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THE EXTENT OF THE STIMULATED ELECTRICAL POTENTIAL DECAY UNDER PHOSPHORYLATING CONDITIONS AND THE H^+/ATP RATIO IN *RHODOPSEUDOMONAS SPHAEROIDES* CHROMATOPHORES FOLLOWING SHORT FLASH EXCITATION

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SUMMARY

1. In chromatophores from *Rps. sphaeroides*, the stimulation by ADP and P_i of the electric potential decay indicated by the carotenoid shift is greater than the stimulation of the decay of the pH change indicated by the colour change of added cresol red under similar conditions. This difference is attributed to H^+ consumption during the synthesis of ATP. The ratio of H^+ translocated across the membrane to ATP synthesized was estimated to be approximately 1.7 H^+/ATP.

2. The stimulation of the electrical potential decay by ADP and P_i was found to be a constant fraction (10 %) of the total decay when the flash intensity was varied. No 'critical' or 'threshold' potential was observed.

3. The stimulated electrical potential decay after a second flash, given within a few seconds of the first, was related to the amplitude of the electrical potential produced by the second flash (10 %) but neither to the dark time between the flashes, nor to the total extent of the electrical potential above the dark level. These results are consistent with two hypotheses (a) the chromatophores are a mixed population of vesicles, only a small fraction (10 %) of which possess an active ATP synthesizing system (b) the activity of the ATP synthesizing system, though driven by a proton motive force, is controlled by electron transport processess. If alternative (a) is correct then the overall single turnover flash yield of 1 ATP per 1470 bacteriochlorophyll measured in (1) would mean that the yield of the active vesicles is approximately 10 ATP per 1470 bacteriochlorophyll or 30 ATP per vesicle.

4. The stimulation of the electrical potential decay by ADP and P_i is approximately 40 % less in antimycin-treated chromatophores. It is shown that this is probably a consequence of antimycin-inhibited H^+-release on the inside of the chromatophore vesicles following a flash.

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INTRODUCTION

In the previous paper [1] we have presented evidence to show that ATP synthesis by light may be coupled to the efflux of protons from the chromatophore interior and so it is of some importance to measure the ratio of ATP synthesis to the number of charges translocated. The chemiosmotic hypothesis [2] predicts a whole number stoichiometry. It was not possible to use the techniques which Schroeder et al. [3], and Junge et al. [4], have applied to the chloroplast system. The method of Schroeder et al., is inapplicable because in chromatophores phosphorylation after a period of illumination ceases very quickly (within 20 ms of darkening) [5], compared with the slower decay of the chloroplast high-energy state. The method of Junge et al., can not be used since the flash yield of ATP is difficult to measure accurately in chromatophores owing to a comparatively high rate of dark ATP/P_i exchange. We have therefore used a method for determining the stoichiometry based entirely on rapid spectroscopic measurements. The electrical potential decay was measured by the carotenoid shift decay and the decay of the external pH change by cresol red [1] absorption changes following short flash excitation, under phosphorylating and non-phosphorylating conditions.

It is well known that the ATPase reaction of chloroplasts is not completely reversible [6–8] and Melandri et al., have recently shown that the same is true in chromatophores from *Rps. capsulata* [9]. The rate of ATP hydrolysis was found to be significantly greater in illuminated chromatophores and chloroplasts. The light-stimulated ATPase activity in both chloroplasts and chromatophores is modified by treatment with uncoupling agents, suggesting that the enzyme may be controlled by the membrane high-energy state. Junge [10] has proposed that in chloroplasts the electric potential across the membrane may be of regulatory influence since ATP synthesis and concommitant stimulation of the 515 nm shift disappear below a "critical" electric potential. Boeck and Witt [11], however, observed no "critical" potential and Graber and Witt [12] have shown that the biphasic nature of the decay of the 515 nm shift depends on the pH gradient across the thylakoid membrane. The decay of the chromatophore carotenoid shift is similarly biphasic under phosphorylating conditions [1] and in this communication we examine some of the possible explanations of this observation.

METHODS

Growth of bacteria, preparation of chromatophores and biochemical assays were carried out as in the accompanying communications [1, 13]. The simultaneous measurements of the flash-induced carotenoid shift and cresol red change were made with a double-beam spectrophotometer [13]. The measuring wavelengths were 523 and 575 nm, respectively. The suspending medium for such experiments contained in 10 % sucrose, 50 mM KCl and 8 mM $MgCl_2$. The bacteriochlorophyll concentration was of the order of 10^{-5} M and the final pH was adjusted to 7.9 in the sample cuvette. Orthophosphate and ADP were added to a final concentration of $2.5 \cdot 10^{-4}$ and $2 \cdot 10^{-4}$ M, respectively.

RESULTS AND DISCUSSION

H^+ translocated per ATP synthesised in Rps. sphaeroides chromatophores

In an accompanying paper [1] we have shown that the decay kinetics of the carotenoid shift and of the pH change indicated by cresol red following short flash activation are similar, suggesting that the electric field produced by the electron transport reactions is dissipated by the efflux of H^+ through the chromatophore membrane. The stimulation of the decay rate after the addition of ADP and P_i is greater for the carotenoid shift than for the cresol red change presumably because H^+ is consumed during ATP synthesis [14] (Table I):

$$ADP + P_i + nH^+ \rightarrow ATP + H_2O$$

where $n = 1$ at pH 8.0. We may use this data to estimate the ratio of H^+ translocated per ATP synthesised by the *Rps. sphaeroides* chromatophore suspension. It is assumed that charge separation during electron transport is delocalised by proteolytic reactions at the outer and inner membrane interfaces [15]. It follows that the outward charge translocation through the ATPase (x) following a flash is given by the equation

$$x = ra \tag{i}$$

where $r = H^+$ translocated/ATP synthesised, a = yield of ATP from a single flash, whereas the net production of H^+ accompanying ATP synthesis (y) is given at pH 8.0 by

$$y = a(r-n) = a(r-1) \tag{ii}$$

Eliminating a from equations (i) and (ii) we obtain equation (iii):

$$r = \frac{x}{x-y} \tag{iii}$$

TABLE I

H^+/ATP RATIOS FOLLOWING FLASH ACTIVATION OF *RPS. SPHEROIDES* CHROMATOPHORES

Each sample of chromatophores shown in column 1 was prepared from a different batch of bacteria. The carotenoid shift and cresol red signal were measured simultaneously on a double beam spectrophotometer. The experimental conditions were similar to those shown in Fig. 1. The values shown in the second and third columns of the table are the measured phosphorylation-dependent acceleration of the carotenoid shift or cresol red absorbance change decay 400 ms after the flash expressed as a percentage of the initial amplitudes generated by the flash. Each value is the difference between two experiments of 64 or 128 flashes one in the presence and one in the absence of ADP (10^{-4} M). Phosphate at $2.5 \cdot 10^{-5}$ M and antimycin at $5 \cdot 10^{-7}$ M were present in all experiments. The H^+/ATP ratios were calculated as described in the text.

Chromatophore preparation	x Stimulation by ADP and P_i of carotenoid shift decay (% of total decay)	y Stimulation by ADP and P_i cresol red signal decay (% of total decay)	$\frac{x}{x-y} = r$ $r = H^+/ATP$
A	6	0	1.0
B	4	2	2.0
C	6	3	2.0
D	6	2.5	1.7
E	8	3.5	1.8
Mean	6	2.2	1.7

Values of x were measured from the stimulated carotenoid shift decay in the presence of ADP and P_i, 400 ms after a single-turnover flash (see ref. 1). Values of y were taken from similar measurements on the decay of the cresol red change.

Values of r from five different chromatophore preparations are given in Table I. A mean value, $r = 1.7$ is fairly consistent with an ATPase type II of the chemiosmotic hypothesis [2], where 2 H^+ are translocated per ATP synthesised.

Upon flash excitation, 1 H^+ per approximately 100 bacteriochlorophyll are translocated across the chromatophore membranes [16]. Under phosphorylating conditions an average of 6 % of the total translocated charge passes back across the ATPase (Table I). If 1 ATP is synthesised per 2 H^+, we should predict a flash yield of 1 ATP per approximately 3300 bacteriochlorophyll. This compares with the experimentally determined mean value of 1 ATP per 1470 bacteriochlorophyll [13]. The agreement is poor but likely within the experimental error in view of the difficulties encountered in the measurement of the [^{32}P]ATP yield.

Dependence of the stimulation of the carotenoid shift decay by ADP on the amplitude of the carotenoid shift

Fig. 1 shows the stimulation of the carotenoid shift by ADP and P_i (see ref. 1) in the presence and absence of antimycin A as a function of the initial amplitude of the shift varied through the flash intensity. The highest values of the initial amplitude were obtained with groups of saturating flashes (maximum of 4) at 1 ms intervals. In both cases the stimulation of the decay in the presence of ADP and P_i is linearly dependent on the initial amplitude and there is no detectable "critical electric poten-

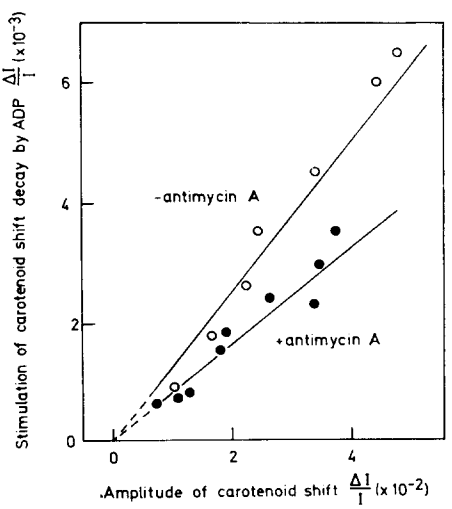

Fig. 1. The dependence of the stimulation of the carotenoid shift decay by ADP and P_i on the amplitude of the carotenoid shift. From a series of experiments similar to that shown in Figs 1 and 2 in ref. 1. The phosphorylation-dependent carotenoid shift decay in the presence (●) or absence (○) of antimycin was calculated as the difference of amplitudes under phosphorylating and non-phosphorylating conditions 400 ms after the flash. Antimycin, where indicated, was added to a final concentration of $5 \cdot 10^{-7}$. Chromatophores containing $1.25 \cdot 10^{-5}$ M bacteriochlorophyll were suspended in 10 ml medium. For further details, see Methods and text.

tial" [10]. Charge translocation accompanying ATP synthesis ceases after approximately 300 ms even through the remaining electric potential is sufficient to drive further H^+ efflux.

In the presence of antimycin A, the same initial amplitude of the carotenoid shift produces about 40 % less stimulation of the decay with ADP and P_i following a flash. It should be noted that this concentration of antimycin was sufficient to completely inhibit photophosphorylation of ADP under continuous illumination but only inhibit the single flash yield of ATP synthesis by approximately 20 % [13]. A five-fold increase in the antimycin A concentration produced no further inhibition of the phosphorylation-dependent charge translocation.

We have shown in ref. 1 that the stimulation of the decay of the carotenoid shift in the presence of ADP and P_i is related to charge translocation accompanying the synthesis of ATP but why does the stimulation only account for approximately 10 % of the total decay? One possibility is that the ATP synthesis reaction driven by the high energy state produced by the flash, passes through a rate limiting step which is slow compared with the $\approx$ 60 ms half-time of the initial phosphorylation-dependent charge translocation.

This however, is disproved by double flashes with an intervening dark period varied between 300 ms and 4.0 s. The intensity of the second flash was controlled with neutral density filters such that the amplitude of the carotenoid shift produced by the

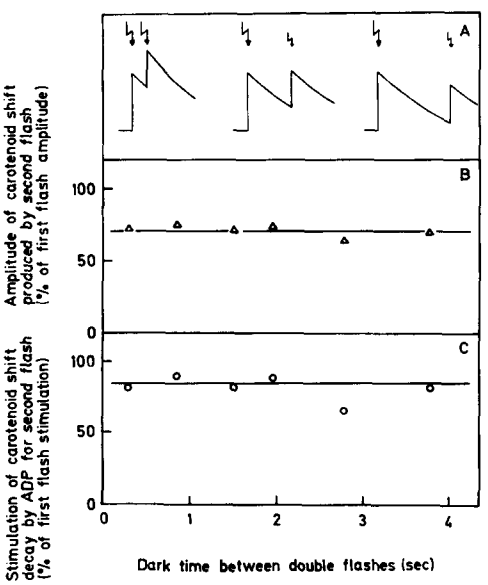

Fig. 2. Dependence of the stimulation of the carotenoid shift decay by ADP and P_i after the second flash on the dark time between double flashes (C). From a series of experiments similar to that in Fig. 1 except that antimycin was present in all experiments at $5 \cdot 10^{-7}$ M. (A) is a diagrammatic representation of the experimental technique. The line drawings show the kinetics of the carotenoid shift measured at 523 nm and the arrows give the time of the flashes. The amplitude of the second flash-induced carotenoid shift (shown in B) was kept constant by using appropriate neutral density filters (Schott NG 5). (C) The stimulation of the carotenoid shift decay by ADP and P_i after the second flash of the pair as a function of the dark time between the flashes.

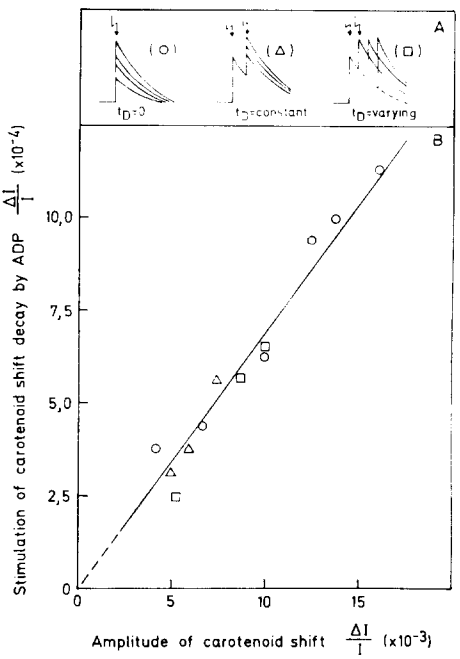

Fig. 3. Dependence of the stimulation of the carotenoid shift decay by ADP and P_i on the amplitude of the flash-induced signal by varying different parameters. From a series of experiments similar to that in Fig. 1 except that antimycin was present in all experiments at $5 \cdot 10^{-7}$ M and bacteriochlorophyll concentration was $6.8 \cdot 10^{-5}$ M. (A) illustrates diagrammatically the different types of experiments. t_D is the dark time between double flashes. The symbol (○) represents the stimulation of the carotenoid shift decay by ADP for single flash, (△) for the second flash fired 500 ms after the first flash. The flash intensity was varied with appropriate grey filters (Schott NG 5). The symbol (□) represents the stimulation of the carotenoid shift decay by ADP for the second flash without attenuation but after varying the dark time between the double flashes. For further details see text.

second flash was kept constant (Figs 2A and 2B). For each dark time the experiment was performed in the presence and absence of ADP and the degree of stimulation of the decay of the shift after the second flash was plotted in Fig. 2C. It may be seen that the stimulation of the carotenoid shift by ADP after the second flash is independent of the dark time between the first and second flashes.

Fig. 3 shows the results of three types of experiment; (i) the stimulation of the carotenoid shift decay by ADP and P_i is plotted against the amplitude of the carotenoid shift generated by a single pulse (data similar to those in Fig. 2) (ii) double pulse experiments in which the time between the flashes is held constant at 500 ms. The intensity of the second pulse is varied with grey filters and the stimulated decay in the presence of ADP and P_i is plotted against the amplitude of the carotenoid shift produced by this flash (iii) the second pulse is given at variable time after the first without intensity attenuation. Fig. 3A illustrates diagrammatically each of these types of experiment. The stimulation of the decay of the carotenoid shift in the presence of ADP and P_i was in all cases linearly related to the amplitude of the carotenoid shift produced by the flash. The stimulated decay was apparently independent of the total value of the membrane potential produced by the flash. The data shown in Figs 1–3

are not easily reconciled with a simple energetic control of the ATP synthesis after a flash in a homogeneous chromatophore suspension.

We must first consider the possibility that the chromatophores are a mixed population of vesicles, only some 10 % of which possess active ATP synthesising enzymes. The chromatophores lacking active ATPase would show only the basal ionic permeability insensitive to the addition of ADP and P_i (see also ref. 1). The carotenoid shift decay of chromatophores possessing ATPase enzyme would be entirely stimulated in the presence of ADP and P_i to a half-time of about 60 ms. Both types of vesicles are assumed to possess functional electron transport chains. Such a mixed population would give rise to a biphasic decay in the net carotenoid shift signal. When the flash intensity is varied (Fig. 1) the proportion of the membrane potential produced in the "active" and "inactive" chromatophores would remain constant so that the stimulation of the decay of the carotenoid shift in the presence of ADP and P_i would be proportional to the amplitude of the carotenoid shift. The membrane potential of the "active" chromatophore would decrease to almost zero within 300 ms after a flash so that the ADP and P_i dependent acceleration of the carotenoid shift decay after a second flash (Figs 2 and 3) would depend on the amplitude of the carotenoid shift after the second flash. If the chromatophore population is heterogeneous, the independence of the stimulation of the carotenoid shift decay in the presence of ADP and P_i on the total value of the membrane potential is only apparent, due to the slow decay of the "inactive" vesicles. In this case the overall single turnover flash yield of 1 ATP per 1470 bacteriochlorophyll [13] is misleading. The "active" vesicles would synthesise 10 ATP per 1470 bacteriochlorophyll per flash or assuming 4700 bacteriochlorophyll per vesicle [13], 30 ATP per active vesicle per flash.

In terms of the chemiosmotic hypothesis, this explanation is not unattractive since in whole bacteria the chromatophores are probably part of a continuous membrane structure across which energy coupling takes place [17]. It would not be necessary for every "chromatophore" to possess an ATPase since the high energy state is delocalised across the entire bacterial membrane. There are, however, observations which can be interpreted as inconsistent with the idea of a mixed population: (i) electron micrographs of *Rps. sphaeroides* show that the chromatophores are well supplied with the 90 Å particles whose properties relate with the coupling factor protein of the ATPase [18]. Of course it is not known whether all the visible ATPase particles are "active"; (ii) titration of the stimulation of the carotenoid shift in the presence of ADP and P_i with venturicidin shows a sharp inhibition by one molecule of antibiotic per 300 bacteriochlorophyll [1]. Assuming 4700 bacteriochlorophyll per chromatophore [13] and assuming that one venturicidin binds to one ATPase we may calculate an upper limit of 15 ATPase per chromatophore; (iii) work with whole bacteria is complicated by their resistance to inhibitors and their endogenous adenine nucleotides and substrates but preliminary experiments have shown that the decay of the flash-induced carotenoid shift of *Rps. sphaeroides* cells is biphasic (unpublished observations). Between 10 % and 50 % of the decay is fast ($t_{\frac{1}{2}} \simeq 200$ ms) and the remainder is slow ($t_{\frac{1}{2}} \simeq 2.0$ s). The proportion of the extent of the fast and slow phases varies with dark incubation time and oxygen concentration and we can not be sure that the fast and slow decays represent phosphorylating and non-phosphorylating ion flux.

If the chromatophores are homogeneous and if all have active ATPase systems

the data shown in Figs 2 and 3 require further consideration. The amplitude of the carotenoid shift produced by a flash is a measure of the membrane potential which, in the presence of antimycin, is generated by electron transport between cytochrome $c \rightarrow P\text{-}870 \rightarrow X$ [19]. The stimulation of the carotenoid shift decay in the presence of ADP and P_i is related to the extent of this reaction following a flash but not to the absolute value of the membrane potential and pH gradient above the dark level. This interpretation is at variance with the predictions of the chemiosmotic hypothesis. There is perhaps some interaction between the electron transport processes and the ATP synthesising apparatus, other than the proton motive force. Since we have shown that the membrane potential and pH gradient provide the energy for the synthesis of ATP we suggest that there may be a regulatory effect of electron transport on the activity of the ATPase. The nature of this interaction is not known.

The effect of antimycin on the trans-membrane pH gradient and correlation with phosphorylation-dependent charge translocation

It was noted above that the stimulation of the decay of the carotenoid shift in the presence of ADP and P_i is less in antimycin-treated chromatophores for the same flash induced initial amplitude of the carotenoid shift (see Fig. 1). It is of interest to see whether this decreased efficiency in the presence of antimycin could be accounted for by a decreased rate of electron transport driven H^+ release on the inside phase of the chromatophores following flash activation. H^+ binding on the outside of the chromatophores as indicated by rapid alkalinisation of added phenol red is only marginally inhibited by antimycin [20].

We have made use of the nigericin-type antibiotic dianemycin. The rationale for the experiment is that the decay of the cresol red change after a short flash will only be accelerated by dianemycin if the rapid H^+ binding on the outside of the chromatophores is associated with H^+ release on the inside, i.e. if a pH gradient exists across

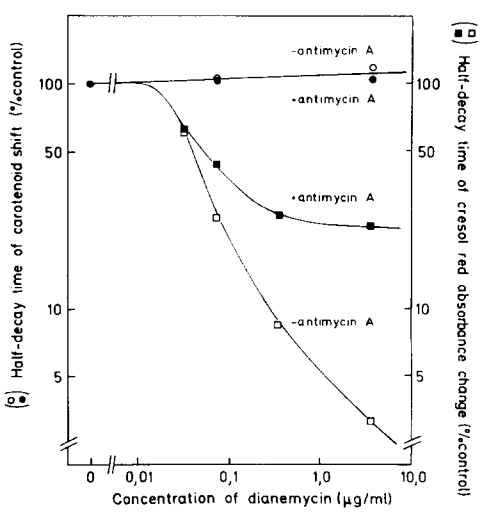

Fig. 4. The dependence of the half-decay times of the carotenoid shift and cresol red absorbance change on dianemycin concentration in the presence and absence of antimycin A. The closed symbols (●, ■) represent the antimycin-treated samples ($5 \cdot 10^{-7}$ M) and the open symbols (○, □) the untreated samples. For other details, see Methods.

the membrane. This method presupposes that the K^+ gradient across the membrane is zero – a condition which is most probably met in our experiments, with chromatophores made and suspended in 50 mM KCl.

In the absence of dianemycin, the addition of antimycin A has little effect on the half-decay time of the cresol red and carotenoid changes. Fig. 4 shows that at high dianemycin concentrations the decay of the cresol red change is much slower in the antimycin treated samples. The carotenoid shift decay is hardly affected over this range of dianemycin concentration in either the presence or absence of antimycin.

In antimycin-treated chromatophores the build-up of Δ pH following flash excitation is evidently delayed, i.e. the release of H^+ on the inside of the vesicles limits the dianemycin-catalysed H^+ efflux following flash-induced rapid H^+ binding. Since the stimulation of the carotenoid shift in the presence of ADP and P_i is abolished by rapid dissipation of the pH gradient [1] it seems likely that the decrease in the stimulation of the decay of the carotenoid shift in the presence of ADP and P_i in antimycin treated chromatophores is a consequence of the inhibited Δ pH formation.

In continuous illumination antimycin A almost completely abolishes ATP synthesis and prevents the lowering of the steady-state level of the carotenoid shift by ADP and P_i [21]. This is presumably because the inhibitor decreases the proton motive force to a value which is insufficient to drive the continued synthesis of ATP and the associated H^+ efflux through the ATPase. We do not agree with M. Baltscheffsky's suggestion [21] that only the antimycin sensitive component of the carotenoid shift is an indicator of the chromatophore high energy state since: (i) following flash excitation, concentrations of antimycin sufficient to completely inhibit electron transfer between the *b* and *c* type cytochromes only inhibit the stimulated decay of the carotenoid shift in the presence of ADP and P_i by 40 % and this is adequately explained by the above experiment; (ii) the decay of the carotenoid shift in the presence or absence of antimycin is completely accelerated by uncoupling agents and valinomycin. When high concentrations of these compounds are used the decay of the shift becomes monophasic [22], strongly suggesting that the carotenoid shift is a response to a single phenomenon.

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