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STUDIES ON WELL COUPLED PHOTOSYSTEM I-ENRICHED SUBCHLOROPLAST VESICLES

OPTIMIZATION OF FERREDOXIN-MEDIATED CYCLIC PHOTOPHOSPHORYLATION AND ELECTRIC POTENTIAL GENERATION

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Optimal conditions for cyclic photophosphorylation and electric potential generation have been established in well coupled Photosystem (PS)I-enriched subchloroplast vesicles supplemented with ferredoxin. Using NADPH and oxygen as redox-poising agents, it is shown that accurate redox poising of the cyclic system is required for optimal electron transfer. The molar ratio of NADPH to oxygen, rather than their concentrations, regulates the rate of cyclic photophosphorylation. In the present experimental system, the actual redox potential of ferredoxin is of crucial importance for optimal cyclic electron transfer and energy transduction. Under conditions for optimal redox poising of the cyclic system, a relatively strong expression of the flash-induced slow electric potential component was found, as monitored by the absorption changes of carotenoids and of oxonol VI. The function and regulation of cyclic electron transfer in stroma lamellae membranes in vivo are discussed in view of the lateral heterogeneity of redox components in chloroplast membranes.

Introduction

Investigation of the primary photosynthetic energy-transducing reactions would benefit from the availability of relatively simple and stable membrane vesicles in which the native components are largely conserved. In a previous paper [1], we described the isolation and characterization of well coupled and stable PS I-enriched vesicles, derived from spinach chloroplasts after mild digitonin treatment. On the basis of chlorophyll content these vesicles show a 2-fold enrichment in P-700, plastocyanin and ATPase as compared to chloroplasts, in line with the increased activities of PS I-associated electron transfer and cyclic photophosphorylation [2]. They have no PS II activity and contain all the components involved in native cyclic electron transfer except ferredoxin, in amounts originally present in the stroma lamellae.

Abundant information concerning the mechanism of photosynthetic energy transduction during cyclic electron transfer around PS I has been obtained by using artificial mediators like phenazine methosulfate, diaminodurene, anthraquinones and naphthaquinones [3–5], including experiments with

Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DBMIB, 2,5-dibromo-3-methyl-6-isopropylbenzoquinone; DNP-INT, 2-iodo-6-isopropyl-3-methyl-2′,4,4′-trinitrophenyl ether; sDSPD, disulfodisalicylidenepropane-1,2-diamine; Chl, chlorophyll; Qbc, plastohydroquinone-plastocyanin oxidoreductase: cytochrome *b-c* complex containing bound plastoquinone and Rieske (FeS) protein; PS, photosystem.

PS I-enriched membrane preparations [6,7]. However, the use of artificial redox mediators is of limited relevance for studying the native system because of electron bypass of important segments of the native electron-transfer chain like the Qbc (often indicated with cytochrome b_6/f [8] or cytochrome *b*-563/*f* [9]) redox complex and because of artificial proton translocation by some mediators (phenazine methosulfate, diaminodurene) [3,4,6]. Cyclic electron transfer via ferredoxin, the native catalyst which is easily lost during isolation of broken chloroplasts, does not have these disadvantages [3,4,10–13] and would therefore be preferable for the intended investigations.

Dark electron transfer through the Qbc redox complex with appropriate redox mediators can also give insight into the mechanism of photosynthetic energy transduction. A promising progress in this field is the recent finding that an isolated cytochrome 'b_6-*f*' complex reincorporated into lipid vesicles, which catalyzes H^+ translocation and membrane potential formation [8], can be reduced by ferredoxin [9].

Studies carried out under anaerobic and PS II-inhibiting conditions with added reductants [4–6,14–17] point to the importance of an appropriate redox balance of the added cyclic mediator for obtaining maximal activities for cyclic electron transfer. Evidence was presented that in vivo both oxygen (autooxidation of ferredoxin) and NADPH (reduction of ferredoxin via ferredoxin-$NADP^+$ oxidoreductase) have important regulatory functions in PS I-associated electron transfer [10,18–22]. Arnon and Chain [10,18] showed that both NADPH and oxygen can enhance (cyclic) photophosphorylation in broken chloroplasts supplemented with ferredoxin while PS II activity was more or less inhibited.

In this paper we describe the optimization of cyclic photophosphorylation (i.e., of electron transfer) in PS I vesicles using NADPH and oxygen as redox-poising agents. Under optimal conditions for redox poising we have also studied the electric potential generation after single-turnover flashes using intrinsic and extrinsic electric potential probes, i.e., the electrochromic carotenoid and oxonol VI absorption changes, respectively.

Materials and Methods

PS I-enriched vesicles were isolated from market spinach as described previously [1]. The reaction mixture contained 5 mM Tes-KOH buffer (pH 7.8), 2.5 mM KH_2PO_4, 20 mM NaCl, 20 mM KCl and 5 mM $MgCl_2$. ATP synthesis was measured in the presence of 0.5 mM ADP; in the case of adenylate kinase (EC 2.7.4.3) activity 5–20 μM P^1,P^5-diadenosine-5′-pentaphosphate was added. Chlorophyll concentrations in phosphorylation and flash experiments were 12.5 and 50 μg Chl/ml, respectively. All experiments were carried out at 20°C in a special cylindrical black Delrin cuvette (1.8 ml) provided with a Clark oxygen electrode, the actinic light being provided from the bottom [23]. The oxygen concentration was varied by prebubbling the medium with pure nitrogen gas. After PS I vesicles were incubated with ferredoxin for 3 min in the reaction mixture to allow proper reconstitution, illumination started after addition of NADPH. The PS I vesicles were preincubated with the electron-transfer inhibitors 5 min before illumination was started. In phosphorylation experiments the reaction was stopped after 2–5 min illumination by addition of 100 μl of 5 M perchloric acid to 400 μl of the mixture. After neutralization with 100 μl of 5 M KOH and centrifugation the ATP concentration was assayed by the coupled luciferase method [24].

Flash-induced absorption changes were measured with a laboratory-built fast-responding dual-wavelength spectrophotometer [25]; ΔA_{515} and ΔA_{590} were recorded against the references 540 and 603 nm, respectively. Oxonol VI shows an absorbance increase and decrease in maximal extent at 625 and 590 nm, respectively; the isosbestic wavelength is at 603 nm. We have measured the oxonol VI response as a positive absorption change by measuring at 603–590 nm. Saturated flash activation was provided by a General Electrics FT-230 xenon flash tube (2 kV) firing 5-μs half-amplitude flashes (tail-depressed) and filtered through a filter cutting off below 695 nm (Schott, Mainz). The signals of 25 flashes fired at a frequency of 0.125 Hz were averaged. On-line processing and triggering were mediated with a microprocessor-minicomputer system as described before [25]. In continuous-illumination experi-

ments the light guide was connected to a tungsten iodide light source (250 W) and filtered through a filter cutting off below 695 nm and two heat-absorbing filters (Calflex C).

Luciferin, luciferase and ADP, containing less than 0.1% ATP, were obtained from Boehringer, ferredoxin from Sigma, DBMIB, DNP-INT and sDSPD were kindly provided by Professor A. Trebst. Oxonol VI (bis[3-propyl-5-oxoisoxazol-4-yl]pentamethineoxonol) was synthesized and kindly provided by Professor W.G. Hanstein.

Results

When PS I vesicles are supplied with both ferredoxin and NADPH under semiaerobic conditions a slow oxygen consumption occurs that is linear in time and independent of illumination, as shown in Fig. 1. The rate of oxygen uptake is variable in different preparations and is not related to the rate of cyclic photophosphorylation (i.e., electron transfer) (results not shown). This oxygen uptake reflects the well known autooxidation of reduced ferredoxin [10,26] which is gradually inhibited after addition of 2 mM sDSPD. This inhibitor blocks electron transfer in the ferredoxin-$NADP^+$ oxidoreductase region [27].

Since ferredoxin mediates the native cyclic electron transfer [6,10,26] around PS I, and PS II activity is absent in our membrane preparation, the amount of electrons in the cyclic system should be under the control of input from NADPH and output towards oxygen. We optimized this regulation measuring cyclic photophosphorylation as a function of oxygen and NADPH concentrations and light intensity. The results are shown in Figs. 2 and 3, respectively. From Fig. 2 it is obvious that if either NADPH or oxygen is present, the other component must be added for proper adjustment of cyclic electron transfer. In all experiments clear optima are found for the concentrations of oxygen and NADPH. Obviously, accurate redox poising of the system is necessary for optimal cyclic electron transfer, in particular at the lower NADPH and oxygen concentrations. It seems that suboptimal activities result from either overreduction or overoxidation of the cyclic system and the optima are found at about the same molar ratio of NADPH to oxygen. The ratio is about 5 in this system. As shown in Table I, the NADPH/oxygen ratio determines the rate of ATP formation, rather than their concentrations.

Fig. 3 illustrates that light intensity also shows optimum rather than saturation characteristics. We observed that both the NADPH/oxygen ratio and the oxygen consumption (cf. Fig. 1) are rather independent of light intensity (results not shown). We tentatively conclude that the lower activities at the higher light intensities in Fig. 3 are caused by disturbance of the redox balance in the cyclic system around the supposedly rate-limiting step involving the Qbc redox complex.

We have tested the sensitivity of optimal cyclic photophosphorylation for various inhibitors. Table II shows that sDSPD is a relatively weak inhibitor

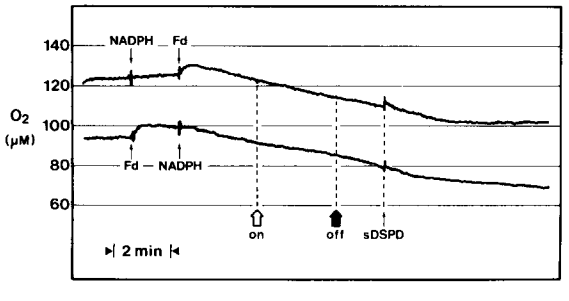

Fig. 1. Ferredoxin-dependent and light-independent oxidation of NADPH by molecular oxygen in PS I vesicles. The reaction medium (see Materials and Methods) contained in addition PS I vesicles at a chlorophyll concentration of 12.5 μM; NADPH, ferredoxin (Fd) and sDSPD were added to final concentrations of 0.5 mM, 4 μM and 2 mM, respectively. The sample was illuminated as indicated.

TABLE I

CYCLIC PHOTOPHOSPHORYLATION IN PS I VESICLES AS A FUNCTION OF VARIOUS CONCENTRATIONS OF NADPH AND OXYGEN AT A CONSTANT MOLAR RATIO

Experimental conditions were as in Fig. 2.

[NADPH] (μM)	[Oxygen] (μM)	ATP synthesis (nmol/min per mg Chl)
25	5	125
125	25	110
250	50	118
500	100	122
750	150	110
1000	200	115

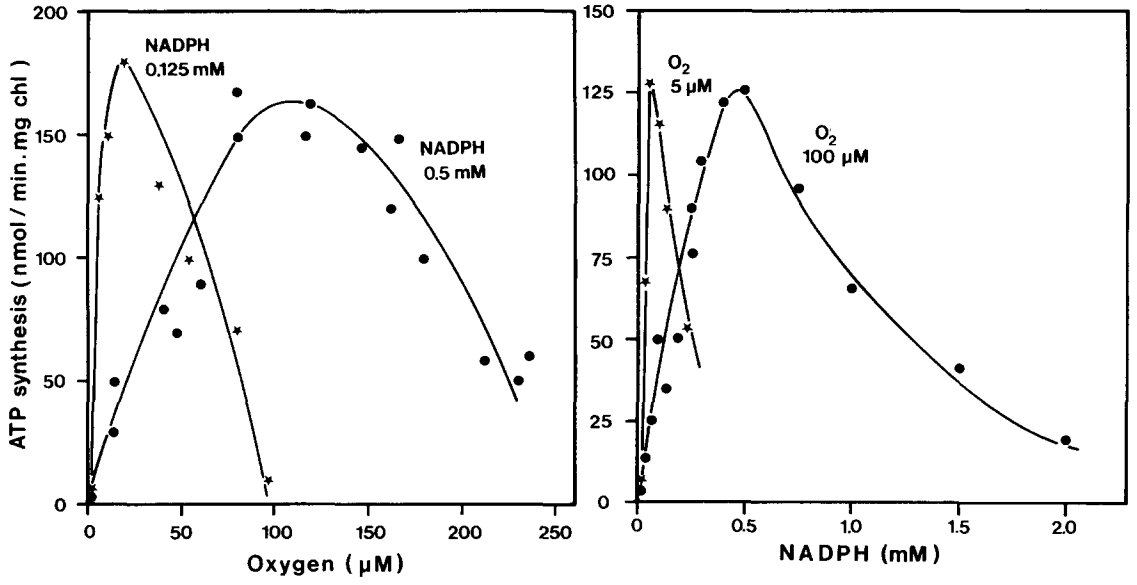

Fig. 2. Cyclic photophosphorylation by PS I vesicles as a function of the concentrations of oxygen and NADPH. The reaction medium (see Materials and Methods) contained in addition 4 μM ferredoxin and concentrations of NADPH and oxygen as indicated. Light intensity was 18 mW/cm^2.

(cf. Fig. 1) and that inhibitors of components in the Qbc redox complex are only partly effective. The inhibitor effects of the latter components are in harmony with their effects on the electrical potential generation induced by light flashes (not shown). In the literature, a wide variety in the effectiveness of these inhibitors is reported [4,6,10,16,17,22,27]. Further studies are required for establishing the involvement of alternative electron pathways [13,28] and/or optimal conditions for inhibition potencies.

Under control for optimal redox poising of the ferredoxin-mediated cyclic system we have studied the electric potential generation after single-turnover flashes using the electric potential-

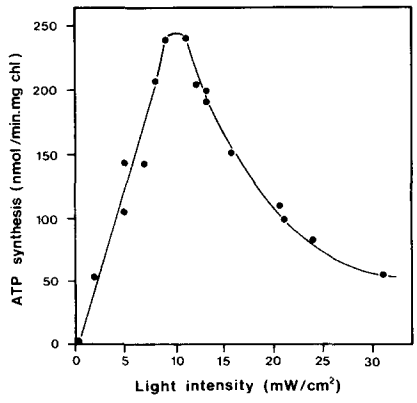

Fig. 3. Cyclic photophosphorylation by PS I vesicles as a function of light intensity at optimal redox poising. Experiments were performed as in Fig. 2, except that 0.5 mM NADPH and 100 μM oxygen were present.

TABLE II

EFFECT OF VARIOUS ELECTRON-TRANSFER INHIBITORS ON CYCLIC PHOTOPHOSPHORYLATION IN PS I VESICLES UNDER OPTIMAL CONDITIONS FOR CYCLIC ELECTRON TRANSFER

Reaction conditions were as in Fig. 3; the light intensity was 10 mW/cm^2. The inhibitors were tested in different PS I vesicle preparations. The noninhibited activities varied between 200 and 300 nmol ATP/min per mg Chl.

Inhibitor		Inhibition of ATP synthesis (%)
sDSPD	(1 mM)	30
	(2 mM)	60
Antimycin A	(5 μM)	40
DBMIB	(10 μM)	45
DNP-INT	(5 μM)	35
DCMU	(5 μM)	0

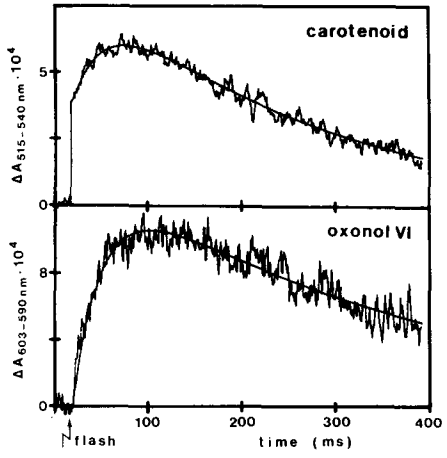

Fig. 4. Transient absorption changes of electric potential indicators induced by single-turnover flashes in PS I vesicles. The flash experiments (see Materials and Methods) were performed under conditions of optimal redox poising (Fig. 3). The concentration of oxonol VI was 0.5 μM, giving no uncoupling under these conditions.

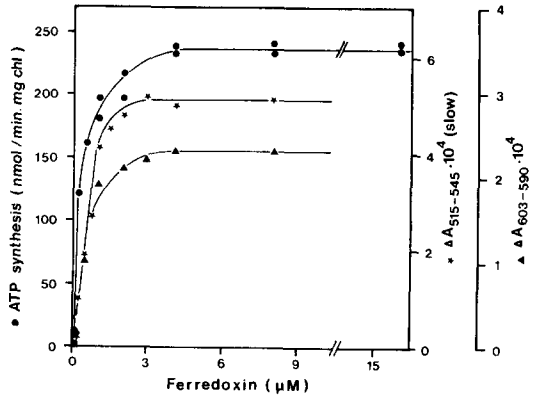

Fig. 5. Cyclic photophosphorylation and electric potential generation in PS I vesicles as a function of the concentration of ferredoxin. The conditions for phosphorylation and electric potential experiments were as in Figs. 3 (light intensity 10 mW/cm^2) and 4, respectively. The carotenoid and oxonol VI responses are indicated as the maximal extents at 60–80 ms after the flash.

sensitive carotenoid (ΔA_{515} [29]) and oxonol VI (ΔA_{590} [30]) absorption changes (Fig. 4). As compared to broken chloroplasts [30], PS I vesicles show a very prominent slow-rising component in the overall carotenoid response with similar kinetics to those of the oxonol VI response. Remarkably, in this reconstituted system the carotenoid response is qualitatively similar to that in whole algae [31] and highly intact chloroplasts [11,15,27]. Moreover, in PS I vesicles this flash-induced response remains identical for at least 30 min. The half-rise time of the slow potential component [31], indicated by ΔA_{515} (slow) and ΔA_{590} (approx. 14 ms), is somewhat longer than those reported for various chloroplast preparations and algae (3–7 ms) [11,15,27,32–34]. This seems to correlate with correspondingly slower ATP synthesis and electron transfer in our PS I vesicles (unpublished results). In a short communication [35], we have reported that the slow electric potential component is much more sensitive to uncouplers, ionophores and other membrane-affecting treatments than the fast component (the latter being sensed only by the carotenoids).

In Fig. 5 the effects of increasing ferredoxin concentrations on steady-state cyclic photophosphorylation and the extent of single-turnover electric potential generation are presented. Clearly, both phenomena are saturated in parallel by ferredoxin. The saturating concentration of about 4 μM is similar to that required for $NADP^+$ photoreduction [10], NADPH-induced PS II fluoresence [22] and cyclic photophosphorylation [10] in broken chloroplasts but significantly smaller than values (50–150 μM) reported by others [4,16,17].

Discussion

In this paper we have established the optimal conditions for cyclic photophosphorylation (i.e., electron transfer) and electric potential generation in PS I vesicles after reconstitution of the native ferredoxin-mediated cyclic system. Our results stress the importance of careful redox poising in order to obtain optimal functioning of the cyclic energy-conserving system.

Fig. 2 and Table I indicate that the molar ratio of NADPH to oxygen rather than their concentrations determines the rate of cyclic electron transfer. Because it is assumed that in the absence of PSII activity ferredoxin links cyclic electron transfer with the oxidation of NADPH and reduction of oxygen, these results suggest that the redox state of ferredoxin is of crucial importance. In intact chloroplasts the redox state of ferredoxin is

also regulated by the concentration and reduction rate of $NADP^+$. Ziem-Hanck and Heber [21] showed that a pulse of oxygen (7 μM) was sufficient to optimize cyclic electron transfer under nitrogen and saturated CO_2 atmosphere. We assume that under these conditions, when the reducing power towards ferredoxin exerted by NADPH is diminished, in particular by $NADP^+$, the optimal oxygen concentration for cyclic electron transfer is reduced to low values comparable with the low NADPH experiments in Fig. 2. Other pools of redox mediators, like thioredoxin [36], may also contribute to the redox balance of the system. However, the ferredoxin step need not be the rate-limiting step under all conditions, e.g., at higher light intensities (Fig. 3).

Our results can be accommodated in the earlier observations with respect to the affinities of ferredoxin and ferredoxin-$NADP^+$ oxidoreductase for oxygen and NADPH, respectively [10,18,37,38]. On the other hand, several reports point to much lower K_m values in chloroplasts for these components [39,40], which would hamper the type of titration experiments as reported here for PS I vesicles.

We have shown that in the stroma lamellae vesicles NADPH can act as electron donor for the cyclic system under physiological conditions. Because stroma lamellae are enriched in PS I and contain little if any PS II [1,2], this points to a predominant function of these membranes in vivo in PS I-associated cyclic photophophorylation using NADPH as electron donor, while linear electron transfer would be restricted to the grana lamellae. As a consequence, lateral shuttles of reducing equivalents from grana to stroma lamellae via some electron-transfer component would be of less crucial importance than suggested [41–44]. This view is supported by the findings of Leegood et al. [33,34], who showed that in the agranal chloroplasts of bundle-sheath cells of a C_4 plant PS II activity was inadequate to poise cyclic electron transfer and presented evidence that NADPH, produced during malate decarboxylation, provides the necessary electrons. As a consequence, in vivo stroma lamella membranes alone would (co)regulate the ATP/NADPH ratio in the stroma, in particular when NADPH is accumulated. NADPH accumulation has been observed in intact chloroplasts when the availability of ATP limits NADPH oxidation during CO_2 fixation [37].

The results agree with the view that in intact chloroplasts pseudocyclic electron transfer is necessary to poise the electron-transfer chain for obtaining an appropriate balance between linear and cyclic electron transfer resulting in optimal ATP production necessary for CO_2 fixation [10,19–21].

We found a relatively strong expression of the flash-induced slow component of the carotenoid response, ΔA_{515}(slow), and of the oxonol VI response, ΔA_{590}, in PS I-enriched and ferredoxin-supplemented vesicles at optimal redox poising for cyclic photophosphorylation (i.e., electron transfer). This is at variance with suggestions that the slowly generated electric field, which is responsible for these responses, is only visible in very intact systems like intact chloroplasts and algae [11,31]. The slow electric field is, in parallel with phosphorylation activity, dependent on the concentration of ferredoxin (Fig. 5), in accordance with studies on intact chloroplasts, in which an increase in native cyclic electron transfer goes hand in hand with an increased extent of ΔA_{515}(slow) [11,15,38]. This stresses the importance of the Qbc redox complex, the electron acceptor for ferredoxin in the chain, for the generation of this electric field.

In order to resolve the involvement of the fast electric potential component (monitored by the carotenoids) and the slow electric potential component (monitored by the carotenoids and oxonol VI) in energy transduction, we have investigated the differential influence of various chemical and physical treatments on these components. This will be the subject of forthcoming papers.

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