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PHOTOPHOSPHORYLATION AND RELATED PROPERTIES OF REAGGREGATED VESICLES FROM SPINACH PHOTOSYSTEM I PARTICLES

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SUMMARY

The small Photosystem I particles prepared from spinach chloroplasts by the action of Triton X-100 (TSF 1 particles) reaggregate into membrane structures when they are incubated with soybean phospholipids and cholate and then subjected to a slow dialysis. The membranes so formed are vesicular in nature and show the capability of catalyzing phenazine methosulfate-mediated cyclic photophosphorylation at rates which are usually about 20 % of those observed with chloroplasts, but higher rates have been obtained. When coupling factor is removed from the chloroplasts by treatment with EDTA, a requirement for coupling factor can be shown for the subsequent ATP formation. The uncouplers carbonylcyanide 3-chlorophenylhydrazone, valinomycin, Triton X-100 and NH^+_4 are effective with the reformed vesicles, which do not show the typical light-induced pH gradient observed with chloroplasts. Incubation of the TSF 1 particles with phospholipids alone allows for the formation of membrane vesicles, but such vesicles are only slightly active in ATP formation. In most properties investigated, the reformed membrane vesicles resemble the original chloroplast membrane so far as phenazine methosulfate-mediated cyclic photophosphorylation is concerned, which indicates a high degree of selectivity in the reaggregation process. The major difference between chloroplasts and the reformed vesicles is the failure of the latter to show a light-induced pH gradient.

INTRODUCTION

In their general features, the processes of oxidative phosphorylation in mitochondria and photophosphorylation in chloroplasts are very similar. In both cases the formation of ATP is coupled to sequential electron transfer reactions in an organized membrane which serves not only to facilitate the electron transfer reactions, but also to accomplish a separation of ions to produce a potential gradient [1, 2]. In some as

Abbreviations: TSF 1, the photosystem 1 particle obtained from chloroplasts by the action of Triton X-100; MOPS, morpholinopropane sulfonic acid; CCCP, carbonylcyanide 3-chlorophenylhydrazone; CF_1, coupling factor from chloroplasts, HEPES, *N*-2-hydroxyethylpiperazine-N^1-2-ethanesulfonic acid.

yet unknown mechanism, the potential gradient so generated is coupled to the formation of ATP through the mediation of a complex protein known as coupling factor, F_1 for mitochrondria and CF_1 for chloroplasts according to the nomenclature of Racker [3].

Both mitochondria and chloroplasts contain a membrane system which consists of a series of vesicles, and it is such osmotically active vesicles which allow for the formation of an ion gradient. Racker et al. [4, 5] have shown that, in the case of mitochondria, a vesicular membrane structure is essential for oxidative phosphorylation. In their pioneering work with mitochondrial systems, they were able to reconstitute a vesicular membrane system from submitochondrial particles by means of slow dialysis of the mitochrondrial fragments incubated in the presence of phospholipids and cholate. Such preparations not only demonstrated ATP-P_i exchange activity, but also showed the capability of oxidative phosphorylation.

Despite the biochemical similarity of mitochondria and chloroplasts, to date there has been no reported reconstitution of chloroplast fragments to produce an active photophosphorylation system. The early experiments of Huzisige et al. [6] reported a reconstitution of the electron transport chain from isolated Photosystem I and Photosystem II fragments prepared from spinach with digitonin, but the rate was very low. Arntzen et al. [7] reported reconstitution experiments which gave improved activity, and further showed that the Photosystem I particles from stroma lamellae were not capable of recombination with grana Photosystem II. The most recent experiments of Ke and Shaw [8] were with isolated Photosystem I and Photosystem II particles (the TSF 1 and TSF 2a particles which are both small individual particles) and reported an activity of 67 μmol NADP photoreduced using diphenylcarbazide as the electron donor. Plastocyanin was a requirement and lecithin was reported to serve as a binding agent for the two particles.

Reconstituted vesicles prepared by Carmeli and Racker [9] from materials extracted from spinach leaves were shown to catalyze P_i- ATP exchange. Treatment of spinach chloroplasts with cholate extracted coupling factor, other proteins and lipids, but the chlorophyll was removed; consequently it was not possible to test for photophosphorylation activity.

In this report we present data showing that the isolated Photosystem I particle from spinach prepared by the action of Triton X-100, the TSF 1 particle previously described [10] can be reaggregated under controlled conditions in the presence of phospholipids and cholate to form vesicles which are active in phenazine methosulfate-supported photophosphorylation.

EXPERIMENTAL

Chloroplasts and other preparations. The methods used for the preparation of spinach chloroplasts and the subchloroplast fragments with Triton X-100 have been previously described [10]. The fragments so produced are designated as TSF-1 and TSF-2 corresponding to the two photosystems of the chloroplast. Chlorophyll was determined by the method of Vernon [11]. Coupling factor from spinach leaves was prepared by the method of Strotmann et al .[12].

Cyclic photophosphorylation. Cyclic photophosphorylation using phenazine methosulfate as the cofactor was initially measured using ^{32}P incorporation into ATP. The reaction mixture of 2.0 ml contained the following components in μmol:

100 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, pH 7.8, 10 KCl, 7 $MgCl_2$, 2 ADP, 0.15 phenazine methosulfate and 10 inorganic phosphate containing ^{32}P at a level of 10^6 cpm for the experiments which used the ^{32}P assay for the determination of ATP formed. The reactions were initiated with illumination from a tungsten light source at $7.5 \cdot 10^5$ erg/cm^2 per s. The illumination time was 30 s, since the rate of ATP formation decreased after this period. The reaction was stopped by the addition of 0.2 ml 20 % trichloroacetic acid, and the esterified phosphate was determined by the ^{32}P method of Avron [13]. The radioactivity was counted with a Nuclear Chicago Scintillation Counter (PPO, POPOP, Dioxane cocktail.)

After it was determined that there was agreement between the ^{32}P assay and the luciferin assay for ATP formation, the majority of the analyses were performed using the more sensitive luciferin assay. This assay utilizes the spectrophotometer and reagents provided by E. I. DuPont De Nemours Co. of Wilmington, Delaware. The use of the luciferin assay for ATP determination is described by Lyman and DeVincenzo [14]. The photophosphorylation reaction was conducted in cuvettes exposed at 20 °C to tungsten light as indicated above. The reaction mixture contained the following components (in μmol) in a volume of 2.0 ml: 100 HEPES buffer, pH 7.8, 10 KCl, 7 $MgCl_2$, 0.5 ADP, 5 inorganic phosphate and 0.15 phenazine methosulfate. The reaction was terminated by the addition of 0.2 ml of 20 % trichloroacetic acid and 0.02 ml of the resultant mixture was added to 2.0 ml of morpholinopropane sulfonic acid (MOPS) buffer, pH 7.4. From this dilution, 0.01 ml was injected into the cuvette containing 0.7 mM luciferin and 100 units of luciferase in 0.1 ml of solution. The instrument was calibrated against known concentrations of ATP, which allowed the concentration of ATP in the unknown to be directly determined. Dark controls were established for each sample analyzed and the reported activities are the differences between the illuminated and dark reaction systems. Because of the presence of some ATP (or some other reacting substance) in the commercial samples of ADP used, we have had to decrease the concentration of ADP in the photophosphorylation reaction mixture to a level of 0.5 μmol to reduce the readings on dark controls to reasonable levels.

Photosystem I activity. The fractions were assayed for Photosystem I activity by means of the photoreaction of diphenylcarbazone according to the directions of Vernon and Shaw [15].

Reconstitution of membrane vesicles. The general approach of slow dialysis in the presence of phospholipids and cholate as developed by Racker's group [4, 5] was used. We utilized commercial soybean phospholipids after homogenization for 5 min at 100 W output on a Branson sonic oscillator. The sonication mixture contained 0.5 g of phospholipids in 40 ml of dialysis buffer.

TSF 1 particles were prepared from spinach chloroplasts as previously described [10] and the protein and chlorophyll concentrations determined. Phospholipids and cholate were added to TSF 1 suspensions contained in 2 ml of 0.01 M HEPES buffer, pH 7.8, in various amounts. The most active preparations in terms of photophosphorylation activity were generally obtained from mixtures containing 5 mg phospholipids, 4 mg cholate and 1 mg TSF 1 protein. The resultant mixture was placed in thin-walled dialysis tubing and dialyzed against 100 vols of 10 % methanol plus 0.01 M HEPES buffer, pH 7.8. The dialysis buffer was changed twice during the first 8 h and the third buffer solution left for 12 h.

Reagents. From Sigma Chemical Co. we obtained the HEPES buffer, carbonylcyanide 3-chlorophenylhydrazone (CCCP), cholic acid, ADP, ATP and soybean phospholipids. Phenazine methosulfate was obtained from Aldrich Chemical Co. and the valinomycin and MOPS buffer were purchased from CalBiochemicals.

Comparison of ^{32}P and luciferin assay. The data presented below have been determined with the luciferin assay for ATP, which is more rapid than the usual ^{32}P assay. Excellent agreement was found between the two methods. Chloroplasts exhibited a rate of 334 μmol ATP/h per mg chlorophyll by the ^{32}P method and 336 by the luciferin method. Reaggregated vesicles gave values of 60 and 57, respectively, for the two methods.

RESULTS

The approach used by Racker's group [4] for the reconstitution of mitochondrial particles into vesicles which were active in oxidative phosphorylation was also effective with chloroplast particles. We have taken the small subchloroplast Photosystem I particles isolated from spinach chloroplasts [10] and subjected them to slow dialysis in the presence of soybean phospholipids and cholate to produce well formed, distinct vesicles. Electron micrographs of the various fractions so obtained show there is a requirement for phospholipids for the formation of vesicles, and the data presented below in Table I show that cholate is required to obtain active vesicles.

TABLE I

PHOTOPHOSPHORYLATION ACTIVITY OF VESICLES RECONSTITUTED FROM SPINACH TSF 1 PARTICLES

Cyclic phosphorylation was measured in the presence of phenazine methosulfate as described in the section on Methods. For these experiments the coupling factor was not removed prior to the Triton X-100 treatment leading to the preparation of the TSF 1 fraction.

Experiment No.	Conditions	Activity (μmol/h per mg chlorophyll)
1	Chloroplasts	745
	TSF 1 undialyzed	1
	TSF 1 dialyzed with phospholipids and cholate	320
2	Chloroplasts	790
	TSF 1 undialyzed	1
	TSF 1 dialyzed with cholate	18
	TSF 1 dialyzed with phospholipids	7
	TSF 1 dialyzed with phospholipids plus cholate	81
	As above minus phenazine methosulfate	3

The various membrane fractions obtained are shown in Figs 1–5. The undialyzed TSF 1 preparation shown in Fig. 1 lacks any structural elements, and shows the background of particulate material that is characteristic of TSF 1 preparations [10]. Dialysis in the presence of phospholipids and cholate in the ratio of 5 mg phospholipids : 4 mg cholate: 1 mg TSF 1 protein resulted in an aggregation of the

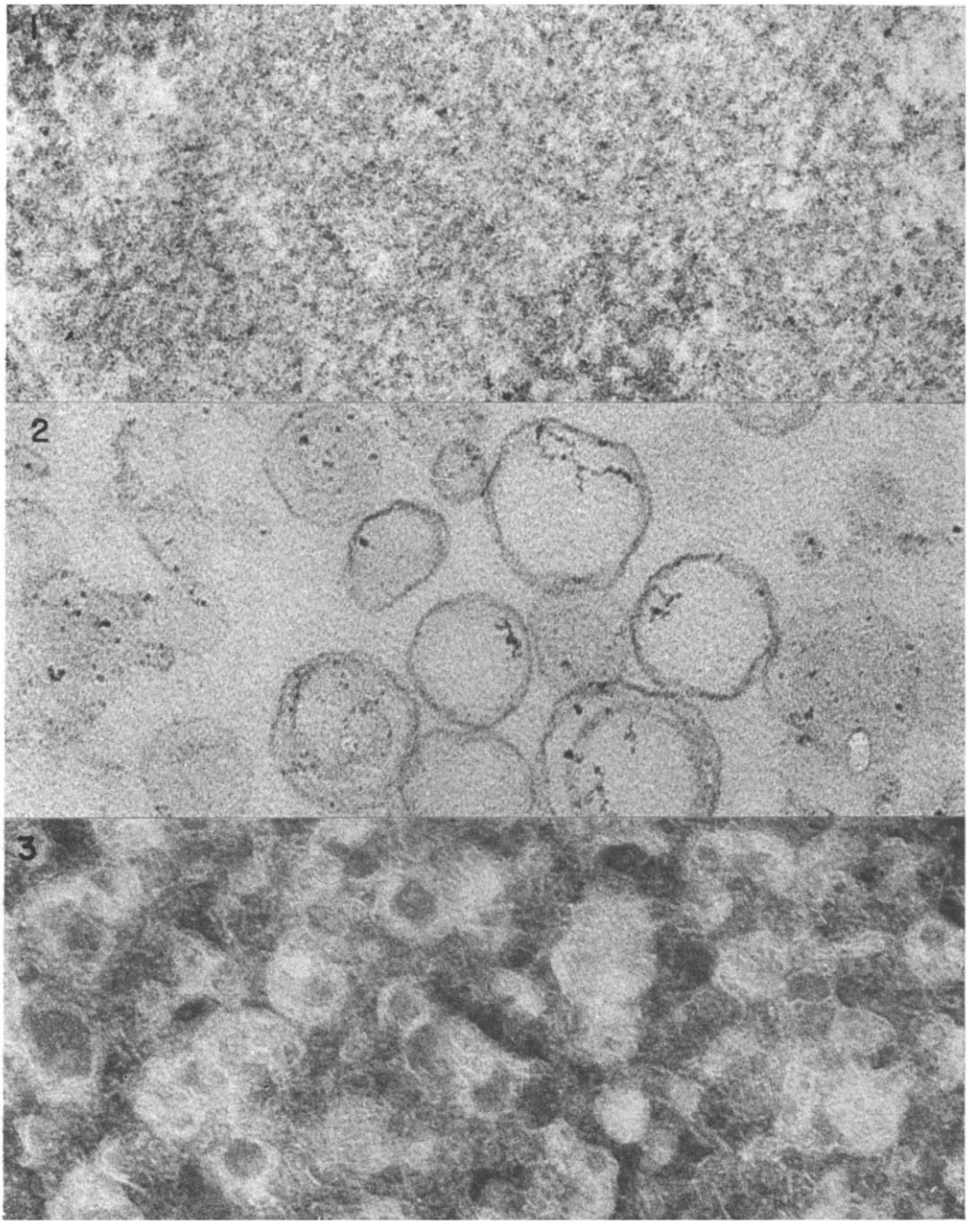

Fig. 1. Thin section of undialyzed TSF 1 preparation from spinach chloroplasts (× 90 000). The thin section technique does not allow the definition of the individual particles shown previously by negative staining [18].

Fig. 2. Thin section of TSF 1 preparation from spinach chloroplast following addition of soybean phospholipids (5 mg) and cholate (4 mg) to the TSF 1 preparation (1 mg protein) and dialysis against 10 % methanol plus 0.01 M HEPES buffer pH 7.8. (× 90 000).

Fig. 3. Negative staining of the reaggregated vesicles described in Fig. 2 (× 90 000).

TSF 1 material into membranes which formed distinct vesicles. Fig. 2 shows a thin section electron micrograph of such vesicles, which occur either as single units or as aggregates sometimes containing two or more concentric vesicles. The size of the reformed vesicles is in the range of 0.2–0.4 microns.

The vesicular nature of the dialyzed material is further shown in Fig. 3, which is an electron micrograph prepared by negative staining. Figs 4 and 5 show similar preparations which were made by dialysis in the presence, respectively, of cholate alone or phospholipids alone, showing that the presence of phospholipids is required for the formation of vesicles during the dialysis procedure.

The vesicles shown in Fig. 2 are representative in terms of size. We have not examined the effect of different and specific phospholipids upon the aggregation of the particles into membrane vesicles, but from the information gained with mitochondria it would appear that there would be a low degree of specificity for phospholipids to allow for the formation of the vesicles themselves.

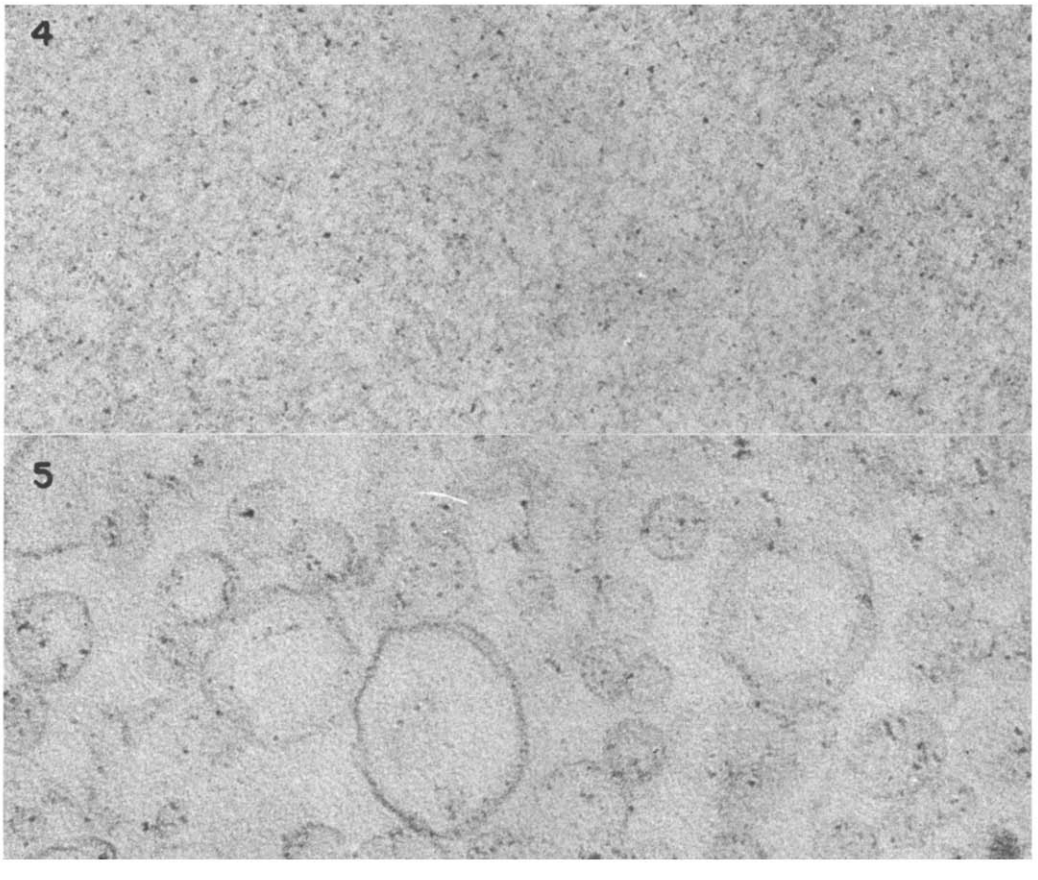

Fig. 4. Thin section of spinach TSF 1 fraction (1 mg protein) incubated with cholate (4 mg) but no phospholipids prior to dialysis (× 90 000).

Fig. 5. Thin section of spinach TSF 1 fraction (1 mg protein) incubated with soybean phospholipids (5 mg) but no cholate prior to dialysis (× 90 000).

This study has been directed toward the requirements for the formation of reaggregated membranes from isolated subchloroplast particles and an examination of the properties of such membranes. We have used phenazine methosulfate-stimulated photophosphorylation as a model system to study, since activity in this process would involve the functional electron transport initiated by the photochemical reaction in which an electron is ejected from the excited P700 molecule to produce $P700^+$, which electron transport is coupled to the esterification of ADP to form ATP. The phenazine methosulfate-mediated cyclic photophosphorylation system is the simplest system for ATP formation, involving only phenazine methosulfate as a cofactor, which can react directly with the reduced form of the primary electron acceptor for Photosystem I and then traverse the membrane in a "cofactor shuttle" to produce a proton gradient [16].

As shown in Table I, the vesicles formed by the slow dialysis of TSF 1 particles in the presence of phospholipids and cholate are active in phenazine methosulfate-mediated cyclic photophosphorylation. In experiments of this type, photophosphorylation activity has been consistently observed, with the activity of the reaggregated vesicles generally being about 20 % of the original chloroplasts. We observe essentially no activity with the undialyzed TSF 1 preparation. As shown in Figs 4 and 5, the presence of phospholipids alone is sufficient to allow for the formation of the vesicular membrane structure, but the data in Table I show that such vesicles have only minimal activity. Thus, the presence of cholate in the dialysis medium is necessary for the higher activities we routinely observe.

The experiments described in Table I were performed with chloroplasts which had not had their coupling factor removed by EDTA treatment. To show a requirement for the coupling factor in the re-aggregated TSF 1 vesicles, similar experiments were performed with chloroplasts which had first been treated with 1 mM EDTA to remove the coupling factor [17]. The reaggregated vesicles from such preparations showed a requirement for coupling factor and also the presence of Mg^{2+}. Since it is difficult to remove all the coupling factor from the chloroplast membrane by EDTA washing, and since any residual coupling factor would appear in part in the TSF 1 fraction, it is likely that there was some coupling factor contained in the reaggregated vesicles in those experiments in which it was not added back during the dialysis procedure.

Table II also shows the Photosystem I activities of the various preparations, using the diphenylcarbazone reaction as a measure of the activity. As was shown earlier [14], the TSF 1 particles show greater activity than do the original chloroplasts when plastocyanin is present with the TSF 1 particles. In the vesicles formed by dialysis, the activities are less. Thus, in both cases where the chlorophyll is present in a condensed membrane system, there is less diphenylcarbazone activity than in the TSF 1 preparation. It is also interesting to note the inverse relationship between the diphenylcarbazone and photophosphorylation activities. The reason for this is not immediately apparent, but it is consistent with the concept that the rate of electron flow through an ordered electron transfer system, coupled to a phosphorylating system, would be less than that observed with isolated particles.

The effects of uncouplers on photophosphorylation activity in the reaggregated vesicles are shown in Table III. As expected, ATP formation was inhibited by the addition of either CCCP, Triton X-100, NH_4^+, or valinomycin in the presence of K^+.

TABLE II

REQUIREMENT FOR COUPLING FACTOR FOR PHENAZINE METHOSULFATE-MEDIATED PHOTOPHOSPHORYLATION ACTIVITY IN VESICLES RECONSTITUTED FROM TSF 1 PARTICLES

For these experiments the major portion of the coupling factor was removed from the chloroplasts by washing with EDTA [17] prior to treatment with Triton X-100. Where indicated, coupling factor was present in the dialysis medium at a concentration of 5 mg phospholipid, 4 mg cholate, 1 mg TSF 1 protein and 0.6 mg CF_1 protein. The fractions were also analyzed for Photosystem I activity using the diphenylcarbazone assay with plastocyanin added [14].

Conditions	ATP formed (μmol/h per mg chlorophyll)	Diphenylcarbazone reaction (μmol/h per mg chlorophyll)
Chloroplasts	596	1,460
TSF 1 undialyzed	1	1,470
TSF 1 dialyzed in presence of 10^{-4} M $MgCl_2$	43	654
TSF 1 dialyzed in presence of coupling factor	73	473
TSF 1 dialyzed in presence of coupling factor and 10^{-4} M $MgCl_2$	120	351

TABLE III

EFFECT OF UNCOUPLERS ON PHENAZINE METHOSULFATE-MEDIATED PHOTOPHOSPHORYLATION ACTIVITY OF VESICLES RECONSTITUTED FROM SPINACH TSF 1 PARTICLES

The chloroplasts from which the TSF 1 was prepared were washed with 1 mM EDTA to remove coupling factor and the reconstituted vesicles prepared as in Table II. The reaction volume for the photophosphorylation assay was 2.0 ml. K^+ was present at a concentration of 10 mM. The vesicles described in Experiment 2 were prepared from the chloroplast sample reported in Experiment 1.

Experiment	System	Added uncoupler (μmol)	μmol ATP/h per mg chlorophyll
1	Chloroplasts	None	334
		1 CCCP	44
		2 CCCP	9
		2 NH_4 Cl	128
		0.002 Valinomycin	155
		0.006 Valinomycin	77
2	Vesicles	None	60
		13 Triton X-100 (0.008 %)	6
		1 CCCP	2
		2 NH_4 Cl	11
3	Vesicles	None	200
		1 CCCP	97
		2 CCCP	28
		0.002 Valinomycin	93
		0.006 Valinomycin	93

It should be noted that the concentration of CCCP required to uncouple the vesicles is greater than that required for the intact chloroplasts. Thus, the reaggregated vesicles have generally the same response to these uncoupling agents as the original chloroplast membrane, which indicates a high degree of integrity of the reformed membrane.

The time courses of the photophosphorylation reactions observed with chloroplasts and reaggregated vesicles are shown in Fig. 6. The reformed vesicles do show a somewhat different pattern, with a break in the curve in the region of 30 s reaction time. Subsequent to this there appears a slow, continuing reaction. The reformed vesicles do have an active ATPase activity, which we have not examined in detail, but we suppose this is responsible for the slow rate of ATP formation observed after the initial 30-s period. To minimize this effect, we have selected 30 s as the reaction time in our experiments on ATP formation.

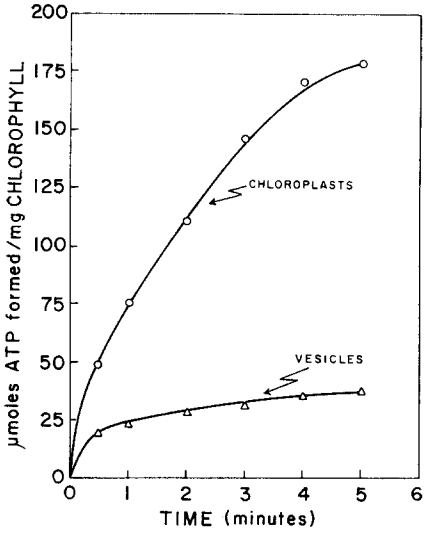

Fig. 6. Time course of ATP formation in phenazine methosulfate-mediated cyclic photophosphorylation catalyzed by the reaggregated membrane vesicles from spinach TSF 1 particles. The assay conditions are given in the section on Methods. Both phospholipids and cholate were added to the incubation mixture.

The pioneer work of Jagendorf and Uribe [1] revealed that illuminated chloroplasts accumulate protons, with the formation of a proton gradient across the membrane. This phenomenon has been extensively studied by others [18], since it represents one aspect of the energy storing mechanism of the chloroplasts leading to ATP formation. We did not observe a similar proton gradient with illuminated reformed vesicles. We have not studied this reaction in detail with the vesicles to determine if it would be possible by various experimental manipulations to regain the ability to produce a light-induced proton gradient. The relationship of a demonstrated light-induced proton gradient to the formation of ATP in such reconstituted systems is discussed below.

DISCUSSION

In an earlier series of experiments [19] it was observed that the Photosystem I particles prepared from spinach, the TSF 1 particles, tend to spontaneously reaggregate. The present experiments show that when such reaggregation occurs in the presence of phospholipids and cholate, discrete vesicles are formed, and that such vesicles have the capability of performing cyclic photophosphorylation in the presence of phenazine methosulfate. This cofactor of cyclic photophosphorylation is unique in that light intensity curves with intact chloroplasts show that phenazine methosulfate-mediated photophosphorylation does not saturate with light intensity as do other photophosphorylation reactions [13, 20]. Trebst explains this in terms of the phenazine methosulfate serving as both an acceptor for electrons from the reduced primary acceptor of Photosystem I as well as a lipophilic compound which can traverse the membrane and release protons upon the inside of the vesicle when it becomes oxidized by the oxidized form of P700. Thus phenazine methosulfate-mediated cyclic photophosphorylation is the most simple form of photophosphorylation. It nevertheless requires (a) a functional reaction center P700, (b) an intact vesicle which has osmotic integrity, (c) a sidedness of the membrane which allows for the generation of ionic gradients as a consequence of the electron transfer reactions and (d) a functional coupling factor complex associated with the membrane and which can convert the electrochemical energy stored in the membrane system into ATP formation.

The reformed vesicles described in this study satisfy all these requirements, which indicates that the reaggregation process is taking place with a high degree of selectivity, so that all the requirements listed above are met. The integrity of the reformed membrane is indicated by the fact that, even though a proton gradient is not detected in the light, known uncoupling agents are active on the membrane. It is interesting that although the presence of cholate in the dialysis medium is not required for the formation of membrane vesicles, which appear normal in electron micrographs, such vesicles are relatively inactive for ATP formation. This indicates a possible role of the cholate in the structure of the membrane itself, which is important for the formation of ATP. This merits further investigation.

The TSF 1 particle is prepared through the action of Triton X-100 upon the native chloroplast membrane. Unpublished data from our laboratory indicate that the particle derives in part from the stroma lamellae (which contains mostly if not exclusively Photosystem I) and in part from the solubilization of some Photosystem I complex from the grana region of the membrane. In any event, the fraction so obtained is enriched in the Photosystem I chlorophyll-protein complex, but does contain other chloroplast components, including some residual 22 and 24 kdalton polypeptides which are characteristic of the light harvesting complex [21, 22]. When coupling factor is not removed with EDTA washing, the polypeptides of this protein also appear in the TSF 1 fraction. Even with EDTA washing, it is not possible to remove all the coupling factor. Consequently, considering the TSF 1 fraction which reaggregates to form the vesicles we have been studying, it is not possible to draw any conclusions concerning whether some particular protein, or all of the proteins in the TSF 1 preparation, is required for the membrane formation to proceed. The data do show, however, that it is possible to fractionate a native chloroplast membrane with

detergents to produce a small particle containing a functional Photosystem I, and then cause these particles to reaggregate in a selective manner so that vesicles are formed which are active in photophosphorylation and thus have all the requirements for this process. The work described here opens up the way for a more systematic study of the requirements for the formation of such membrane vesicles, both in terms of the proteins from the native chloroplast membrane and in terms of the role of phospholipids in the membrane formation process. Along with this, it can allow a more definitive study concerning the manner in which the coupling factor functions in an osmotically competent reformed vesicle.

The recent experiments of Knowles and Racker [23] show that it is possible to form ATP with a Ca^{2+}-ATPase purified from sarcoplasmic reticulum under conditions where it is not possible to form an ion gradient across a membrane. They propose the energy for ATP formation in their system is derived from the interaction of Ca^{2+} with the protein, which interaction leads to a conformational change in the protein resulting in energy storage. This observation is not inconsistent with the need for an ion-transporting mechanism associated with vesicles to efficiently and continuously produce ATP in a functioning organelle. The structure and organization of the protein(s) in the coupling factor are important in each case and are responsive to the ionic environment to which they are exposed. The proton gradient observed in native chloroplasts could be due to an ion transporting mechanism which is required to allow the coupling factor and perhaps some other membrane component as well to cycle between different configurational forms and allow for a continuous generation of ATP.

The fact that reformed vesicles do not demonstrate a light-induced proton gradient does not necessarily indicate that the chemiosmotic process of ATP formation is not operative in such vesicles. It is possible to dissociate the observable proton accumulation from ATP generation in chloroplasts treated with digitonin to produce small particles [24], in which case no proton accumulation is noted in the light. Similar particles prepared from lettuce by the action of digitonin [25] or from spinach by sonication [26] showed only small amounts of proton accumulation, and this was abolished without disturbing the photophosphorylation activity by adding NH_4^+ Ammonium salts and valinomycin behaved in a synergistic manner to cause uncoupling in such systems.

In the case where no proton accumulation is observed, this could be explained by a lowered buffering capacity inside the vesicles or the lack of penetrating counterions to neutralize the electric charge which would build up very quickly. In the case of the lettuce subchloroplast particles [25] or the spinach particles prepared by sonication [26] it appears that the low level of protons accumulated is caused by a reduced permeability to either Cl^- or NH_4^+, thus allowing a membrane potential to be formed rapidly [27]. In any event, the lack of a demonstrable proton accumulation in the present case is not unique in photosynthetic systems, and can be explained by existing theories of chemiosmotic coupling to ATP formation.

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