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FORMATION OF ATP BY THE ADENOSINE TRIPHOSPHATASE COMPLEX FROM SPINACH CHLOROPLASTS RECONSTITUTED TOGETHER WITH BACTERIORHODOPSIN

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SUMMARY

The energy-linked ATPase complex has been isolated from spinach chloroplasts. This protein complex contained all the subunits of the chloroplast coupling factor (CF_1) as well as several hydrophobic components. When the activated complex was reconstituted with added soybean phospholipids, it catalyzed the exchange of radioactive inorganic phosphate with ATP. Sonication of the complex into proteoliposomes together with bacteriorhodopsin yielded vesicles that catalyzed light-dependent ATP formation. Both the $^{32}P_i$-ATP exchange reactions and ATP formation were sensitive to uncouplers such as 3-*tert*-butyl-5,2′-dichloro-4′-nitrosalicylanilide, bis-(hexafluoroacetonyl)acetone and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone, that act to dissipate a proton gradient. The energy transfer inhibitors dicyclohexylcarbodiimide, triphenyltin chloride and 2-β-D-glucopyranosyl-4,6′-dihydroxydihydrochalcone were also effective inhibitors of both reactions.

INTRODUCTION

Reconstitutively active oligomycin or DCCD-sensitive ATPase complexes from mitochondria, yeast and bacteria [1–3] have several properties in common. They all contain a water-soluble factor which catalyzes a DCCD-insensitive ATP hydrolysis and a mixture of water soluble and hydrophobic proteins (F_0) which confer DCCD sensitivity to F_1. The F_1-F_0 complex when reconstituted into liposomes catalyzes a DCCD-sensitive $^{32}P_i$-ATP exchange and ATP-driven proton translocation.

A DCCD-sensitive ATPase complex, from which the majority of impurities have been removed has been isolated recently from mitochondria [1]. In SDS-acryl-

Abbreviations: CF_1, the water-soluble coupling factor of the chloroplast ATPase complex; DCCD, dicyclohexylcarbodiimide; 4′-DOP, 4′-deoxyphlorizin, (2′-β-D-glucopyranosyl-4,6′-dihydroxy-dihydrochalcone); FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; F_o, the hydrophobic portion of the ATPase complex; F_1, the water-soluble coupling factor of the mitochondrial ATPase complex; P_i, inorganic orthophosphate; SDS, sodium dodecyl sulphate; S-13, 3-*tert*-butyl-5,2′-dichloro-4′-nitrosalicylanilide; 1799, bis-(hexafluoroacetonyl)acetone.

amide gels it contains the five subunits of F_1, oligomycin sensitivity conferring protein and three additional major bands with apparent molecular weights of 32 000, 23 000 and about 11 000. The latter appears to correspond to the DCCD-reactive proteolipid described by Cattell et al. [4]. Attempts to isolate this mitochondrial proteolipid in a native and biologically active form have been unsuccessful thus far. We have therefore turned to the study of the ATPase complex from chloroplasts [5] in the hope that it may be more amenable to resolution. Indeed, solubility and stability properties of the chloroplast complex are quite different. In contrast to the mitochondrial proteolipid which is insoluble in ethanol, the chloroplast proteolipid can be extracted with 80 % ethanol without loss of the DCCD binding capacity [6].

It is the purpose of this paper to report on the properties of the chloroplast ATPase complex and its reconstitution together with bacteriorhodopsin into liposomes.

EXPERIMENTAL PROCEDURES

Materials and Methods

Chloroplasts were prepared by differential centrifugation of a homogenate of market spinach leaves as described previously [7]. However, the medium for grinding was 0.35 M NaCl, 0.05 M NaP_i, pH 7.4, and 2 mM NaEDTA, and for the suspension was 0.2 M sucrose, 3 mM $MgCl_2$, 3 mM KCl and 0.05 M Na^+/Tricine, pH 8. Chlorophyll concentration was determined in 80 % acetone by the procedure of Arnon [8]. Chloroplast suspensions at a chlorophyll concentration of 4 mg/ml were stored at −70 °C before use. All steps were performed at 0–4 °C.

The extraction of chloroplasts with 2 % cholate was performed as described by Carmeli and Racker [5] with the exception that no bovine serum albumin was added. After stirring on ice for 15 min, the suspension was centrifuged at 229 000 × *g* for 60 min. The straw-colored supernatant was adjusted to 33 % saturation with a saturated solution (23 °C) of $(NH_4)_2SO_4$ (pH 8) and allowed to stand 20 min on ice. The precipitate was removed by centrifugation at 12 000 × *g* for 10 min. The supernatant was adjusted to 39 % of saturation with a saturated solution of $(NH_4)_2SO_4$ (pH 8), allowed to stand 20 min on ice and the precipitate collected at 12 000 × *g* for 10 min. Excess fluid was removed from the pellet with a small piece of filter paper. After dissolving the pellet in a small volume of a buffer composed of 0.2 M sucrose, 0.02 M Na^+/Tricine (pH 8), and 3 mM $MgCl_2$ it was frozen in small aliquots in liquid nitrogen and stored at −70 °C. In some experiments other protein fractions were prepared at slightly different ammonium sulfate saturation as indicated.

Mg^{2+}-dependent ATPase and $^{32}P_i$-ATP exchange were assayed according to Carmeli and Racker [5] except that the reaction medium contained 80 mM Na^+/Tricine (pH 8), 12 mM $MgCl_2$, 12 mM ATP, 0.25 % defatted bovine serum albumin and (only during exchange) 20 mM $^{32}P_i$ (150–250 mCi/mol).

Vesicles used in the exchange reactions were reconstituted by the cholate dilution procedure [9]. Crude soy phospholipids were acetone washed [10], phosphatidylethanolamine and phosphatidylcholine were purified from bovine mitochondria by the procedure of Kagawa et al. [11]. A chloroform solution of lipids was dried under nitrogen and sonicated to clarity in a solution containing 1.4 % sodium cholate. 0.2 M sucrose, 0.02 M Na^+/Tricine (pH 8) and 3 mM $MgCl_2$. A solution containing 5 mg ATPase protein, 20 mg of the indicated lipids and 7 mg cholate per ml was incubated 20 min on ice then diluted 50-fold into the reaction medium.

Vesicles containing bacteriorhodopsin were reconstituted by sonication in a bath-type sonicator [12]. Crude soybean phospholipids (20 mg/ml) were sonicated until clear (about 15 min) in a buffer containing 0.2 M sucrose, 0.02 M Na^+/Tricine (pH 8) and 3 mM $MgCl_2$. In a final volume of 0.5 ml containing 150 μg bacteriorhodopsin, 1.5 mg of sonicated lipids, and 1.15 mg of the ATPase complex, the mixture was sonicated an additional 6 min, yielding a clear suspension of purple vesicles.

ATP formation was measured in the presence of hexokinase to eliminate $^{32}P_i$-ATP exchange, as described previously [13]. The reaction mixtures were illuminated in a shaking water bath (22 °C) at a light intensity of about $8 \cdot 10^5$ ergs/cm^2 per s. The light was passed through 10 cm water heat filter and a yellow plastic filter (500–650 nm). Light intensity was measured with a Yellow Springs Instrument Co. model 65 radiometer.

Protein was determined by the method of Lowry et al. [14]. Polyacrylamide gel electrophoresis in the presence of SDS was performed as described by Weber and Osborn [15]. The materials for the gel electrophoresis were obtained from BioRad. Lipid content was determined by hydroxamate formation [16]. Cholic acid was purified by recrystallization [10] and the content of cholic acid in the isolated ATPase was determined using radioactively labelled cholic acid [11].

RESULTS

Isolation of the DCCD-sensitive ATPase

The results of a typical fractionation are shown in Table I. The ratio of lipid to protein in the fraction was reduced to about one-fifth of that of the chloroplasts. Thin-layer chromatography of the lipids revealed that most of the pigments were removed by the procedure but the composition of the remaining lipids was similar to that of chloroplasts. Gel electrophoresis revealed at least 13 polypeptides (Fig. 1a), five of which correspond to subunits of CF_1 (Fig. 1b). Most preparations were con-

TABLE I

EXTRACTION AND PRECIPITATION OF EXCHANGE FRACTION

Magnesium-dependent ATPase and $^{32}P_i$-ATP exchange were activated in freshly isolated chloroplasts by treatment for 15 min with 25 mM dithiothreitol. The activated chloroplasts were then extracted with 2 % cholate and fractionated as described under Experimental Procedures. The cholate extract was diluted to 0.7 % cholate and incubated 20 min before reconstitution by the cholate dilution technique.

Source	Protein (mg)	Lipids (mg)	Mg^{2+}-ATPase (nmol/min per mg)	$^{32}P_i$-ATP exchange (nmol/min per mg)
Chloroplasts*	512	397	515	120
Cholate extract	312	120	39**	5**
Ammonium sulfate precipitate (33–39 %)	46	9	380***	50***

* 128 mg chlorophyll.
** Assayed without added phospholipids.
*** Assayed after reconstitution into liposomes.

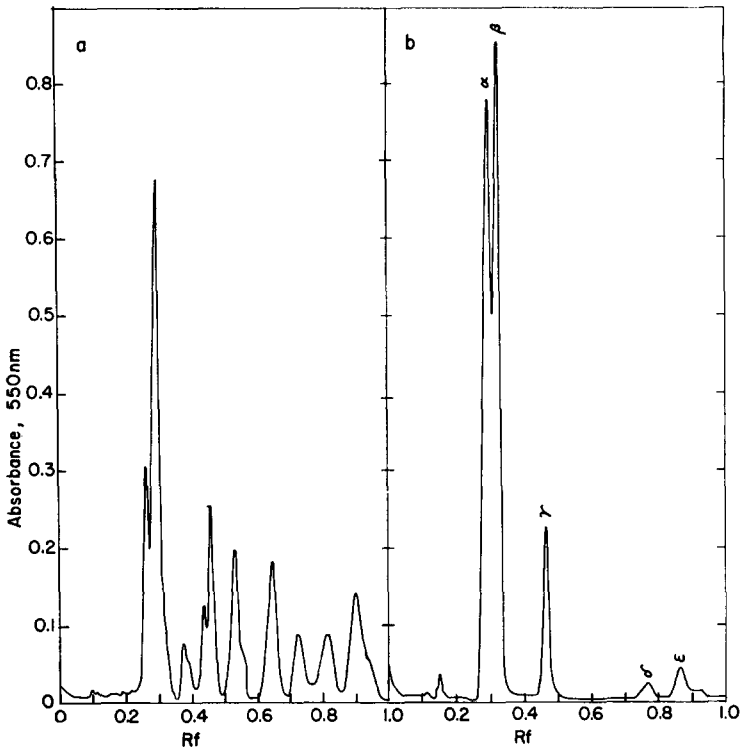

Fig. 1. Spectrophotometric tracing of proteins separated by polyacrylamide gel electrophoresis in the presence of SDS. (a) The ATPase complex and (b) purified CF_1, obtained by chloroform treatment of chloroplasts as described by Younis et al. [18] was analyzed by electrophoresis of 20 μg of the indicated protein.

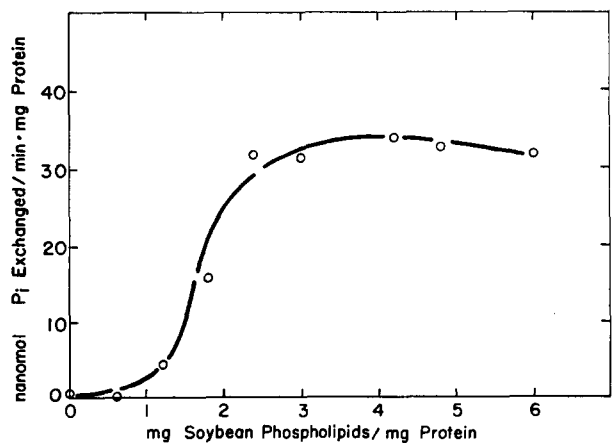

Fig. 2. Dependence of $^{32}P_i$-ATP exchange on added phospholipids. A buffered solution (pH 8) containing 5 mg protein and 7 mg sodium cholate per ml and the indicated concentrations of crude soy phospholipids was incubated for 20 min on ice before dilution 50-fold into the reaction solution. After incubation for 10 min at 37 °C $^{32}P_i$-ATP exchange was measured as described under Experimental Procedures.

taminated with ribulose-diphosphate carboxylase. The subunits of this enzyme overlap in SDS-acylamide gel electrophoresis with the β and ε subunits of CF_1. When suspended in buffer, at about 10 mg/ml, the fraction contained about 4 mg cholate per ml and about 0.01 M NH_4^+.

The fractionated complex was stable at −70 °C for at least 10 weeks. At 4 °C the complex lost $^{32}P_i$-ATP exchange activity quite rapidly although some preparations retained about 50 % activity after 1 week.

Reconstitution of $^{32}P_i$-ATP exchange

As shown in Fig. 2, the $^{32}P_i$-ATP exchange required phospholipid for activity. Similar to the experience with the mitochondrial complex rather high concentrations of phospholipids were needed for maximal activity. Neither pure phosphatidylcholine nor phosphatidylethanolamine alone were very effective but a mixture of both was almost as effective as the crude lipid mixture (Table II).

As shown in Fig. 3, the $^{32}P_i$-ATP exchange was linear with time both at 30 and 37 °C. The effect of the MgATP concentration on the rate of exchange is shown in Fig. 4a. We confirmed that exchange activity is maximal when Mg^{2+} and ATP are equimolar [5]. At 12 mM MgATP used throughout this study, more than 95 % of the initial ATP remained at the end of incubation.

ADP inhibited the exchange activity as shown in Fig. 4b. The nucleotide was added as the magnesium complex to prevent a secondary inhibition which occurs when the free nucleotide complexes with Mg^{2+} decreasing the concentration of the substrate MgATP (see ref. 5).

The $^{32}P_i$-ATP exchange was inhibited by uncouplers that act as proton ionophores as shown in Table III. The exchange reaction was completely stopped in the presence of $1.25 \cdot 10^{-3}$ M of the energy transfer inhibitor 4'-deoxyphlorizin which also fully inhibited ATPase activity. DCCD inhibited ATPase activity only about 30 % at $5 \cdot 10^{-5}$ M although this concentration inhibited exchange almost completely (data not shown).

Reconstitution of photophosphorylation driven by bacteriorhodopsin

Lipid vesicles containing bacteriorhodopsin, the proton pump of *Halobacterium halobium*, were prepared as described previously [17]. The resolved ATPase was

TABLE II

EFFECT OF DIFFERENT PHOSPHOLIPIDS ON $^{32}P_i$-ATP EXCHANGE

Proteoliposomes were reconstituted by cholate dilution and assayed for $^{32}P_i$-ATP exchange as described under Experimental Procedures.

Phospholipid composition	$^{32}P_i$-ATP exchange (nmol/min per mg protein)
Soybean phospholipid	49.1
Phosphatidylethanolamine	8.9
Phosphatidylcholine	6.5
Phosphatidylethanolamine : phosphatidylcholine (1 : 1)	31.2
Phosphatidylethanolamine : phosphatidylcholine (3 : 1)	29.2
Phosphatidylethanolamine : phosphatidylcholine (1 : 3)	13.3

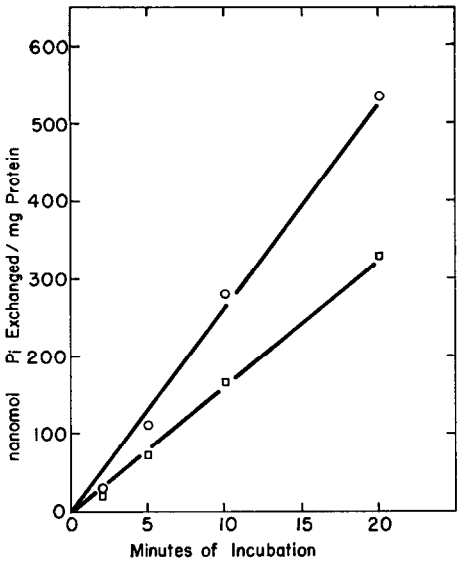

Fig. 3. Effect of temperature on the rate of the $^{32}P_i$-ATP exchange reaction. Proteoliposomes reconstituted by cholate dilution were incubated at either 30 or 37 °C. Individual samples were removed and assayed after incubation for the indicated time as described under Experimental Procedures. ○—○, 37 °C; □—□, 30 °C.

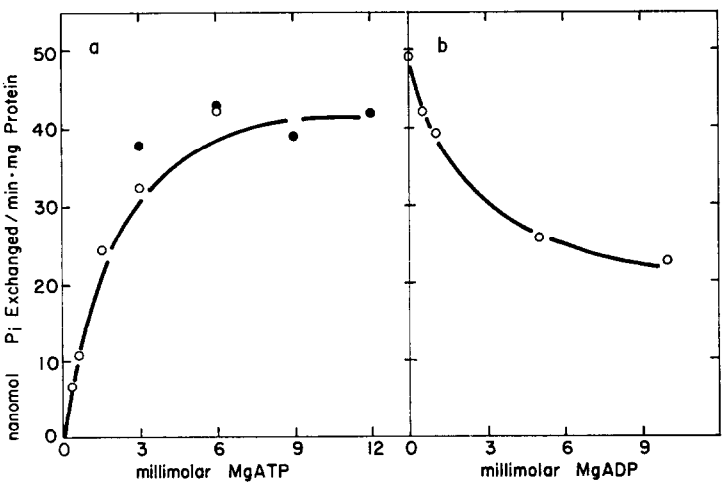

Fig. 4. Effect of substrates and reaction products on the rate of the $^{32}P_i$-ATP exchange. (a) The indicated concentration of an equimolar solution of $MgCl_2$ and ATP/NaOH (pH 8), were added to a reaction solution containing 0.08 M Tricine/NaOH (pH 8), 0.02 M Na_2HPO_4 (250 mCi/mol) and 0.25 % bovine serum albumin. Proteoliposomes were formed by cholate dilution directly into the reaction solution (closed circles are from a second experiment). (b) An equimolar solution of $MgCl_2$ and ADP/NaOH (pH 8), was added at the indicated concentrations to a reaction solution containing 0.08 M Tricine/NaOH (pH 8), 12 mM $MgCl_2$, 12 mM ATP, 0.02 M Na_2HPO_4 (250 mCi/mol) and 0.25 % bovine serum albumin. $^{32}P_i$-ATP was measured as described under Experimental Procedures.

TABLE III

EFFECT OF UNCOUPLERS AND ENERGY TRANSFER INHIBITORS ON $^{32}P_i$-ATP EXCHANGE

Exchange vesicles were formed by cholate dilution just prior to addition of the indicated uncoupler or inhibitor as described under Experimental Procedures.

Additions		$^{32}P_i$-ATP exchange	
		nmol/min per mg protein	Inhibition (%)
Experiment 1			
None		43	0
Triphenyl tin	($5 \cdot 10^{-6}$ M)	18	58
	($1 \cdot 10^{-5}$ M)	15	65
	($4 \cdot 10^{-5}$ M)	0.3	99
Experiment 2			
None		38.1	0
S-13	($1 \cdot 10^{-6}$ M)	12.2	68
	($2 \cdot 10^{-6}$ M)	8.2	78
	($4 \cdot 10^{-6}$ M)	2.3	94
1799	($1 \cdot 10^{-5}$ M)	23.9	37
	($2 \cdot 10^{-5}$ M)	12.9	66
	($4 \cdot 10^{-5}$ M)	2.0	95
FCCP	($1 \cdot 10^{-6}$ M)	36.5	4
	($2 \cdot 10^{-6}$ M)	30.4	20
	($4 \cdot 10^{-6}$ M)	20.8	45
	($1 \cdot 10^{-5}$ M)	1.7	96

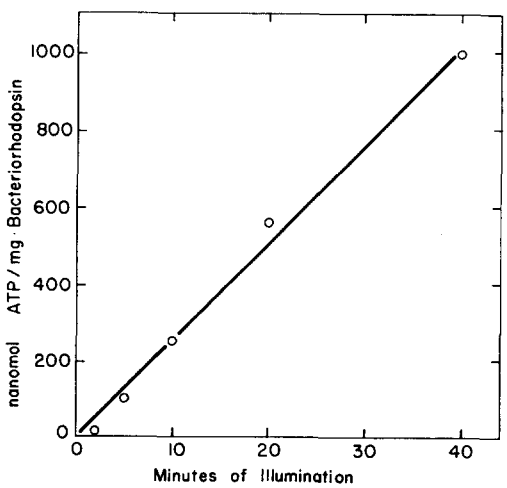

Fig. 5. ATP formation during illumination of reconstituted vesicles containing bacteriorhodopsin and the ATPase complex. Proteoliposomes were formed by sonication and assayed as described under Experimental Procedures.

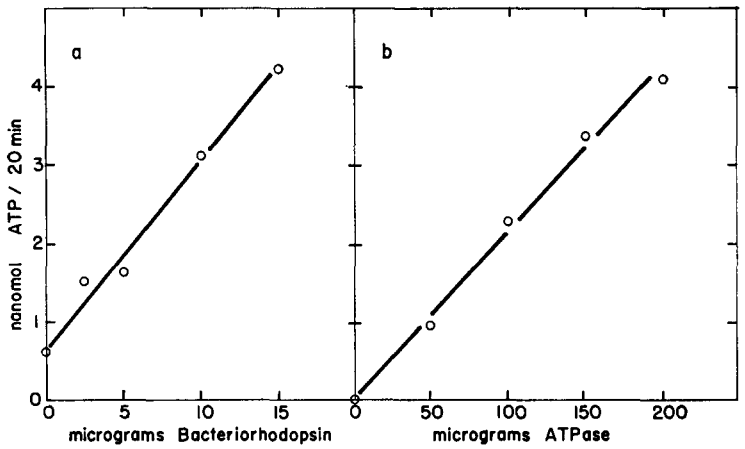

Fig. 6. The rate of ATP formation by reconstituted proteoliposome with varying amounts of ATPase complex and bacteriorhodopsin. (a) The amount of bacteriorhodopsin was varied by adding bacteriorhodopsin and 53 μg of the ATPase complex to sonicated phospholipid vesicles followed by a 6 min additional sonication. (b) The amount of ATPase complex contained in the proteoliposomes was varied in the presence of 15 μg of bacteriorhodopsin prior to final sonication as indicated above.

then introduced by additional sonication for 6 min. The rate of ATP formation was higher at 6 min sonication than at either 3 or 9 min, probably due to lack of incorporation of the complex and damage to the proteins, respectively. This sensitivity to sonication is similar to that of the ATPase complex of mitochondria [17]. When the

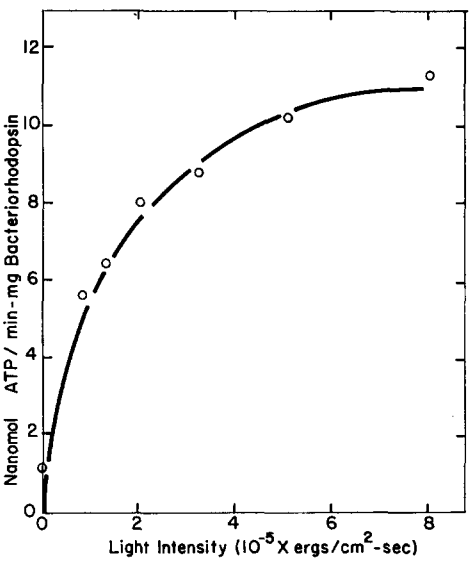

Fig. 7. Dependence of ATP formation on illumination intensity in reconstituted ATPase bacteriorhodopsin vesicles. Light intensity was varied by means of calibrated screens inserted between the light source and the samples.

TABLE IV

EFFECT OF UNCOUPLERS AND ENERGY TRANSFER INHIBITORS ON ATP FORMATION IN RECONSTITUTED BACTERIORHODPSIN VESICLES

Proteoliposomes were prepared by sonication as described under Experimental Procedures and uncouplers or inhibitors were added before illumination. After 20 min illumination the proteins were precipitated with 5 % trichloroacetic acid and removed by centrifugation and the supernatant analyzed as described [17].

Additions	nmol glucose 6-phosphate/min per mg bacteriorhodopsin	Inhibition (%)
Complete system in light	31	0
Complete, dark control	0	100
Complete plus DCCD ($5 \cdot 10^{-4}$ M)	0	100
Complete plus FCCP ($5 \cdot 10^{-5}$ M)	3	91
Complete plus 4'-DOP ($1.25 \cdot 10^{-3}$ M)	6	80
Without bacteriorhodopsin	0	100

reconstituted vesicles were illuminated in the presence of 2 mM $MgCl_2$, 1 mM ADP, 10 mM Na_2HPO_4 (250 mCi ^{32}P/mol), 10 mM glucose, 20 units hexokinase, they catalyzed the net formation of glucose 6-phosphate which was directly proportional to illumination time (Fig. 5).

Over the range of concentrations tested the phosphorylation rate was proportional to the amount of both bacteriorhodopsin and the ATPase complex (Figs. 6a and 6b) and a high light intensity was required (Fig. 7). Unlike the $^{32}P_i$-ATP exchange, the light-dependent net ATP formation did not require activation by dithiothreitol. This is entirely analogous to observations in chloroplasts. However, like the $^{32}P_i$-ATP exchange, the photophosphorylation was inhibited by uncouplers and energy transfer inhibitors (see Table IV).

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