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THE LOCALIZED COUPLING OF BACTERIAL PHOTOPHOSPHORYLATION

EFFECT OF ANTIMYCIN A AND *N,N*-DICYCLOHEXYLCARBODIIMIDE IN CHROMATOPHORES FROM *RHODOPSEUDOMONAS SPHAEROIDES*, Ga, STUDIED BY SINGLE TURNOVER EVENT ANALYSIS

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1. The inhibition by antimycin A of the cyclic electron transfer has been studied in chromatophores from *Rhodopseudomonas sphaeroides* Ga following an approach based on the analysis of the relaxation kinetics of the reaction center optical changes in pulsed light. The recovery kinetics of the bacteriochlorophyll redox state have been found to be clearly biphasic. The half-times of the fast phase (13 ms) and slow phase (about 400 ms) were not modified by antimycin in a range of concentrations from 0.1 to 9 μM. On the other hand the percentage extent of the fast phase, which reflects the rate of the cyclic electron transfer, was monotonically decreased by increasing concentrations of the inhibitor. This indicates that antimycin decreases progressively the fraction of the photosynthetic units, active in cyclic electron transfer. 2. The ATP yield per flash observed under conditions of controlled inhibition of electron flow was strongly dependent upon the amount of active redox cycles. On the other hand, the amplitude of the carotenoid band shift, which has been demonstrated unequivocally to be correlated to the ATP yield per flash in uninhibited chromatophores, was not affected by antimycin up to a 40% inhibition of electron flow. 3. The effect of a progressive limitation by DCCD in the number of active ATP synthetase complexes on flash-induced phosphorylation has been examined. The decrease in ATP yield observed over a wide range of flash frequencies is related simply to the ATPase activity and to phosphorylation in continuous light, irrespective of the value of the membrane potential, which appears to be stabilized by this inhibitor. 4. As a whole, the results obtained at low concentrations of antimycin and under conditions of partial inhibition by DCCD evidence a localized coupling between the redox reactions and phosphorylation.

Introduction

In chromatophores of facultative phototrophic bacteria, a cyclic photosynthetic system operates which is believed to translocate protons from the outer water phase into the inner compartment. Studies with hydrophilic pH indicators added in the suspending medium have demonstrated that two protons are taken up per photocycle [1,2]: these protons are thought to be released into the inner water phase, although direct evidence of this event is still unavailable [1]. The translocation process is electrogenic and gives rise to an electrostatic potential [3–5], positive inside, the extent and the kinetic behavior of which can be conveniently monitored by the measurement of the electrochromic shift of endogenous carotenoids [3,6].

Abbreviations: DCCD, dicyclohexylcarbodiimide, BChl, bacteriochlorophyll.

The flow of electrons through the cycle, which occurs in absence of exogenous electron donors and acceptors, can be accurately controlled by activating the photochemical primary reactions with trains of microsecond flashes, short enough to allow only one single turnover of the system per flash (for review see, for example, Ref. 7). The light-driven proton pump in bacterial chromatophores is therefore fairly well characterized and offers possibly the most easily controllable system available at present.

The high-potential protons translocated into the inner compartment are believed to drive ATP synthesis, catalyzed by an ATP synthetase complex, plugged through the membrane, the structure and the function of which have been proved to be very similar to the one operating in oxidative and photosynthetic phosphorylation in other prokaryotes and in eukaryotes [8]. The synthesis of one molecule of ATP is coupled to the translocation of two positive charges (most likely protons) from the inner to the outer compartment [9].

Following the orthodox chemiosmotic model of energy transduction, the coupling between the light-driven H^+-translocating reactions and the synthesis of ATP should be delocalized throughout the whole chromatophore volume, since no diffusion barrier is postulated between the proton-releasing redox sites on the inner membrane surface, the inner aqueous bulk phase and the proton-conducting channel of ATP synthetase. However several experimental observations in chromatophores [10–13], in chloroplasts [14] and in respiratory systems of aerobic bacteria [15] and of mitochondria [16–19], do not meet the theoretical expectations of this model and are indicative of a short-range coupling between redox and ATP-synthesizing reactions. Most of these experiments have been performed under steady-state conditions or during the transients from resting to active electron flow. In the work described in this paper we have evaluated quantitatively the inhibition by antimycin A of the cyclic electron flow of *Rps. sphaeroides* chromatophores, following a relaxation kinetics approach described previously [20]; the results have been compared with the effect of the inhibitor on the extent of the transmembrane electric field and on photophosphorylation, evaluated during trains of single-turnover flashes with the sensitive luciferase technique. Alternatively, the number of active ATP synthetase complexes was progressively reduced by inhibition with DCCD, and the effect of this treatment on flash-induced phosphorylation was observed. The results indicate clearly a strong dependence of phosphorylation upon the number of electron transport chains and of active ATP synthetases present on the membrane, irrespective of the value of the membrane potential, and support fully the previous conclusions of localized interactions during light-induced phosphorylation.

Materials and Methods

Chromatophores from cells of *Rps. sphaeroides,* strain Ga, were prepared as described in Ref. 21. Bacteriochlorophyll (BChl) content was measured in acetone-methanol extracts, following the procedure described in Ref. 22.

Photosynthetic electron transfer reactions were induced by xenon flashes (20 μs half-width) of nearly saturating intensity (at least 90%). The actinic light was screened by two layers of 88A Wratten filters plus a 715 nm cut-off Schott glass filter.

Flash-induced phosphorylation was monitored at 25°C with detection of the luciferin-luciferase luminescence as described in Ref. 13. The assay medium contained in a final volume of 2 ml: 100 mM sodium glycylglycine (pH 7.75), 10 mM magnesium acetate, 1 mg/ml bovine serum albumin, 8 mM potassium phosphate (pH 7.75), 0.1 mM sodium succinate, 20 μM ADP, 20 mM KCl. 4 μM nigericin, 60 μM luciferin (Sigma), 1.2 mg crude firefly lantern extract (Sigma FLE-50) and chromatophores corresponding to 16 μm BChl. When antimycin was present at least 10 min of preincubation in the dark was allowed before starting the experiments.

Inhibition with DCCD was performed as described in Ref. 23: chromatophores, suspended at 0.92 nmol BChl/ml, were incubated for 1 h at room temperature with concentrations of DCCD in ethanol, varying from 70 to 350 nmol/μmol BChl. The amount of ethanol added was kept constant and appropriate controls were run. After this treatment the particles were stored at −16°C,

in order to maintain the degree of inhibition stable for several hours.

ATPase activity was measured at 30°C, at pH 8, as described in Ref. 24, using the colorimetric assay of Taussky and Schorr [25]. Oligomycin was added at a concentration of 10 μg/ml and was incubated for 5 min before the ATPase assay. Phosphorylation in continuous light was measured by the incorporation of [^{32}P]orthophosphate into ATP, as described in Ref. 21.

Measurements of the carotenoid band shift induced by trains of single-turnover flashes were carried out with a dual-wavelength spectrophotometer at 503–486 nm under the same assay conditions. The calibration of the electrochromic effect with K^+ diffusion-potential pulses was performed as described in Ref. 10.

The rates of the electron transport reactions were evaluated from the reaction-center absorption changes, produced by trains of flashes fired from 20 to 640 ms apart. The measurements were performed at 605 nm: an absorption coefficient of 19.5 $mM^{-1} \cdot cm^{-1}$ was utilized for the calculations [26]. The photomultiplier of the spectrophotometer was screened by a 605 nm narrow-band interference filter. The amplified signals were stored and averaged by a Tracor Northern digital signal analyzer, NS-570A. In these experiments the sample was supplemented with 2 μM valinomycin to avoid any interference of electrochromic effects with the reaction-center absorption changes.

The recovery kinetics of the reaction-center absorption changes between flashes in a train was analyzed utilizing computer programmes based on standard nonlinear least-squares fitting procedures [27].

Results

Effect of antimycin A

It has been shown in a previous paper [20] that the rate of cyclic electron transport can be reliably and sensitively extimated in chromatophores from photosynthetic bacteria in pulsed light from the relaxation kinetics of the redox state of the reaction center ($[BChl]_2$), when the chromatophore suspension is activated by a train of closely spaced single-turnover flashes. If the level of $[BChl]_2$ left oxidized immediately before a flash, when the system is in an oscillatory pseudo-steady-state, is plotted versus the dark time between flashes, recovery kinetics of the reaction-center redox state are obtained which display a distinctly biphasic dependence on multipulse frequency. The slower of the two phases has a half-time comparable to that of the back-reaction of the photosynthetic system and has been attributed, in the absence of added inhibitor, to those reaction centers from which cytochrome c_2 has been dislocated [20]. The rate of the fast phase has been demonstrated to be that of cyclic electron flow in pseudo-steady-state, since it responds to addition of phosphorylating substrates in a way consistent with the occurrence of photosynthetic control [20]. The extent of the fast phase, relative to the total photooxidizable $[BChl]_2$, is a measure therefore of the fraction of active coupled photosynthetic cycles present in the chromatophore population. The $t_{1/2}$ of the fast phase evaluated with this approach (13 ms) is markedly longer than that reported for cytochrome c_2 rereduction in a single turnover (2 ms) [28]; this discrepancy is probably due to the redox poise of the system which could be not optimal for the maximal rate of electron flow under our experimental conditions (aerobic and supplemented with 0.1 mM succinate). The necessity of coupling photophosphorylation with the aerobic reaction of luciferase in parallel experiments has, on the other hand, prevented us from achieving better control of the redox poise (i.e., in anaerobiosis and in the presence of redox mediators).

Following this approach we have studied the effect of increasing concentrations of antimycin A (in a range from 0.1 to 9 μM) on the kinetic parameters of the reaction-center rereduction. As shown in Fig. 1, the data fitting demonstrated that the half-times of the two phases of the recovery kinetics were not affected by antimycin A in the whole range of concentrations used, the fast and the slow phases having half-times of 13 and about 400 ms, respectively. On the other hand the amplitude of the slow recovery phase was progressively increased at increasing concentrations of antimycin A. This phase in antimycin-inhibited chromatophores includes both the slow rereduction of $[BChl]_2$ by the back-reaction, and the leak through the antimycin block, which has been shown to occur with half-times of 300–450 ms, depending

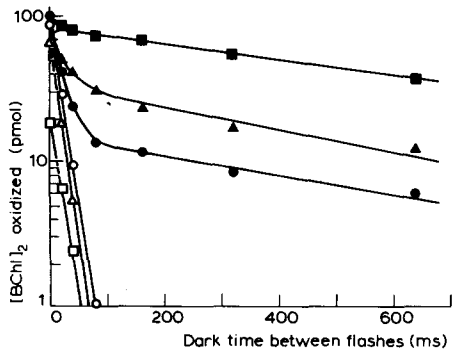

Fig. 1. The effect of antimycin A on the relaxation kinetics of the reaction center bacteriochlorophyll. ●——●, control; ▲——▲, plus 0.6 μM antimycin A; ■——■, plus 9 μM antimycin A. The open symbols correspond to the fast phases of the relaxation kinetics, as analyzed by the best-fit procedure. The experimental conditions are described under Materials and Methods.

on the redox conditions [29].

The extent of the residual fast phase, relative to the total amount of $[BChl]_2$ available for photooxidation, is plotted in Fig. 2 as a function of antimycin A concentration. The total $[BChl]_2$ in the chromatophore sample (corresponding to the experimental point at zero dark time in Fig. 1) has been estimated from the peak value of the optical signal induced by a high-frequency train of flashes in the presence of a saturating concentration of antimycin [20]. According to these results, 50% inhibition of electron flow is obtained with 1.5 μM antimycin under our experimental conditions (the presence of 1 mg/ml bovine serum albumin, which binds antimycin quite effectively, decreases, however, the inhibitory effect of this antibiotic very significantly [10]).These data indicate clearly that the partial inhibition of the ubiquinol-cytochrome c_2 oxidoreductase by antimycin A results in a complete block of a portion of the population of the photosynthetic units, which increases progressively at increasing concentrations of the inhibitor; the remaining units still operate at a normal, unaffected rate, even when more than 80% of the total population of oxidoreductase complexes is blocked.

The effect of antimycin on ATP synthesis in pulsed light has been examined by supplementing the medium with luciferin and luciferase in parallel sets of experiments, in which either the number of flashes per train (at constant frequency) or the frequency of photoactivation was varied. When a chromatophore suspension, partially inhibited by antimycin A, is activated by a train of 7–8 flashes, fired 20–640 ms apart, a clear inhibitory effect can be demonstrated on photophosphorylation (Fig. 3). Quite remarkably, ATP formation is inhibited by the same degree both when the light pulses are very closely spaced ($t_d = 20$ ms, a dark time comparable with the relaxation of electron transport (cf. Fig. 1)), or when a long time is allowed between flashes.

Fig. 4 shows the amount of ATP produced by sequences of flashes fired 320 ms apart. Antimycin A had no detectable effect on the induction kinetics (always about three turnovers of the photosynthetic apparatus were needed to reach the full rate

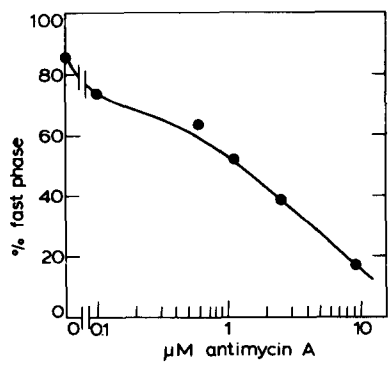

Fig. 2. The dependence of the amplitude of the fast recovery phase (expressed as a percentage of the total $[BChl]_2$ available for photooxidation) upon the concentration of antimycin A.

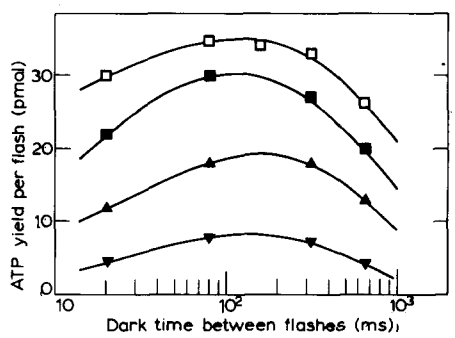

Fig. 3. The inhibition of flash-induced ATP synthesis by antimycin A at different dark times between flashes. □——□, control; ■——■, ▲——▲, ▼——▼: 0.1, 0.6 and 2.6 μM antimycin A.

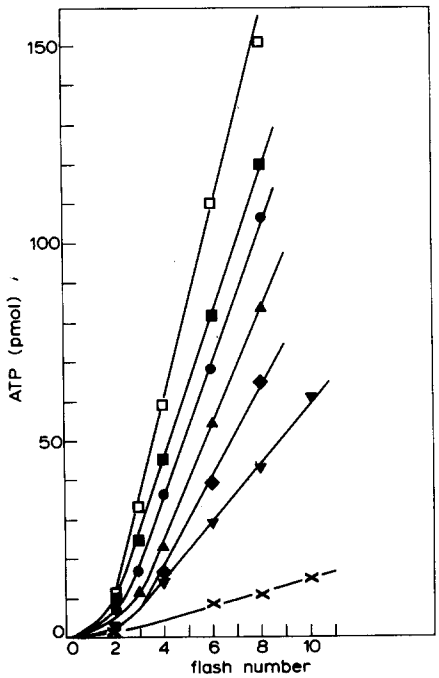

Fig. 4. The onset of photophosphorylation following trains of single-turnover flashes at increasing concentrations of antimycin A. Dark time between flashes: 320 ms. □——□, control; ■——■, ●——●, ▲——▲, ◆——◆, ▼——▼, ×——×: 0.1, 0.35, 0.6, 1.1, 2.6 and 9 μM antimycin A.

of phosphorylation), but reduced progressively and markedly the ATP yield per flash. Even at very low concentrations of antimycin an effective decrease in the amount of ATP produced per flash was found, in agreement with the decreased number of working photosynthetic units, as judged from the relative amplitude of the fast phase in the relaxation kinetics of $[BChl]_2$.

The amplitude of the carotenoid band shift immediately following the flash, over the same range of subsaturating concentrations of antimycin A is illustrated in Fig. 5 as a function of the number of flashes ($t_d = 320$ ms); the peak values reported in the figure are very close to the time-averaged extent of the signal, since in the 320 ms between two subsequent flashes the band shift extent did not decay by more than 10%, also in the presence of antimycin A.

In sharp contrast with the parallelism existing between the decreased number of active photosynthetic units and the concomitant inhibition of photophosphorylation at low concentrations of antimycin, no significative effect can be observed on the carotenoid signals up to a concentration of the inhibitor which causes a 40% block of the electron flow.

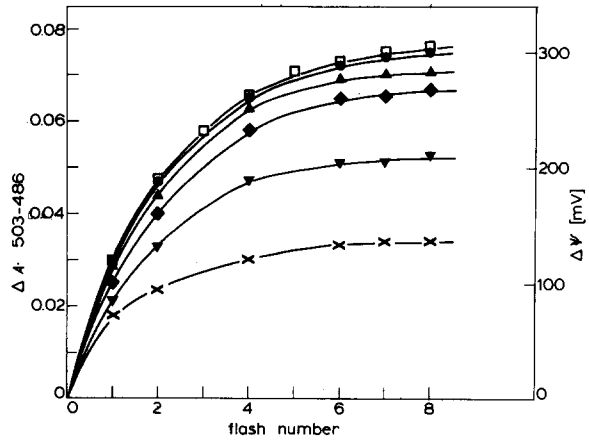

Fig. 5. The effect of antimycin A on the amplitude of the carotenoid signal produced by sequences of flashes. Conditions and symbols as in Fig. 4. The absorption band shift was calibrated for $\Delta\psi$ with K^+ diffusion pulses in the dark [10].

The results described are summarized in Fig. 6, in which the ATP yield per flash, the amount of $[BChl]_2$ active in cyclic electron transfer and the amplitude of the carotenoid band shift are compared as a function of the concentration of antimycin A. It is quite evident that the progressive

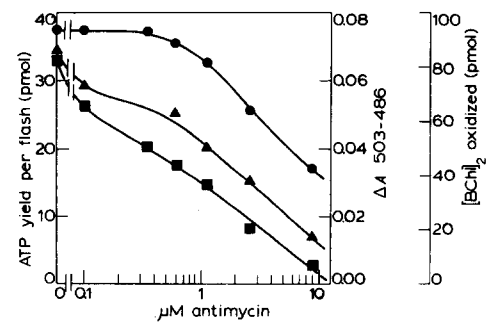

Fig. 6. Comparison of the effects of antimycin A on the ATP yield per flash (■——■), on the number of reaction centers $[BChl]_2$ available for cyclic electron transfer (▲——▲) and on the amplitude of the carotenoid band shift (●——●).

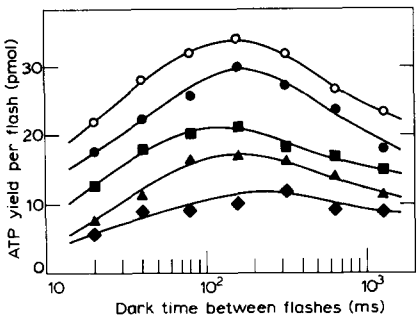

Fig. 7. The inhibition of flash-induced ATP synthesis by DCCD at different dark times between flashes. O——O, control; closed symbols, increasing amounts of DCCD (70, 140, 210 and 320 nmol DCCD/μmol BChl).

inhibition of phosphorylation reflects the decrease in the number of photosynthetic cycles operating, while no correlation exists, at low concentrations of the inhibitor, between the ATP yield per flash and the energetic state of the membrane monitored by the carotenoid signal.

Effect of N,N'-dicyclohexylcarbodiimide

With a similar and, in a way, complementary approach the effect of the progressive inhibition of the ATP synthetase on flash-induced phosphorylation has been examined. For this study DCCD was chosen, since this inhibitor was demonstrated to bind covalently to an acyl residue of the proteolipid and thus to inactivate irreversibly single

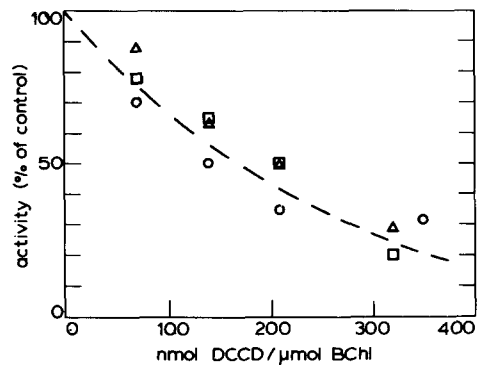

Fig. 8. Parallel inhibition by DCCD of ATPase (O——O), photophosphorylation in continuous light (□——□) and ATP yield per flash (△——△) during phosphorylation induced by trains of flashes.

ATPase complexes. The effect of partial inhibition by DCCD on photophosphorylation, as a function of flash frequency, is shown in Fig. 7. In perfect agreement with the effect of antimycin A, a given concentration of DCCD decreases the ATP yield per flash by a constant degree, independent of the dark time between flashes. Suitable controls run in parallel have demonstrated that DCCD, at the concentrations used, does not affect at all the rate of electron flow, and exerts a stabilizing and stimulatory effect on the carotenoid signal. The decrease in ATP yield follows closely the inhibition of ATPase and of photophosphorylation in continuous light (Fig. 8). It appears therefore that the amount of ATP formed per flash is strictly related to the number of active ATP synthetase complexes present in the membrane. Again, this parallelism is found both when the ATPase turnover is limiting (high flash frequencies or continuous light) or when the dark time between flashes is long enough to allow electron flow (cf. Fig. 1) and ATP synthesis (cf. Ref. 13, Fig. 4) to go to completion.

Discussion

In the work presented here the rate of electron flow, the extent of the electrostatic energetic state of the membrane and the rate of photophosphorylation in pulsed light have been comparatively analyzed under conditions of partial inhibition by antimycin A.

The response of the relaxation kinetics of $[BChl]_2$ to inhibition of electron flow is easily explained by a statistical interpretation of the inhibitory action of antimycin on the ubiquinol-cytochrome c_2 oxidoreductase complex, consistent with the tight binding of antimycin. These data indicate therefore that the relevant parameter of electron flow, which can be directly compared with the rate of photophosphorylation, is the amount of $[BChl]_2$ involved in the fast recovery phase, i.e., the number of photosynthetic electron transport chains (functional associations of reaction centers and cytochrome c_2 reductase complexes) still operating upon partial inhibition by antimycin. The data demonstrate also the very strict redox coupling between single reductase and reaction-center complexes.

The interference of light-induced absorption changes, due to electrochromic effects, with the spectroscopic signal of the reaction center [30] has led us to carry out the experiments on the relaxation kinetics of $[BChl]_2$ in the presence of KCl and valinomycin. This does not affect, however, our analysis of the partial inhibition of electron flow: separate experiments (not shown), performed on chromatophores from *Rps. capsulata* Ala pho^+, a strain impaired in carotenoid biosynthesis and lacking the light-harvesting Complex II, therefore expecially suitable for measurements of electron transport reactions, demonstrated that the effect of antimycin on the relaxation kinetics of the reaction center, in the absence of uncoupling agents, was quite similar to that observed in chromatophores from *Rps. sphaeroides* Ga. The fast and the slow recovery phases were in fact unmodified in rate, while the relative amplitude of the faster phase was depressed in the presence of subsaturating concentrations of the inhibitor; only a slight acceleration of the fast phase was induced by valinomycin. It was moreover demonstrated in a previous paper that the rate of electron transfer in *Rps. capsulata* Ala pho^+ under aerobic phosphorylating conditions is practically coincident with that in membranes uncoupled by nigericin plus valinomycin [20]. This observation is at variance with that reported by Prince et al. [31], who showed that carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) affected also the amount of cytochrome c_2 left reduced before a flash in a train. Again, this discrepancy might be due to the different redox poise that in our conditions limits the rate of electron flow in uncoupled chromatophores [20]. If it is assumed that this behavior of chromatophores of *Rps. capsulata* Ala pho^+ holds also for *Rps. sphaeroides* Ga, it is possible to relate the amplitude of the fast phase of $[BChl]_2$ reduction, although in the presence of valinomycin, to the amount of ATP produced per flash, under our experimental conditions.

The results obtained monitoring the amplitude of the carotenoid band shift in pseudo-steady-state under conditions of limited electron transfer show that the energetic state of the membrane (which is essentially only electrostatic, there being nigericin present at saturating concentration) is practically insensitive to low concentration of antimycin. This behavior is reflecting a rapid delocalization of the field generated by localized charge separation in chromatophores vesicles [6], in which a reduced number of photosynthetic units is turning over.

The most significant finding from the present experiments is concerned with the lack of relationship between the membrane potential, as seen by carotenoids, and the amount of ATP produced per flash under conditions of clearly defined inhibition of electron flow. A one-to-one correlation between the extent of the electrochromic shift and the ATP yield per flash had been clearly demonstrated by previous experiments [13] in uninhibited chromatophores. The data of Fig. 5 prove, however, that in chromatophores brought into a pseudo-steady-state by a train of flashes a direct correlation also exists between the number of active photosynthetic units and the ATP yield per flash, since a decreased ATP yield can be observed in chromatophores partially inhibited by antimycin A in which the extent of the band shift is not reduced. This correlation prevails, at low inhibitor concentrations, over the dependence of the ATP yield on the membrane potential, which per se remains unaffected by antimycin A up to a drastic reduction of the number of operating photosynthetic chains. Only at higher concentrations of antimycin is the dependence on the electrostatic state of the membrane reflected in the lower ratio 'ATP produced/active photosynthetic units' observed.

It was previously demonstrated that the lower amounts of ATP produced by the first turnovers of the photosynthetic apparatus are due to energy limitation [13]. The observation that no further lag was induced by subsaturating concentrations of antimycin during the onset of phosphorylation (Fig. 3) can therefore be considered as a proof, independent of any consideration of the extent of the carotenoid shift, of the low sensitivity of the membrane potential to partial inhibition of electron flow.

The response of flash-induced ATP formation to the progressive inactivation of the ATP synthetase complexes is quite consistent with the general conclusions drawn above. The limitation of the number of active ATP synthetases in the chromatophore population, as evaluated from the inhibition of the ATPase activity, is faithfully re-

flected also in the yield of ATP per flash, measured at a flash frequency (3 Hz) which allows complete relaxation of the fast phase of electron transport between flashes (see also below). DCCD treatment, on the other hand, does not cause uncoupling, but rather stabilizes the membrane potential, which in control chromatophores, under our experimental conditions, remains at a level competent to drive ATP synthesis for at least 10 s [13]. However, a slight inactivation of ATPase corresponds to an immediate parallel decrease in the ATP yield, indicating that in photophosphorylation the delocalization of energy transduction is a very limited phenomenon.

It is generally believed that in continuous light the turnover rate of ATP synthetase represents a kinetic limiting step, as compared to electron transport, in the overall process of photophosphorylation. This certainly is not the case when the chromatophores enter in an oscillatory pseudo-steady-state generated by a train of flashes fired at low frequency. For this reason it becomes extremely important to analyse the inhibition by antimycin or by DCCD with respect to the flash frequency. The experimental results show that the degree of inhibition is practically independent of the frequency for both inhibitors: antimycin A, which limits the number of active electron transport chains and therefore the number of protons translocated per turnover and per vesicle; or DCCD, which limits the rate of utilization of high energy protons.

The present data confirm therefore that in bacterial chromatophores the coupling between redox reactions and phosphorylation is more direct than that postulated by the three-phase chemiosmotic model. The formation of an energetically competent membrane potential seems in fact to be a necessary but not sufficient condition for ATP synthesis, which appears to be severely conditioned by the functional interaction of electron transport chains and ATP synthetases. Evidence for this conclusion has been reached also in biological systems other than chromatophores, through a series of observations already published (cf. Refs. 10–19), based on independent approaches to the problem. The results discussed in this paper are also fully consistent with previous data from our laboratory [10,11,32], which demonstrated a localized coupling between electron transfer and ATP synthesis under continuous illumination. This situation has been here resolved in single turnovers of the photosynthetic apparatus, under conditions of controlled electron flow.

The best extimates available indicate that the average number of reaction centers per chromatophore ranges from a minimum of 11 [33] to a maximum of 40–50 [34]. A corresponding situation pertains also for to ATP synthetase, which appears to be present to a large degree per vesicle on the basis of evidence obtained by electron microscopy [35], inhibitor titration [36] or direct measurements of ATP/$2e^-$ ratios [20]. Such a distribution of the functional units per vesicle is absolutely not compatible with the observed effects on phosphorylation of a limitation of the number of active electron transfer chains or ATP synthetases, if the results are interpreted in terms of delocalized coupling. On the other hand, delocalization of charge distribution is clearly demonstrated by the observed insensitivity of the membrane potential to partial inhibition of electron flow.

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