-1}$ for the major component in vivo [8].

Light-induced absorbance changes of chromatophore suspensions were monitored at room temperature with a Perkin Elmer spectrophotometer (model 356) in the split beam mode. Infrared actinic illumination (78 $W \cdot m^{-2}$) was provided by a tungsten-halide, 650 W source after filtration through a narrow-band interference filter (B-IR 888, Balzers). The photomultiplier was shielded from actinic light with the help of complementary colour and interference filters. The standard chromatophore suspension, in a cuvette of 1 cm optical path, contained: 45 mM Tricine/NaOH (pH 8.0); 30 μM 2,6-dichlorophenolindophenol (DCIP) or 30 μM 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD); 1.6 mM sodium ascorbate; 1 mM KCN, and 2–7 μM

bacteriochlorophyll (as chromatophores). Departures from this basic mixture are described under the particular experiments.

A similar reaction mixture, containing 30 μM DCIP and 6.6 μM bacteriochlorophyll, was used to determine the apparent K_m values for oxygen during the light-dependent oxygen uptake. The samples were kept at 25 °C and illumination was provided by a slide projector equipped with a 150 W, tungsten-halide lamp and a broad-band infrared filter (IR-total, Balzers). After additional filtering through 10 cm of water, the light intensity reaching the samples was 49 $W \cdot m^{-2}$. The oxygen content of the reaction mixture was measured with an oxygen-electrode system (Biological Oxygen Monitor, model 53, YSI) connected to a strip-chart recorder (Servograph REA 310, Radiometer). The apparent K_m values were computed from the recorder traces, which fitted the integrated form of the Michaelis-Menten equation as long as oxygen concentration did not fall below 30 μM.

Light-dependent ATP formation by isolated chromatophores was estimated in 3-ml reaction mixtures containing 32.5 mM Tricine/NaOH (pH 8.0), 25 mM glucose, 0.5 mM ADP, 5 mM HK_2PO_4, 5 mM $MgCl_2$ and 1 μM bacteriochlorophyll (as chromatophores). When anaerobic conditions were required, 4.6 units/ml of glucose oxidase (EC 1.1.3.4) were also added and 2.0 ml of paraffin oil were layered on the aqueous phase. The reaction mixtures were placed in open test-tubes (11 mm internal diameter) and allowed to stand in the dark at 25 °C for 3 min. Then they were illuminated as in the assay of oxygen uptake. ATP formation was stopped by adding 1.5 ml of 3 M perchloric acid to each sample. After 10 min at 0 °C, 2-ml aliquots of the acidified mixtures were transferred to test-tubes containing 5 ml of 0.5 M sodium arsenate/H_2SO_4 (pH 7.4). The samples were then adjusted to pH 7.4$\pm$0.02 with 2 M KOH, filtered to remove $KClO_4$, and assayed for ATP by a luciferin-luciferase method [9].

RESULTS AND DISCUSSION

Photooxidation of $DCIPH_2$

When $DCIPH_2$ is used as the terminal electron donor for oxygen photoreduction in suspensions of *Rsp. rubrum* chromatophores, the formation of DCIP results in an increase of the absorbance of the reaction mixture around 600 nm [10]. In the presence of an excess of sodium ascorbate, which is not a good direct donor for oxygen photoreduction but which regenerates $DCIPH_2$ and sustains therefore a constant rate of oxygen uptake [2–4], the absorbance of the mixture stops increasing after a few seconds and reaches a steady value in the light. (Fig. 1). The light-induced change is reversible and the original value of absorbance in the dark is restored shortly after the actinic light is turned off. Besides, under the experimental conditions of Fig. 1, the change may be repeated by a second illumination of the same reaction mixture. The increase of absorbance seems to be caused by a light-induced increase of the concentration of the oxidized form of the donor, which reaches a new steady-state value when the rate of $DCIPH_2$ photooxidation is balanced by the rate of DCIP reduction by ascorbate. Some other results confirm this interpretation: (i) no endogenous light-induced optical changes occur at the same wavelength (586 nm) when $DCIPH_2$ is omitted from the reaction mixture; (ii) no changes are observed, either, when $DCIPH_2$ is replaced by DAD, which is also an effective donor for oxygen photoreduction (4) but which does not show absorption bands in this range of the spectrum; (iii) an

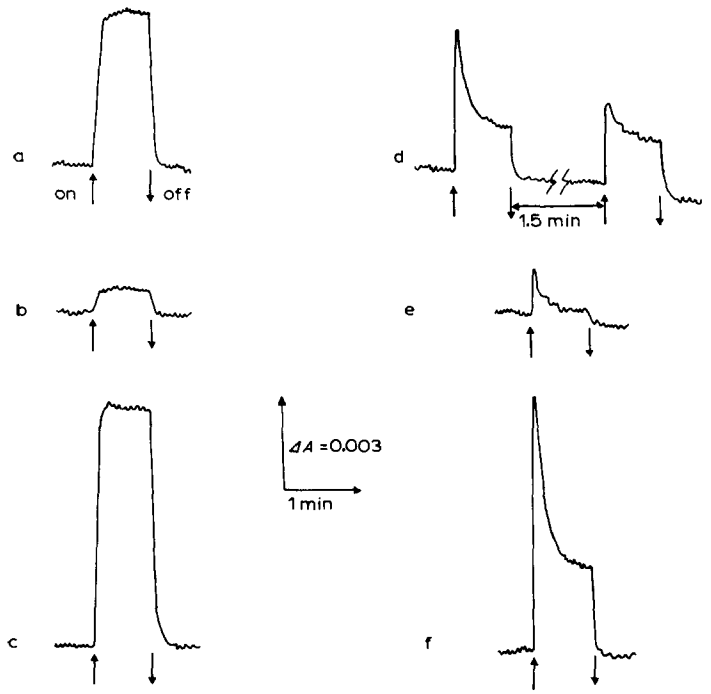

Fig. 1. Light-induced absorbance changes at 586 nm in suspensions of wild-type (a, b, c) and mutant (d, e, f) chromatophores. The DCIP-containing reaction mixture described under Methods was used with the following modifications: a, d none; b, e sodium ascorbate concentration increased to 8 mM; c, f plus 3.3 μM HQNO. All samples contained 5.5 μM bacteriochlorophyll.

increase of ascorbate concentration depresses the extent of the change, as expected from the higher rate of chemical DCIP reduction under these conditions (Fig. 1); (iv) a decrease of $DCIPH_2$ concentration, which results in a lower rate of the aerobic photooxidation of $DCIPH_2$ (4), depresses also the extent of the absorbance change at 586 nm; and (v) the light-elicited absorbance increase is enhanced by HQNO (Fig. 1), an inhibitor of cyclic electron flow [11] which stimulates the net rate of electron transfer from $DCIPH_2$ to oxygen [3, 4].

The corresponding absorbance changes supported by chromatophores isolated from the photooxidase defective strain, *Rsp. rubrum* F11, are more complex: on illumination, the absorbance increases transiently and then decreases to a steady level which is still higher than that of the reaction mixture in the dark (Fig. 1). The extent of the net absorbance increase, measured after the overshoot has taken place, is smaller than the increase observed in suspensions of wild-type chromatophores. Besides, the overshoot cannot be fully repeated by a second illumination (Fig. 1) unless the reaction mixture has been previously kept in the dark for longer than five minutes. In spite of these differences with suspensions of wild-type chromatophores, the optical change at 586 nm seems to be caused also by light-induced variations of the concentration of DCIP in the reaction mixture, because it is not observed when $DCIPH_2$ is omitted or replaced by DAD. In addition, the change responds to the presence of HQNO and to

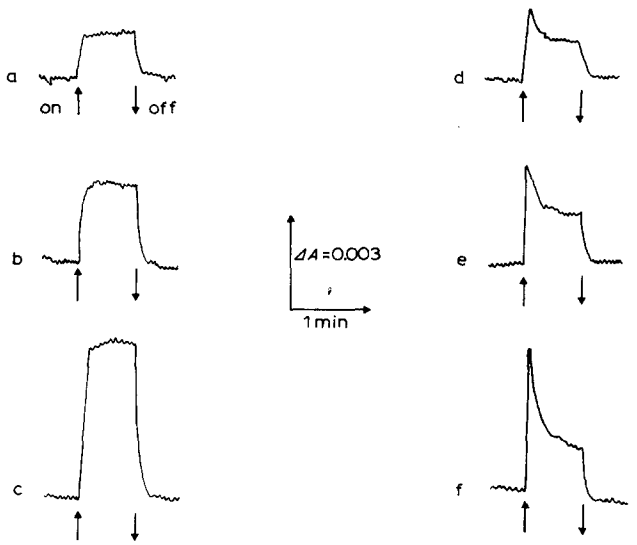

Fig. 2. Effect of the intensity of actinic light on the light-induced absorbance changes at 586 nm. The DCIP-containing reaction mixture described under Methods was used. All samples contained 5.5 μM bacteriochlorophyll, either as wild-type (a, b, c) or as mutant (d, e, f) chromatophores. Actinic-light intensity was 17 (a, d), 28 (b, e) or 78 (c, f) $W \cdot m^{-2}$.

alterations of the concentrations of $DCIPH_2$ and of sodium ascorbate in the same way as the change catalyzed by wild-type chromatophores (Fig. 1).

The light-elicited absorbance changes supported by both mutant and wild-type chromatophores depend on the intensity of actinic light. However, there are differences between the dependence shown by each type of membrane preparation, as illustrated in Fig. 2. The net absorbance increase in suspensions of mutant chromatophores reaches a maximum value at light intensities which are too low to saturate the corresponding change in wild-type chromatophores and the initial transient change characteristic of mutant preparations. Therefore, the differences between both strains become more conspicuous at high intensities of actinic light.

If the absorbance changes at 586 nm reflect light-induced variations of the concentration of DCIP in the reaction mixture, as supported by the results of Fig. 1, the lower extent of the net absorbance increase observed in mutant suspensions indicates a depressed ability to increase the level of DCIP under steady illumination. Since the steady-state rate of oxygen photoreduction is low in mutant chromatophores [5], the small increase of DCIP concentration must be due to a low steady-state rate of $DCIPH_2$ photooxidation. The initial transient increase of DCIP concentration, which overshoots clearly the final steady value, seems to indicate that the electron-transfer step altered by the mutation starts limiting the rate of $DCIPH_2$ photooxidation only after some time of illumination. Once established, the limiting step appears to decay very slowly in the dark, as suggested by the long dark interval of time required before a second illumination may repeat completely the original transient increase (Fig. 1). The existence of a slower step which needs some time in the light to be operative explains also why the light intensity required to elicit the maximum steady level of

DCIP is lower than that required to saturate the initial transient increase (Fig. 2).

The abnormal $DCIPH_2$ photooxidation observed in suspensions of mutant chromatophores seems to be directly related to the lack of photosynthetic growth of the mutant because chromatophores isolated from strain RF110, a spontaneous phototrophic revertant derived from F11 [5], supported light-elicited absorbance changes at 586 nm which were identical to those observed in suspensions of wild-type chromatophores. Moreover, the same was true for all the results which will be described in the following sections. However, the data obtained with the revertant have not been included to avoid unnecessary repetition.

Photooxidation of reaction center bacteriochlorophyll

Exogenous electron donors for the photochemical redox reactions of chromatophores are oxidized, directly or indirectly, by the photooxidized form of reaction-center bacteriochlorophyll, *P*-870, which is the primary donor in bacterial photosynthesis [12]. Therefore, it seemed interesting to test whether *P*-870 photooxidation was also altered in mutant chromatophores.

A blue shift of the absorption band of bacteriochlorophyll near 800 nm is one of the spectral changes caused by *P*-870 photooxidation in whole cells, isolated chro-

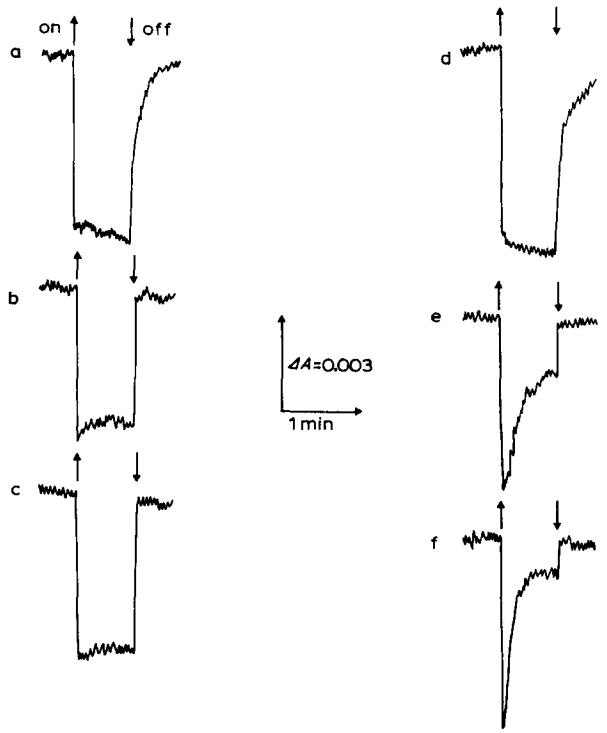

Fig. 3. Effect of DCIP and of HQNO on the light-induced absorbance changes at 810 nm. The DCIP-containing reaction mixture described under Methods, supplemented with 5 μM gramicidin D, was used with the following modifications: a, d DCIP omitted; b, e none; c, f plus 3.3 μM HQNO. All samples contained 2.1 μM bacteriochlorophyll either as wild-type (a, b, c) or as mutant (d, e, f) chromatophores.

matophores and purified reaction-center particles of non-sulfur photosynthetic bacteria [13, 14]. The shift results in a decrease of optical absorbance at 810 nm which may be observed in suspensions of mutant and of wild-type chromatophores (Fig. 3). When exogenous electron donors are not added, both the extent and the kinetics of the light-induced change are similar in both strains. In the presence of $DCIPH_2$, however, the changes elicited by light depend on the type of chromatophores used. As expected from its ability to donate electrons to photooxidized *P*-870, $DCIPH_2$ reduces the net absorbance decrease under steady illumination, but the extent of the reduction is clearly higher in reaction mixtures containing mutant chromatophores (Fig. 3). Besides, the initial absorbance decrease of mutant suspensions overshoots the final steady value in the light, so that the whole change (Fig. 3) resembles the one corresponding to $DCIPH_2$ photooxidation by the same chromatophores (Fig. 1).

It was also observed that DAD, another effective electron donor for oxygen photoreduction [4], behaved like $DCIPH_2$ in eliciting a higher reduction of the 810 nm optical change in mutant than in wild-type chromatophores (not shown). Besides, the establishment of a steady-state level of absorbance in the mutant was also preceded by an overshoot. The similarity of the effects of both electron donors contrasts with the failure of DAD to support the light-dependent absorbance increase at 586 nm and is consistent with our previous conclusion that the 586 nm changes are directly due to the formation of DCIP in the light.

Fig. 3 illustrates also the effect of HQNO on the 810 nm absorbance change which is observed in the presence of $DCIPH_2$. The drug stresses the differences existing between both strains, because it enhances slightly the absorbance decrease in the wild-type and depresses the net change in the mutant. The differential effect of HQNO will be discussed in a later section dealing with the connection between the cyclic and the photooxidase systems.

Gramicidin D, a phosphorylation uncoupler [15], was included in the reaction mixtures of Fig. 3 because the spectral changes due to the light-dependent oxidation of *P*-870 overlap with some other optical changes which are caused by the electric field associated to the energized state of the chromatophore membrane [16] and these latter changes are selectively eliminated by phosphorylation uncouplers, which dissipate the energized membrane state. The omission of gramicidin D resulted in some enhancement of the extent of all changes, but did not alter significantly the results shown in Fig. 3. Therefore it seems that the differences existing between the optical changes supported by the two types of chromatophores reflect differences between the ability to increase $P\text{-}870^+$ levels in the light and not differences in the changes related to energy conservation at the membrane.

In addition to the blue shift of the 800 nm band, the photooxidation of reaction-center bacteriochlorophyll results also in other modifications of the absorption spectrum of chromatophores, mainly in the photobleaching of the bacteriochlorophyll bands near 870 and 605 nm [13]. We investigated the effects of $DCIPH_2$ and DAD on the absorbance changes at 870 nm and found results which were qualitatively similar to those shown in Fig. 3. In the presence of DAD, the photobleaching at 605 nm showed also the differences between mutant and wild-type chromatophores. However, $DCIPH_2$ could not be tested in this spectral range because the large absorbance increase caused by its photooxidation masked completely the smaller photobleaching.

The similarities existing between the absorbance changes due to DCIP and

those due to reaction-center bacteriochlorophyll in suspensions of mutant chromatophores, together with the admitted fact that exogenous electron donors are ultimately oxidized by $P\text{-}870^+$ [12, 13], suggest that the low rate of $DCIPH_2$ oxidation under continuous illumination is the consequence of a low steady-state rate of the photochemical reaction and not the result of an alteration of the ability of the exogenous donor to reduce $P\text{-}870^+$. Thus, the electron-transfer step defective in the mutant appears to be located on the low-potential side of the reaction center, between the primary acceptor and oxygen. Furthermore, the initial high formation of DCIP and $P\text{-}870^+$ indicates that light-driven electron flow may proceed at a high rate for some seconds before the steady-state is finally established, an observation which suggests the existence of a pool of secondary acceptors between the primary acceptor and the site altered by the mutation. In this way, the effect of the mutation on the rate of the photochemical reaction is delayed until the accumulation of the pool in the reduced state impedes the normal reoxidation of the primary acceptor. A direct consequence of this interpretation of the light-induced absorbance changes observed in mutant chromatophores is that the actual site of oxygen reduction should be altered in the mutant. This conclusion is correct as evidenced by the kinetic analysis of the light-

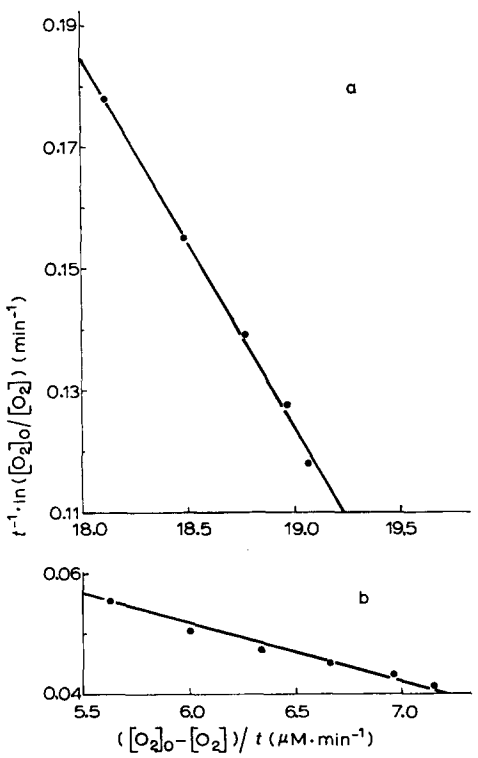

Fig. 4. Effect of oxygen concentration on the rate of light-dependent oxygen uptake by wild-type (a) and mutant (b) chromatophores. The data were obtained as described under Methods and plotted according to Segel [20]. The least square method was used to draw the lines. The correlation coefficients are 0.997 (a) and 0.977 (b). The computed kinetic parameters are: K_m, 16.5 (a) and 111.8 (b) μM; V, 3.2 (a) and 1.8 (b) mol of O_2 per mol of bacteriochlorophyll per min.

dependent oxygen uptake (Fig. 4). The rate of the reaction depends on oxygen concentration according to the equation of Michaelis-Menten and, with the chromatophore preparations used to obtain the data of Fig. 4, apparent K_m values for oxygen of 16 μM (wild-type) and 112 μM (mutant) were found. The standard deviation of K_m values obtained with several preparations was near 15 %. Therefore, the differences between the two strains were clearly significant.

Relation between the cyclic and the photooxidase systems

Previous work on the photoreduction of oxygen by exogenous electron donors in isolated *Rsp. rubrum* chromatophores has indicated that the reaction is mediated by a part of the cyclic system which includes the photochemical reaction center and some other electron carriers [2–4]. On the other hand, the properties of chromatophores isolated from strain F11, which show normal rates of endogenous cyclic photophosphorylation but which are deficient in photooxidase activity [5], suggest strongly that the constituent(s) altered by the mutation does not belong to the cyclic system. These conclusions (and those drawn previously in the present report) support a branched model for electron transfer in which the specific constituents of the photooxidase system would be located in a side chain which would connect a pool cyclic acceptors with oxygen (Fig. 5). As proposed before [4], the exogenous donors would inhibit competitively the normal return of electrons from the photoreduced cyclic acceptors to $P\text{-}870^+$. In the wild-type, an excessive accumulation of reduced acceptors would then be prevented by the side chain, which would reoxidize the acceptors and would be directly responsible for oxygen reduction. Such an aerobic oxidation would not be possible in strain F11, so that the acceptors would accumulate in the reduced state and cause an inhibition of the photochemical reaction which could be partly relieved by the direct autooxidation of some of the acceptors.

This tentative model accounts for the effect of the inhibitor of cyclic electron transfer, HQNO [11], on the light-induced absorbance changes described in this report. It has been found previously [4] that HQNO lowers the apparent K_m values for the exogenous donors and elicits, therefore, an enhancement of the rate of oxygen photoreduction at non-saturating donor concentrations. This result, which may be

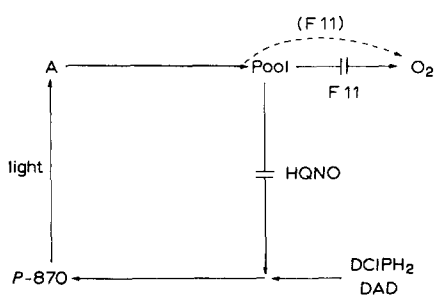

Fig. 5. Postulated model of light-driven electron transport in isolated chromatophores of *Rsp. rubrum*. No attempt has been made to identify specific constituents except for reaction center bacteriochlorophyll (*P*-870). Some steps may involve two or more electron carriers. Strain F11 is blocked in a side chain which connects a pool of secondary acceptors and oxygen. The dotted arrow indicates an alternative site for oxygen reduction in the mutant strain.

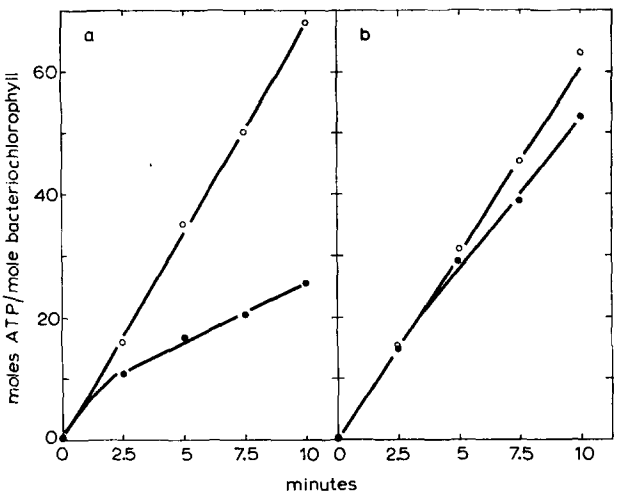

Fig. 6. Differential effect of aerobiosis on the photophosphorylation supported by wild-type (a) and by mutant (b) chromatophores. Phosphorylative activity was measured under aerobiosis (●) or under anaerobiosis (○) in the absence of exogenous redox factors, as described under Methods.

understood as the consequence of the removal by HQNO of a competitive inhibitor of donor oxidation (the endogenous cyclic carriers which reduce $P\text{-}870^+$), is consistent with the stimulation by the drug of the net absorbance changes corresponding to $DCIPH_2$ photooxidation (Fig. 1). At the same time, HQNO should elicit an increase of the pool of reduced acceptors. In the mutant, where the reoxidation of the pool appears to be already limiting the rate of the photochemical reaction, the additional increase would result in a further lowering of the steady-state levels of $P\text{-}870^+$ in the light (Fig. 3).

It has been known for a long time that oxygen inhibits cyclic photophosphorylation in *Rsp. rubrum* chromatophores [17]. Since the inhibition is relieved by added reductants, the effect of oxygen has been attributed to an alteration of the redox state of the chromatophore constituents. As shown in Fig. 6, the inhibition is very small in mutant chromatophores to which no exogenous electron donors were added. This result confirms that the electrons of the cyclic system can be transferred to oxygen through the redox carrier(s) altered in strain F11 and supports, therefore, the branched model presented in Fig. 5. In the absence of exogenous reductants the accumulation of reduced acceptors must be small, but as the endogenous electron pool of the cyclic system is also small, a slow diversion of electrons to oxygen (too slow to elicit detectable oxygen uptake [4]) may be enough to result in an inhibition of photophosphorylation after prolonged illumination. In mutant chromatophores the withdrawal of electrons from the cyclic system is blocked and aerobiosis has little effect on photophosphorylation.

Recent experiments carried out with intact cells [6] are also in agreement with the model of light-driven electron transfer proposed here. In those experiments the cells had an apparently over-reduced photosynthetic apparatus and were unable to increase their ATP levels in the light and to grow phototrophically. Under those conditions, restoration of light-dependent ATP accumulation and phototrophic

growth was dependent on the existence of a functional photooxidase system and on the addition of small amounts of oxygen, too small to elicit detectable growth and ATP accumulation in the dark [6]. Therefore, it appears that the photooxidase system may be used in vivo to avoid also an excessive accumulation of the cyclic acceptors in the reduced state. However, it remains unknown whether that is the physiological role of the system during normal phototrophic growth.

Concluding remarks

The model of electron transfer shown in Fig. 5 seems to provide a reasonable explanation for the properties of the photooxidase defective mutant. Nevertheless, it is only a working hypothesis useful to plan further work on the subject and its verification must await the identification of the actual constituents of the proposed side chain. At this point, we should like to comment that the existence of branched electron-transfer systems in *Rhodospirillaceae* is not a novelty. Recently, the isolation of specific mutants has confirmed that the respiratory system of *Rhodopseudomonas capsulata* contains two different terminal oxidases, connected probably at the level of ubiquinone [18, 19]. One of the redox chains appears to be linked to the cyclic photophosphorylative system [19] and has, therefore, a strong and suggestive resemblance to the side chain of our model. However, neither of the chains of *Rps. capsulata* appears to be required for phototrophic growth and, on the other hand, we have not detected yet any function of the photooxidase system in the dark metabolism of *Rsp. rubrum*.

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