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THE SIZES OF THE PHOTOSYNTHETIC ENERGY-TRANSDUCING UNITS IN PURPLE BACTERIA DETERMINED BY SINGLE FLASH YIELD, TITRATION BY ANTIBIOTICS AND CAROTENOID ABSORPTION BAND SHIFT

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SUMMARY

Several approaches to find out the size of energy-transducing unit in ion transport and photophosphorylation in chromatophores of photosynthetic bacteria (*Chromatium* Strain D, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides*) were applied. Single flash yield (A), titration of ion transport by ionophorous antibiotics (B), and titration of the shift of carotenoid absorption band by antibiotics (C) were the methods used. Method A gave the values of 50–60 as chlorophyll/hydrogen ratio for nonphosphorylating H^+ gradient formation, and the value of 25 for the phosphorylating H^+ change. By Method B, the value of 200–400 was obtained as the molar ratio of chlorophyll to the gradient collapsing agent. By Method C, much larger values (2000–5000) were obtained as the molar ratio of chlorophyll/ionophore, when we measured the effect of valinomycin on the dark recovery rate constants of carotenoid shift. The different biophysical aspects of these "photosynthetic units" are discussed.

INTRODUCTION

The ideas of photosynthetic unit has been discussed in purple bacteria using CO_2 fixation[1] or phosphorylation[2,3] as a physiological parameter. The study of the "reaction center" chlorophyll species[4,5] has also evoked the interest concerning the size and chemical composition of the photosynthetic energy transfer systems. It has been noticed that the "photosynthetic unit" size of purple bacteria determined by flash illumination is smaller than that of green plants and algae. The concentration of "reaction center" chlorophyll molecules and components of the photosynthetic electron transfer chain is also higher (on a chlorophyll basis) in purple bacteria than in green plants. In other words, the molar ratio of chlorophyll/enzyme is generally smaller in photosynthetic bacteria than in green plants and algae.

In this paper, I will discuss the several approaches used to find the unit size for photosynthetic energy transduction in bacterial chromatophores. I have used three techniques to study the case of ion transport or phosphorylation in photosynthetic

Abbreviations: BChl (in tables and figures), bacteriochlorophyll, CCCP, *m*-chloro(carbonyl cyanide) phenylhydrazone.

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bacteria. These are single flash yield experiments, titration of ion transport system by ionophorous antibiotics, and titration of membrane-parameter-sensing indicator by antibiotics.

METHODS

Photosynthetic purple bacteria, *Chromatium* Strain D, *Rhodospirillum rubrum* van Niel Strain 1, *Rhodospirillum rubrum* blue-green Strain (G-9), and *Rhodopseudomonas spheroides* van Niel Strain 2.4.1.C were cultured under illumination and the chromatophores were isolated as described previously[6], using 0.5 M sucrose, 5 mM glycylglycine (pH 7.4) as the isolation medium. Light-induced pH changes were recorded by a combination of a Radiometer 22 pH meter and an Esterline–Angus Speed-Servo recorder. The rate and amount of the H^+ change were titrated by addition of standard acid. H^+ transport under repetitive flashes was studied by the method described previously[6]. The light-induced absorption shift of carotenoid bands in *R. spheroides* was measured either by a DEVAULT–CHANCE[7] laser-activated rapid single-beam spectrophotometer or by a Chance-type double-beam spectrophotometer. Continuous and pulsed light intensities were measured by a Reedeer thermopile and a TRG ballistic thermopile, respectively. Bacteriochlorophyll was determined in ether using the absorption coefficient of WEIGL[8]

RESULTS AND DISCUSSION

The H^+ uptake by chromatophores under continuous illumination and pulsed light is shown in Fig. 1. In the single flash experiment, flashes were sufficiently short (200-μsec duration) to avoid any appreciable recycling of the dark reaction, and the

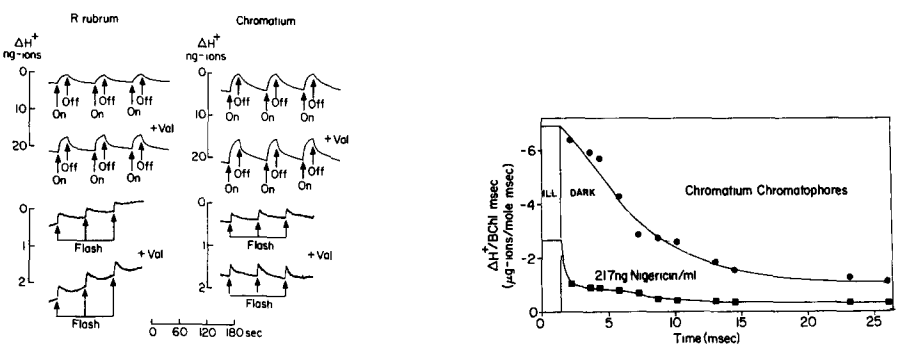

Fig 1 H^+ uptake by continuous illumination and by flashes in *R rubrum* and *Chromatium* chromatophores *R rubrum* chromatophores 17.2 nmoles bacteriochlorophyll per 4 6 ml, *Chromatium* chromatophores 20 9 nmoles bacteriochlorophyll per 4 6 ml, 50 mM KCl, pH 6 5, valinomycin 65 2 ng/ml when used Continuous illumination, tungsten lamp + Wratten 88A filter (> 720 nm) + water layer (4 5-cm thickness), 46 3 kergs/cm^2 sec; flashing illumination, xenon flash (200-μsec duration) + two layers of Wratten 88A filter + water layer (4 5-cm thickness), saturating intensity Temp , 25°.

Fig 2 Analysis of delayed H^+ uptake by repeating flashes *Chromatium* chromatophores 32 2 nmoles bacteriochlorophyll per 4.6 ml, 50 mM KCl, pH 6.5, 21 7 ng nigericin per ml when added Flash duration 1 45 msec, dark intervals varied (see patterns of flashing illumination in ref. 6) Tungsten lamp + Wratten 88A filter (> 720 nm) + water layer (4 5-cm thickness), 46 3 kergs/cm^2·sec Temp , 25°.

dark periods between flashes were sufficiently long (60 sec) to give the maximum yield. To show that point, the time-course of the delayed ion transport, or the ion transport after the flash, was studied under the repeating flash. There we used the light–dark cycles with varied lengths of dark period and calculated the dark relaxation of the ion transport[6] (Fig. 2). Typically, the half-decay time of the order of 10 msec was observed for the H^+ uptake in KCl, longer half-decay time was observed under higher flash intensity. The half-decay time was shortened by nigericin and lengthened by valinomycin.

TABLE I

MAXIMUM SINGLE FLASH YIELD OF H^+ CHANGES UNDER PHOSPHORYLATING AND NONPHOSPHORYLATING CONDITIONS

Experimental conditions similar to those in Fig 1

Medium	*Species*	$-\Delta H^+/BChl$ *per flash (g-ion/mole per flash)*		
		No valinomycin	*Valinomycin (65 2 ng/ml)*	*Number of sets of experiments*
50 mM KCl, pH 6 5	*R. rubrum* wild	0 0156	0 0188	3
	R rubrum blue-green	0 0176	0 0233	1
	Chromatium	0 0147	0 0170	3
Phosphorylating medium (pH 7 8)	*R rubrum* wild	0 040		4

The maximum single flash yields of nonphosphorylating and phosphorylating H^+ changes in three types of chromatophore preparations are shown in Table I. The single flash yield of H^+ change in KCl increased a little in the presence of valinomycin, but this increase was smaller in degree than in the case of continuous illumination. From these values of maximum yield the chlorophyll/hydrogen ratio with a single flash was calculated to be about 50–60 for H^+ transport in KCl. In the case of phosphorylating H^+ change, the chlorophyll/hydrogen ratio of 25 was obtained. These values can be regarded as the "classical" photosynthetic unit size[3], namely $A = m/n \cdot c \cdot f$. A is the size of classical "photosynthetic unit"; m, the number of chlorophyll molecules; n, the number of sites of primary photochemical reaction; c, is the number of sites of energy coupling per electron flow (in the chain or cycle), and f is the energy coupling coefficient, determined by the nature of the chemical reaction(s) involved and the efficiency of energy utilization. In general, $c \geqslant 1$; $0 < f < 1$. Small f is expected for a complex process such as CO_2 fixation, where reducing equivalents (NADPH or NADH) and energy source (ATP) must be supplied. However, in phosphorylation and ion transport, f may be close to unity under optimal conditions.

The effects of the "ionophorous" or transport-mediating antibiotics on the light-induced H^+ gradient formation in bacterial chromatophores and chloroplasts have been studied in many laboratories[9–15]. The acceleration of light-induced H^+ uptake in the presence of valinomycin-type antibiotic and K^+ (or other cations), and the inhibition of the H^+ uptake by nigericin-type antibiotic under similar conditions were the major effects. Increased or decreased ion flux and energy utilization affect other

paths of energy utilization, *e.g.*, phosphorylation and internal bromothymol blue change, to varying degrees[11–16].

The titration of ion transport systems by the ionophorous antibiotics can also be used to determine the size of ion-transporting vesicles. In this titration, it is assumed that the nigericin-type antibiotic binds with the membrane and induces the collapse of the H^+ gradient across the membrane in the presence of K^+ (refs. 15, 17). If this assumption is correct, the chlorophyll/antibiotic molar ratio required to abolish the light-induced ion gradient formation will correspond to the size of the ion-transporting vesicle. Even when we have to consider larger vesicles or larger areas of membrane, and the multiple binding of antibiotic molecules, the ratio will still correspond to the area of vesicle, in which one gradient collapse event or channeling will effectively abolish the H^+ gradient formation The effect of ionophorous antibiotics on the light-induced H^+ change, plotted against the molar ratios of ionophorous antibiotics to chlorophyll is shown in Fig. 3. Very strong and specific inhibition of the ion transport is observed, especially with nigericin and X-206.

The results of titration with different types of chromatophores and antibiotics are summarized in Table II. With the most effective inhibitors, nigericin and X-206, the values of the range of 200–400 were obtained as the molar ratio of chlorophyll to the gradient-collapsing agent. As mentioned earlier, this may be used as the indication of the size or area in which the existence of one gradient-collapsing or ion-exchanging channel will abolish the whole gradient formation.

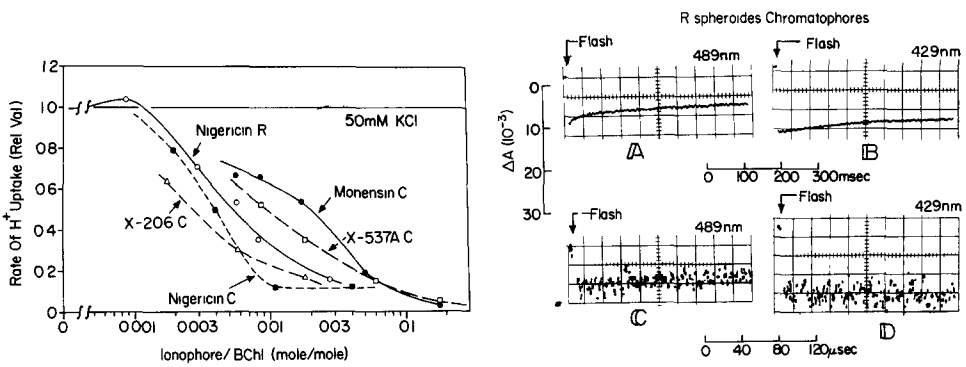

Fig 3. Inhibition of light-induced H^+ uptake by ionophorous antibiotics C and R designate *Chromatium* chromatophores and *R rubrum* chromatophores, respectively 50 mM KCl, pH 6.5 Other details are similar to those in Fig 1

Fig. 4. Flash-induced absorbance changes of *R spheroides* chromatophores Bacteriochlorophyll 57 5 μM, 50 mM KCl, 5 mM glycylglycine, pH 7 4 Rapid single-beam spectrophotometer traces, A and B, 50 msec/div ; C and D, 20 μsec/div. Measuring wavelengths, 489 nm (A and C) and 429 nm (B and D) Path length 3 2 mm Excitation, Q-switched laser flash, wavelength 694 nm; duration about 20 nsec; incident energy $4.34 \cdot 10^{15}$ quanta/cm^2. Temp , 24°

In the last type of analysis, we used the effect of ion transport on the membrane-parameter-sensing indicator. Energetical control of the absorption band shift of carotenoids of purple bacteria has been reported[18–20]. The typical carotenoid shift under continuous illumination has been reported[21–25], and we will not repeat it here. The time-course of the carotenoid change is presented in Fig. 4. In the rapid trace (C), a half-rise time of less than 2 μsec was observed. In the slower trace (A), the decay

TABLE II

LEVELS OF IONOPHOROUS ANTIBIOTICS REQUIRED TO REDUCE THE LIGHT-INDUCED H^+ UPTAKE TO 50%

50 mM KCl or 50 mM NaCl, pH 6 5. Other details are similar to those in Fig 1

Chromatophores	*Antibiotic*	*Level of ionophore to reduce ΔH^+ to 50%*	
		Ionophore/BChl (mmole/mole)	*BChl/ionophore (mole/mole)*
Chromatium chromatophores	X-206	KCl 2 9	345
	Nigericin	KCl 3 9	256
	Dianemycin	KCl 9 8	102
	X-537A	KCl 9 0	111
	Monensin	KCl 19	53
R. rubrum chromatophores	Nigericin	KCl 5 4	185
	Dianemycin	KCl 11	91
	Dianemycin	NaCl 12	83
R spheroides chromatophores	Nigericin	KCl 2 6	383

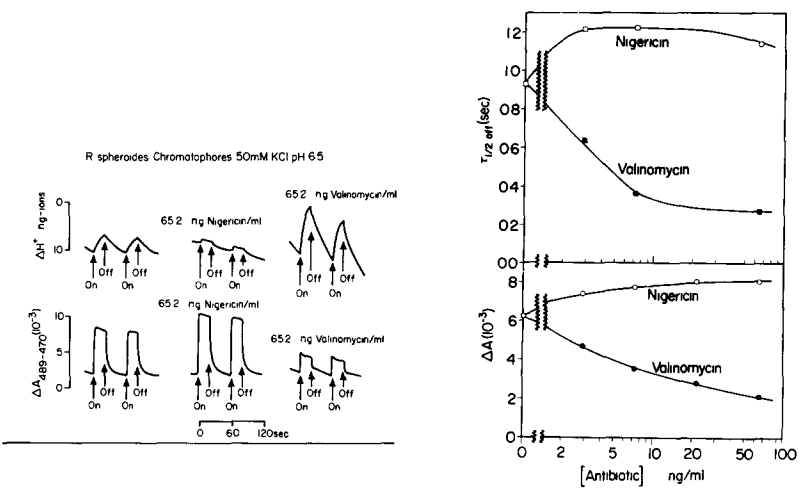

Fig 5 Effects of nigericin and valinomycin on the light-induced H^+ uptake and carotenoid absorption shift in *R spheroides* chromatophores Bacteriochlorophyll 52 1 nmoles per 4 6 ml, 50 mM KCl, pH 6 5 65 2 ng nigericin per ml or 65 2 ng valinomycin per ml were added as indicated Continuous illumination, tungsten lamp + Wratten 88A filter (> 720 nm) + water layer (4 5-cm thickness), 46 3 kergs/cm^2·sec Absorbance changes were recorded by a double-beam spectrophotometer; path length, 5 mm Temp , 24°

Fig 6 Effects of valinomycin and nigericin on the steady-state carotenoid absorbance change by illumination, and on the half-recovery time after illumination *R spheroides* chromatophores, 11 3 μM bacteriochlorophyll, 50 mM KCl, pH 6 5 Absorbance changes were recorded by a double-beam spectrophotometer; path length, 5 mm Other details are the same as in Fig 5

of the carotenoid shift after pulse excitation is seen. This slow dark recovery is very sensitive to the energetical coupling such as ATP formation and ion transport (refs. 18–20; and M. NISHIMURA, unpublished data). In this paper I will present only data concerning the titration of H^+ change and the carotenoid shift by ion-transport-

TABLE III

EFFECTS OF IONOPHOROUS ANTIBIOTICS, UNCOUPLER OF PHOSPHORYLATION AND ENERGY-TRANSFER INHIBITOR ON THE STEADY-STATE CAROTENOID ABSORPTION SHIFT, THE RECOVERY RATE CONSTANTS AND THE HALF-RECOVERY TIME

R spheroides chromatophores, 11 3 μM bacteriochlorophyll, 50 mM KCl, pH 6 5 or phosphorylating medium pH 7 8 Double-beam spectrophotometer recordings or rapid single-beam spectrophotometer recordings (laser pulse-induced change) Other details are similar to those in Fig 5

Medium	*Steady-state* $\Delta A_{489-470\,nm}$ (10^{-3})	*Half-decay time (sec)*	*k* (sec^{-1}) *very rapid phase*	*k* (sec^{-1}) *rapid phase*	*k* (sec^{-1}) *slow phase*	*Transition from rapid phase to slow phase (sec)*
50 mM KCl	6 21	0 928		1 12	0 120	0 92
+ 7 25 ng nigericin per ml	7 71	1 220		0 96	0 095	1 08
+ 2 90 ng valinomycin per ml	4 66	0 636		1 38	0 264	0 79
+ 7 25 ng valinomycin per ml	3 52	0 360		1 96	0 366	0 62
Phosphorylating medium	7 00	1 352		1 01	0 111	1 00
+ 1 45 μM CCCP	5 04	0 270		2 82	no slow phase	no slow phase
+ 1 74 μg oligomycin per ml	14 33	1 608		0 63	0 116	1 50
Laser pulse-induced change 50 mM KCl	10 8		1950 (approx 200 μsec)	0 78 (approx 200 msec)		

mediating agents. Fig. 5 shows the effects of nigericin and valinomycin on the H^+ gradient formation and the carotenoid shift in KCl. On the ion transport, these antibiotics show the expected effects, acceleration by valinomycin and inhibition by nigericin. They induced the opposite effects in the carotenoid shift. Valinomycin induced a decreased steady-state absorbance change of carotenoids and accelerated the dark recovery. Nigericin increased the steady-state absorbance change and slowed down the recovery. The titration curves of absorbance change and half-recovery time by these reagents are shown in Fig 6 The detailed analysis of the dark recovery indicated that the dark process can be approximated by the sum of two exponential decays (*plus* a very rapid decay occurring immediately after flash, with a reaction time of about 200 μsec). The effects of the ionophorous antibiotics, at typical concentrations, on the decay rate constants are indicated in Table III Valinomycin markedly increased the rate constants of dark recovery in both of the rapid and slow phases. Nigericin had the opposite effect. In addition, the effects of an uncoupler of photophosphorylation, *m*-chloro(carbonyl cyanide) phenylhydrazone (CCCP), and an energy-transfer inhibitor, oligomycin, clearly demonstrate the nature of energy-linked behavior of the carotenoid absorption shift Reduced level of the light-induced steady-state carotenoid absorbance change and much accelerated dark recovery were observed in the presence of CCCP No appreciable slow phase was observed. When oligomycin was added, the steady-state carotenoid shift by illumination was much increased The half-recovery time was increased, and the rate constant of the dark recovery in the rapid phase was decreased

From data of this type, with wider concentration range of valinomycin, we calculated the levels of the molar ratio of ionophore/chlorophyll to double the first-order rate constants of the recovery of carotenoid shift (Table IV). The levels of valinomycin required to shorten the half-recovery time of the carotenoid shift to half are also indicated. It is very interesting that the chlorophyll/antibiotic ratio was much larger when we measured the effect of antibiotics on the recovery rate constants of carotenoids than in the case of their effects on the ion transport. In these studies, we used the same preparations of *R. spheroides* in parallel experiments of carotenoid shift and H^+ change. The size of the unit in which one single ionophore molecule can double the decay rate constant, is indeed in the order of a few thousand chlorophyll

TABLE IV

THE LEVELS OF VALINOMYCIN REQUIRED TO DOUBLE THE RATE CONSTANTS OF DARK RECOVERY OF CAROTENOID SHIFT IN *R. spheroides* CHROMATOPHORES

The level of valinomycin required to shorten the half-recovery time to 50% is also shown 11 3 μM bacteriochlorophyll, 50 mM KCl, pH 6 5 Double-beam spectrophotometer recordings, 5-mm path length Other details are the same as in Fig 5

Level of valinomycin	*Ionophore/BChl (mole/mole)*	*BChl/ionophore (mole/mole)*
Level of valinomycin to double k of rapid phase (carotenoid)	$9.0 \cdot 10^{-4}$	1110
Level of valinomycin to double k of slow phase (carotenoid)	$2.2 \cdot 10^{-4}$	4550
Level of valinomycin to reduce over-all $\tau_{1/2}$ to 50%	$5.1 \cdot 10^{-4}$	1960
Level of nigericin to reduce ΔH^+ to 50%	$26 \cdot 10^{-4}$	385

molecules, as compared with the value of 400 in the direct measurement of ion transport of *R. spheroides* particles. Effect of valinomycin on the recovery rate constants was more marked in the slow phase than in the rapid phase. Therefore the titration in the rapid phase gave a smaller value of chlorophyll/ionophore ratio than in the slow phase. Half-recovery time gave a value between two values obtained from the rate constants.

The effect of gramicidin D on the decay of 515-nm shift in chloroplasts also has the large chlorophyll/antibiotic ratio for the effective acceleration of the decay[26]. When we measure the membrane-sensing indicator such as the 515-nm change in chloroplasts or the carotenoid shift in purple bacteria, the ion-transport mediating agent has a much larger surface area of influence than when we measure the stoichiometry of H^+ gradient collapsing. AMESZ AND VREDENBERG[27] have reported that the quantum requirement for the carotenoid absorption shift in *R spheroides* is much smaller than unity. This also suggests that in addition to being energy-harvesting pigment molecules[28,29], carotenoid molecules respond as a membrane-parameter-sensing indicator, and do not behave as stoichiometric electron- or energy-transfer molecules.

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