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THE FUNCTIONAL UNIT OF ELECTRICAL EVENTS AND PHOSPHORYLATION IN CHROMATOPHORES FROM *RHODOPSEUDOMONAS SPHAEROIDES*

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SUMMARY

1. From electron micrographs of chromatophores from *Rhodopseudomonas sphaeroides* and from the estimated bacteriochlorophyll content of the sample a mean value of 4700 bacteriochlorophyll per chromatophore was estimated. The mean diameter of the chromatophore vesicles was 600 Å.

2. The decay of the flash-induced electric potential across the chromatophore membrane measured by the carotenoid band shift was 20 % accelerated by about one valinomycin molecule per 4700 bacteriochlorophyll, i.e. by one ionophore molecule per chromatophore.

3. The inhibition of the flash-induced ATP formation by valinomycin followed a similar pattern to the accelerated decay of the electric potential.

4. The single turnover flash yield of ATP synthesis gave a mean value of one ATP per 1470 bacteriochlorophyll or about 3 ATP per vesicle.

5. With regard to the partitioning of the ionophore between the membrane (85 %) and aqueous phase (15 %) we conclude that one molecule of valinomycin per chromatophore is sufficient to begin to collapse the electrical potential and inhibit ATP synthesis. It is therefore suggested that the membrane potential is an essential component of the energized state which is used for phosphorylation.

The results correspond to those obtained for the 100-fold larger vesicles in chloroplasts (thylakoids) where one molecule of ionophore is also sufficient to quench both events.

INTRODUCTION

Electrical events in chloroplasts can be followed by electrochromism (the shift of pigment absorption bands in an electric field). This has been established by different types of experiment [1–4]. The size of the chloroplast functional unit for

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electrical events and also for photophosphorylation has been estimated by titrating the half-decay time of the flash-induced 515 nm absorbance changes and the ATP formation per flash with the ionophore gramicidin D [1, 5]. It was found that a closed vesicle, the thylakoid, composed of 10^5 chlorophylls, but not an electron transport chain composed of about 500 chlorophylls, is the functional unit of the electrical event and phosphorylation. Some doubt remains, however, since the thylakoid volume is not homogeneous owing to the presence of grana stacks and stroma lamellae.

In chromatophores from photosynthetic bacteria it has also been shown that electrical events are indicated by electrochromic bands shifts [6–8]. In this communication we present evidence to show that in chromatophores from *Rhodopseudomonas sphaeroides*, the intact chromatophore membrane, a smaller structure than the green plant thylakoid by about two orders of magnitude, is also the functional unit of the electrical events and phosphorylation. We have estimated the mean bacteriochlorophyll content of a single chromatophore by direct measurement from electron micrographs of a sample of known bacteriochlorophyll content. The electric potential was measured from the electrochromic shift of the carotenoid absorption bands of the chromatophores. The decay of this potential and also the single flash yield of ATP was titrated with the ionophorous antibiotic, valinomycin, whose K^+-carrying properties are apparently similar in chromatophore membranes and artificial bilayers (see ref. 7). In separate experiments the partition coefficient of the valinomycin between the chromatophores and the aqueous phase was estimated from a biological assay of the supernatant after centrifuging the suspension.

The main purpose of these experiments was to determine whether the electrical effects associated with photo-electron transport in chromatophores are transmembrane phenomena, as earlier observations have suggested [6, 7] and whether the electric fields could behave as an obligate intermediate in the synthesis of ATP [9].

Nishimura [10] has measured the size of the photosynthetic units of chromatophores from *Chromatium*, *R. rubrum* and *Rps. sphaeroides* in titration experiments on the yield of H^+ uptake induced by a 200 μs flash, the steady-state light-induced H^+ uptake and the decay time of the carotenoid band shift, with a range of ionophores. The H^+ uptake data relates to the size of the electron transport unit of the chromatophore. The analysis of the data from continuous illumination experiments is complicated by kinetic considerations, i.e, the competition between the rate of the reactions driven in the forward direction by light and the rate of the dissipative reactions catalysed by the ionophore. Nishimura's observations on the valinomycin titre of the carotenoid shift compare favourably with our own – he found that one molecule of valinomycin per approximately 2000 bacteriochlorophyll was required to stimulate the decay of the flash-induced carotenoid shift by 50 %. He did not relate this data to electrical events across the chromatophore membrane nor make comparable measurements on the functional unit of phosphorylation in flash-light.

METHODS

Preparation of chromatophores

Cells of *Rps. sphaeroides* were grown anaerobically under constant illumination at 30 °C in the medium of Sistrom [11]. The harvested bacteria were used immediately. They were washed in 50 mM KCl, 50 mM *N*-tris(hydroxymethyl)-methyl-

glycine (or in some cases, glycylglycine), 8 mM $MgCl_2$ in 10 % sucrose, final pH 7.4 and broken in a Ribi Cell Fractionator at 12000 lb/inch^2. The chromatophores were sedimented at 140 000 $\times g$ (90 min) after removal of cell debris at 20 000 $\times g$ (15 min) and resuspended in a small volume of the above medium. In some experiments the bacteria were disrupted by grinding with alumina. Bacteriochlorophyll concentrations were estimated from the in vivo extinction coefficients given by Clayton [12].

Chromatophore counting

Chromatophore samples containing a small quantity of bacteriochlorophyll ($3 \cdot 10^{-16}$–$20 \cdot 10^{-16}$ mol) were centrifuged directly on to a copper grid (diameter: 2.4 mm) supported on a formvar film in a Beckmann SW 27 rotor at 25 000 rev./min for 20 h. We assume that this time was sufficient to sediment all the chromatophores, since centrifuging for a longer period gave similar results. The chromatophores were stained on the grid with 0.4 % phosphotungstic acid, pH 7.1, for 1 min [13]. The number of vesicles on the grid was counted on a TEM Elmiskop 1A electron microscope.

Biochemical assays

The incorporation of $^{32}P_i$ into organic phosphate was measured according to the method of Avron [14]. The assay conditions were as follows: 50 mM KCl, 8 mM $MgCl_2$, 50 mM *N*-tris(hydroxymethyl)-methyl-glycine (tricine) or glycylglycine, 0.66 mM ADP, 0.5 mM P_i (containing $8 \cdot 10^5$ counts per min ^{32}P), chromatophores containing approximately 50 nmol bacteriochlorophyll in 2.0 ml of 10 % sucrose at final pH 7.9, 25 °C. In some experiments a glucose (20 mM), hexokinase (25 units) trap was used. The reaction was terminated after 60 or 80 saturating 20-μs flashes (wavelength > 700 nm) at 0.0625 Hz with 0.2 ml of 40 % trichloroacetic acid. The dark incorporation of P_i into organic phosphate under the same conditions as above, except in complete darkness, was subtracted to give the flash yield of ATP synthesis.

Chemicals

Valinomycin was purchased from Calbiochem, San Diego (USA), antimycin A from Boehringer, Mannheim (Germany).

Spectrophotometric measurements

The single beam spectrophotometer and signal averaging system have been described in detail elsewhere [15]. The carotenoid shift was measured at 523 nm with appropriate interference filters. The measuring light intensity was $\approx$ 17 ergs $\cdot$ cm^{-2}. s^{-1}. The photomultiplier was screened from the near infra-red (> 700 nm) flashes with Schott filter BG 38 and Balzers cut-off filter 585 nm. The electrical bandwidth was 6.2 ms^{-1} and the optical pathlength through the sample cuvette was 2 cm. Bacteriochlorophyll concentration was used in the range of 10^{-5} M. The 10 ml reaction medium, if not specially indicated, contained 10 % sucrose, 50 mM KCl, 8 mM $MgCl_2$, 50 mM glycylglycine.

RESULTS

The mean bacteriochlorophyll content of Rps. sphaeroides chromatophores

We have estimated this directly by centrifuging a chromatophore sample of known bacteriochlorophyll concentration on to an electron microscope grid (see Methods section). The electron micrographs show a fairly homogeneous population of vesicles, mean diameter 600 Å, similar to those described by other workers [16]. The chromatophore count, shown graphically in Fig. 1 gives a mean ratio of 4700±1300 bacteriochlorophyll molecules per vesicle. This value compares favourably with a theoretical estimate of 3600–18 000 bacteriochlorophyll per vesicle based on spectroscopic observations of the chromatophore carotenoid shift [7].

The electric potential decay and its acceleration by valinomycin

A short actinic flash induces a 3-phase rise of the chromatophore carotenoid shift [17]. The slowest phase ($\simeq$ 100 ms) due to electrogenic electron flow between the cytochromes *b* and *c* can be abolished by the electron transfer inhibitor antimycin A, while the other 2 phases due to charge separation between cytochrome *c* and *P*-870, and *P*-870 and the primary electron acceptor remain unaffected. In the present experiments we have chosen to carry out the titration experiments in the presence of antimycin A to avoid interference of the slow generation phase in the estimation of the real decay rate of the shift. Certainly, at high valinomycin concentrations, where the decay rate is much faster than the 100 ms rise phase, treatment with antimycin is without effect on the decay processes (Jackson, J.B., unpublished observations). Fig. 2A shows the stimulation of the decay of the carotenoid shift with increasing valinomycin concentration. The half-decay time of the signal is 20 % shorter at an ionophore/bacteriochlorophyll ratio of 1/5000. Different chromatophore preparations show slight variation in the sensitivity to valinomycin. The ionophore/bacteriochlorophyll ratio which gives 50 % acceleration of the carotenoid shift decay has varied in our experiments between limits of 1/1500 and 1/2500.

The question arises as to the effectiveness of the added valinomycin molecules. The distribution of valinomycin between the membrane and the water phase can be

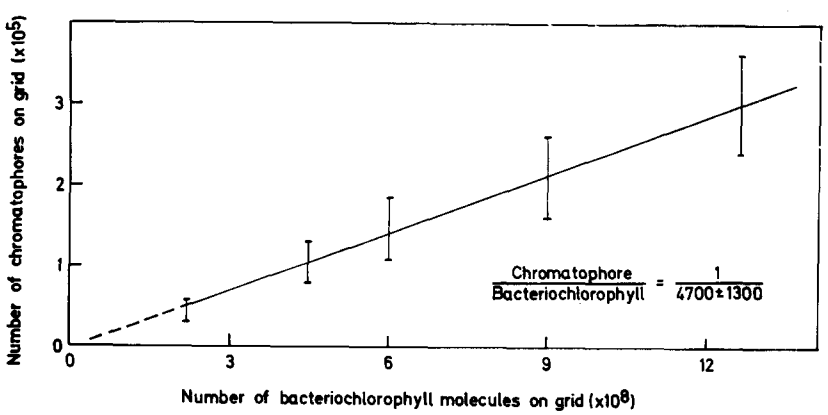

Fig. 1. Relationship between chromatophore number and bacteriochlorophyll content. For details, see Methods.

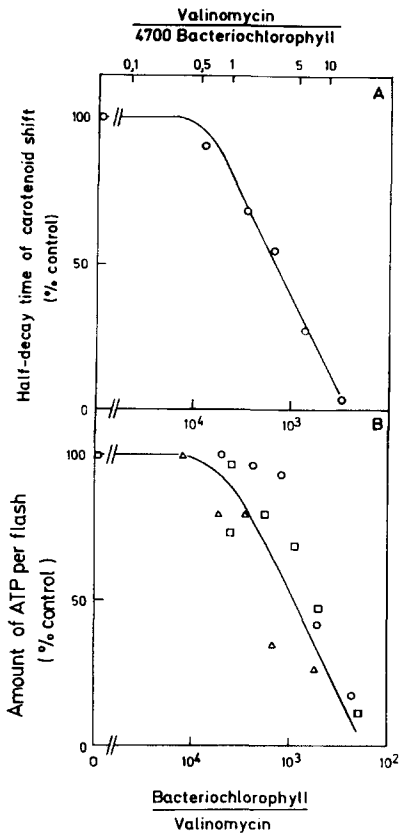

Fig. 2. Dependence (A) of the half-decay time of the electrical potential measured by the carotenoid shift and (B) ATP synthesis on valinomycin concentration. Both reactions were measured with single turnover flashes. The carotenoid shift in (A) was measured at 523 nm. The control sample had a half-decay time of 2.2 s. The different points (○, □, △) in (B) represent different chromatophore preparations. The control values for the yield of ATP synthesis per bacteriochlorophyll per flash (in the absence of valinomycin) were $3.0 \cdot 10^{-3}$, $1.0 \cdot 10^{-3}$ and $1.6 \cdot 10^{-3}$, respectively.

estimated by centrifuging the valinomycin-doped chromatophores and by adding non-treated chromatophores to the supernatant (Table I). The supernatant fraction evidently still contains valinomycin since the carotenoid shift decay of the second sample is accelerated. We calculated the partitioning of the valinomycin between the membranes of the first chromatophore sample and the aqueous supernatant as follows. The relationship between the half-decay time of the carotenoid shift of the first chromatophore sample and the concentration of added valinomycin was first plotted. The valinomycin concentration in the supernatant of the first sample (column 4) was estimated by reading the valinomycin concentration appropriate to the half-decay time of the second sample from the graph. Table I, shows that under our conditions (10^{-5} M bacteriochlorophyll in 10 ml) only 15 % of the added valinomycin is in the water phase. Therefore, the effective concentration of the ionophore is equal to or less than 85 % of the total added amount. This is an upper limit because we cannot be sure of the effectiveness of the bound valinomycin.

TABLE I

The first sample represents the original chromatophores (containing 10^{-5} M bacteriochlorophyll in 10 ml medium) treated with valinomycin. The suspension was centrifuged and fresh chromatophores were added to the supernatant (second sample). The half-time of the carotenoid shift decay was measured as in Methods. The calculation of the data in the fourth column is described in the text, from the relationship between half-decay times of first and second samples (see first, second and third columns).

Total valinomycin concentration of first sample (10^{-8} M)	Half-decay time of carotenoid shift of first sample (s)	Half-decay time of carotenoid shift of second sample (s)	Valinomycin concentration in the supernatant of first sample (10^{-8} M)	Valinomycin in aqueous phase/total valinomycin
–	2.94	2.92	–	–
2.0	0.88	2.70	0.3	0.15
3.9	0.29	2.40	0.5	0.12
9.4	0.17	1.20	1.5	0.16
18.0	0.03	0.59	2.7	0.15

Single turnover flash yield of ATP synthesis

Nishimura [18–20] has measured the yield of ATP synthesis in *R. rubrum* chromatophores following repetitive and single but long (500 μs) flashes. We wanted similar data for the ATP yield after a "single turnover" flash (20 μs) but the pH method of Nishimura [19], proved to be too insensitive. We therefore measured the incorporation of $^{32}P_i$ into organic phosphate following a series of widely spaced (dark time 15–20 s) short flashes (see Methods section). The experiments were frustrated by the dark incorporation of P_i into organic phosphate by our chromatophores [21] and despite dark control samples the experimental error was unavoidably large. To avoid ATP hydrolysis a glucose and hexokinase trap has been used in some experiments. 20–30 % Stimulation of organic phosphate yield was achieved but the inhibition pattern of valinomycin is similar in both cases.

TABLE II

SINGLE FLASH YIELD OF ATP SYNTHESIS

Measured by incorporation of $^{32}P_i$ into organic phosphate. Average of 60 or 80 flashes per preparation at 0.0675 Hz. Of the total $^{32}P_i$ incorporated into organic phosphate in the flash-light experiments approximately 70 % was due to a dark reaction, not dependent on the flashes. This was subtracted with suitable control samples. See Methods section for details.

Chromatophore Preparation	Bacteriochlorophyll / ATP · flash	Chromatophore Preparation	Bacteriochlorophyll / ATP · flash
A	580	H	1740
B	280	I	1000
C	6800	J	2540
D	670	K	380
E	1050	L	630
F	480	M	2600
G	340		
		Mean =	1470

The single flash ATP yield for a number of *Rps. sphaeroides* chromatophore preparations is given in Table II. The mean value of 1 ATP per flash per 1470 bacteriochlorophyll is uncorrected for the reported light-stimulated ATP/P_i exchange [21]. Antimycin A at concentration sufficient to completely inhibit continuous light-induced photophosphorylation [22] lowered the flash yield of ATP by approximately 20 %.

The inhibition of the flash yield of ATP synthesis by valinomycin

In parallel with the determination of the functional electrical unit of the chromatophore we have attempted to find the functional unit of photophosphorylation by titrating the single flash yield of ATP synthesis with the ionophore valinomycin. Fig. 2B shows that the yield of flash-induced ATP synthesis is lowered by 20 % at a valinomycin/bacteriochlorophyll ratio of 1/3100. It is well known that valinomycin only fractionally inhibits photophosphorylation in steady-state illumination [22–24] and yet the present results show almost complete inhibition of the flash yield of ATP. Junge et al. [25], have noted a similar observation in the spinach chloroplast system. It should be noted that the steady-state level of the chromatophore carotenoid shift in continuous illumination is not appreciably lowered by the presence of valinomycin in this concentration range [7].

DISCUSSION

The influence of valinomycin on the decay of the carotenoid shift in chromatophores from *Rps. sphaeroides* is similar to its effect on the electric potential measured across artificial bilayers (see ref. 7). This observation supports the hypothesis that the carotenoid signal is an indicator of a delocalised electric field across the chromatophore membrane. The possibility remained however, that the carotenoids respond in part to charges separated between adjacent electron transport carriers not necessarily anisotropically arranged in the membrane [17]. Fig. 2A shows that the carotenoid shift decay is 20 % stimulated at one molecule of added ionophore per 5000 bacteriochlorophyll and 50 % stimulated at 1 per 1500. The data given in Fig. 1 give a mean value of 4700 molecules of bacteriochlorophyll per chromatophore vesicle. It is clear that a little more than one molecule of valinomycin per vesicle is sufficient to collapse the electric potential. If we consider that 84 % of the added ionophore binds to the membrane (Table II) we find 25 % acceleration of the decay of the carotenoid shift at one bound molecule of valinomycin per chromatophore (or 4700 bacteriochlorophyll). This value may still be an upper limit because we have no indication as to the effectiveness of the bound valinomycin in collapsing the electric field decay. Nevertheless, it is clear that the electric potential (indicated by the carotenoid shift measured a few ms after the flash) is associated with the entire chromatophore membrane and is not localised at the level of the electron transport carriers – in *Rps. sphaeroides* one molecule of cytochrome *c* is associated with $\approx$ 200 bacteriochlorophylls (Jackson, J. B. and Dutton, P. L., unpublished observations and Dutton, P. L., manuscript in preparation). Valinomycin inhibits the flash yield of ATP synthesis at a similar concentration to that required for the acceleration of the decay of the carotenoid shift (Fig. 2B). With due regard to the partitioning of the valinomycin between the chromatophore membranes and the aqueous phase (see below) we may conclude that only one molecule of ionophore per chromatophore is sufficient to inhibit ATP synthesis.

Since each chromatophore possesses $\approx$ 20 electron transport chains and since the flash intensity in our experiments is saturating, it follows that the high energy state leading to the ATP formation must be delocalised across the entire chromatophore membrane and cannot be a localised intermediate associated with the electron transport chain. In view of the well known action of valinomycin in collapsing membrane potentials through electrophoretic K^+ translocation, it is likely that the chromatophore membrane potential is a component of, or is in equilibrium with, the high energy state. The rapid risetime of the fast phase of the carotenoid shift (< 50 ns) [6] argues for an obligate role of the membrane potential in the high energy state leading to ATP synthesis [7].

Following flash illumination, the decay of the chloroplast 515 nm shift is stimulated and ATP synthesis is inhibited by a concentration of gramicidin equivalent to one molecule of ionophore per thylakoid [1, 5]. In chromatophores, treatment with gramicidin does not lead to an accelerated carotenoid shift decay (unpublished observations). Valinomycin, however, is effective. The reason for the gramicidin failure is not clear. It may be that the "pore" mechanism of gramicidin [26], compared with the carrier mechanism of valinomycin [27] cannot operate on the very small vesicles of the chromatophore system. Despite these differences in the ionophore specificity, we have now illustrated the equivalence of the electrical unit and the phosphorylation unit on the one hand in the large vesicular structure of the chloroplast (10^5 chlorophyll molecules) and on the other in the small vesicular structure of the chromatophore ($5 \cdot 10^3$ bacteriochlorophyll molecules).

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