CF_1-DEPENDENT RESTORATION OF ENERGY-LINKED REACTIONS RECONSTITUTED WITH A HYDROPHOBIC PROTEIN FROM SPINACH CHLOROPLASTS

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SUMMARY

The hydrophobic membrane component of chloroplast ATPase (F_o) was purified by extraction of CF_1-depleted chloroplasts with cholate and fractionation of the extract with ammonium sulfate. Vesicles, reconstituted with F_o and phospholipids, catalyzed $^{32}P_i$-ATP exchange when supplemented with CF_1. When bacteriorhodopsin was incorporated into these vesicles light-dependent ATP formation was catalyzed.

INTRODUCTION

Partial reconstitution of the energy conversion apparatus in chloroplasts was first demonstrated by Carmeli and Racker (1). By cholate extraction of chloroplasts, they obtained a fraction which contained CF_1[3], lipids and other proteins, but was depleted in chlorophyll and cytochromes. Recently Winget et al. (2) partially purified and characterized the DCCD-sensitive ATPase complex from chloroplasts. This complex contained CF_1 as well as a hydrophobic portion, F_o. In the present report, we describe a method for isolation of only F_o and its reconstitution with CF_1. Incorporation of the F_o in phospholipid vesicles together with

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3 Abbreviations: CF_1, chloroplast coupling factor; DCCD, N,N'-dicyclohexylcarbodiimide; DTT, dithiothreitol; F_o, hydrophobic fraction from ATPase; FCCP, carbonylcyanide p-trifluoromethoxyphenyl hydrazone; G-6-P, glucose-6-phosphate; PMS, N-methylphenazonium methosulfate; SDS, sodium dodecyl sulfate; S-13, 3-tert-butyl-5,2'-dichloro-4'-nitrosalicylanilide.

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bacteriorhodopsin followed by rebinding of the coupling factor, CF_1, gave vesicles which catalyzed light-dependent DCCD- and uncoupler-sensitive ATP formation.

MATERIALS AND METHODS

ADP, ATP, Tricine, defatted bovine serum albumin and glucose were purchased from Sigma Chemical Company; dithiothreitol was from Calbiochem. Cholic acid from General Biochemicals and soybean phospholipids (Asolectin from Associated Concentrates) were both purified according to the methods described by Kagawa and Racker (3). Growth of Halobacterium halobium and isolation of the purple membranes and bacteriorhodopsin were performed as described by Kanner and Racker (4).

Chloroplasts and EDTA particles were prepared according to the method of Lien and Racker (5). The pellets of chloroplast EDTA-particles, depleted in CF_1 (as checked by their latent ATPase content (5) and PMS-mediated cyclic photophosphorylation activities (6)), were suspended in 0.2 M sucrose, 3 mM KCl, 3 mM $MgCl_2$ and 0.05 M Na+/tricine (pH 8) buffer at a concentration of 4 mg chlorophyll per ml. The particles were extracted with 2% cholate in the presence of 10% of saturation of ammonium sulfate and a clear supernatant obtained by ultracentrifugation as previously described (2).

Proteins were precipitated from the supernatant by adding the appropriate volume of ammonium sulfate solution (pH 8) to the cholate extract at 0-4°C. The mixture was then incubated for 20 min in ice, followed by centrifugation at 12,000 x g for 10 min. The precipitated fractions were redissolved in a medium of 0.2 M sucrose, 0.05 M Na+/tricine (pH 8) and 3 mM $MgCl_2$.

Reconstitution of the protein fractions with phospholipids and bacteriorhodopsin was done as previously described (7). Rebinding of CF_1 was carried out as specified in the legends to tables I and II and figure 2. Exchange between $^{32}P_i$ -ATP was measured at 37 °C in a final volume of 0.9 ml as previously described (2), except $^{32}P_i$ was added after the incubation with CF_1 as indicated in the legend to table I.

ATP formation in vesicles containing bacteriorhodopsin was assayed as described by Racker and Stoeckenius (7).

Electrophoresis of proteins in gels of polyacrylamide with sodium dodecyl sulfate was performed as described by Weber and Osborn (8) with 10% acrylamide.

Proteins were determined by the method of Lowry et al. (9), and chlorophyll was determined spectrophotometrically in 80% acetone extracts according to Arnon (10).

RESULTS AND DISCUSSION

Solubilization and isolation of the hydrophobic part of chloroplast ATPase complex (F_o): Chloroplasts depleted in CF_1 (EDTA-particles) were extracted with 2% cholate and 0.4M $(NH_4)_2SO_4$ in a buffered solution as described under "Materials and Methods."

TABLE I

Protein Fractions Precipitated from the Cholate Extract of EDTA-Particles and the $^{32}P_i$-ATP Exchange when Reconstituted

$(NH_4)_2SO_4$ Saturation	% Protein	$^{32}P_i$-ATP Exchange (nmol ATP/mg prot.-10 min) ug CF_1 Added 0	60	80	100
10-50p	100	2	7	9	12
35-45p	36	12.4	23	27	46

Exchange of $^{32}P_i$ for the γ-phosphate of ATP was measured in proteoliposome vesicles reconstituted from soybean phospholipids and the hydrophobic protein fractions. The hydrophobic protein (7 mg/ml) was mixed with sonicated asolectin (40 mg/ml) in the presence of 7 mg cholate/ml and incubated in ice for 10 min. Twenty microliters of this mixture was diluted with 0.8 ml of a reaction mixture containing 9 mM Mg-ATP, 80 mM Na+/tricine (pH 8) and 0.5% defatted bovine serum albumin. To this, amounts of CF_1 (1 to 1.5 mg/ml) were added and incubated at room temperature for 10 min. The exchange reaction was started by the addition of 0.1 ml of 0.2 M $^{32}P_i$ (specific radioactivity 150 mCi/mol) and incubated at 37°C for 10 min. The reaction was terminated with 5% trichloroacetic acid and the organic ^{32}P was assayed as described in "Materials and Methods".

The bulk of the protein in this extract was precipitated by bringing it to 50% $(NH_4)_2SO_4$ saturation. As shown in table I, phospholipid vesicles formed with this crude protein had a very low rate of P_i-ATP exchange. However, when these vesicles were supplemented with CF_1, the rate was stimulated. Greater specific activity and stimulation by CF_1 was obtained with a fraction precipitated by ammonium sulfate at 35 to 45 percent of saturation. The polypeptide composition of the 35-45p fraction was determined by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate as shown in Fig. 1. When compared with the 10-50p fraction of both CF_1-depleted and intact

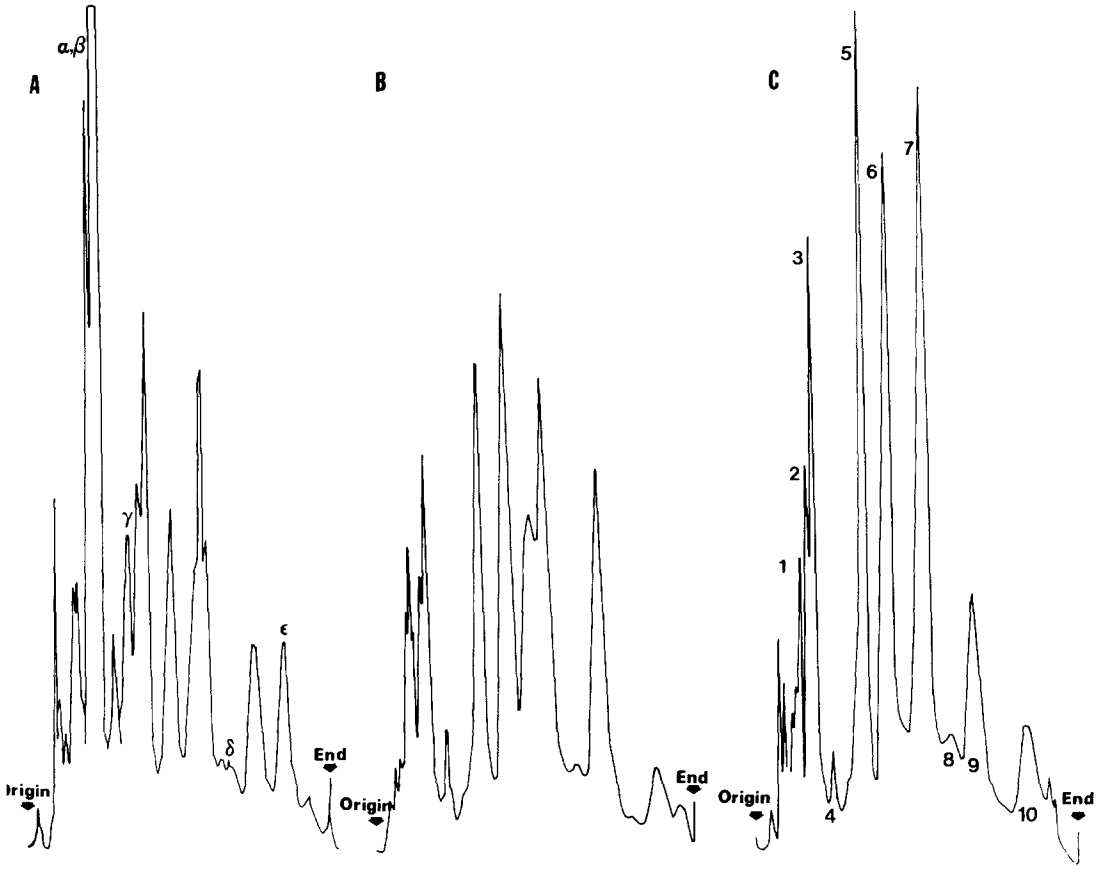

Figure 1: Densitometric tracing of the proteins separated by polyacrylamide gel electrophoresis in the presence of SDS. Gels were loaded with 50 ug of: a) 10-50p fraction of intact chloroplasts b) 10-50p fraction of EDTA-particles c) 35-45p fraction of EDTA-particles (F_o).

chloroplasts, some differences in polypeptide composition are evident. CF_1-polypeptides are greatly reduced in amount in the fractions from EDTA-particles. However, six well defined bands (5 to 10 of Fig. 1C) can be distinguished in the 35-45p fraction. Three of the major bands have apparent molecular weights of 42,000, 32,000 and 24,000 Daltons and the other three are minor bands of 18,000, 15,000 and 11,000 Daltons molecular weight. The minor amounts of high molecular weight polypeptides may be

TABLE II

Reconstitution of F_o-CF_1 in a Light-Dependent Proton Pump Catalyzing ATP Formation

Treatment of Complete System	nmol G-6-P/hr	
	per mg F_o	per mg bacteriorhodopsin
None	71	355
$-CF_1$	32	161
+FCCP (5×10^{-5} M)	20	100
+S-13 (5×10^{-5} M)	18	90
+DCCD (5×10^{-5} M)	19	95
Dark Control	4	20

Sonicated asolectin vesicles (40 mg/ml) were mixed with F_o (7 mg/ml) 1:1 by volume and incubated in ice for 10 min. Bacteriorhodopsin was added at a concentration of one part to 5 parts of the hydrophobic protein and the mixture was sonicated for 6 min. Fifty microliters of this mixture were incubated at room temperature with reagents added in the following order: 70 ug of CF_1 (1 mg/ml), 0.5 ml of an assay medium containing 2 mM ATP, 0.1 M Na+/tricine (pH 8), 0.1 M sucrose, 4 mM $MgCl_2$, 0.1 mM EDTA, 20 mM $^{32}P_i$ and defatted bovine serum albumin (1 mg/ml). The indicated inhibitors were added dissolved in 5 ul ethanol. Fifty microliters of a solution of yeast hexokinase (430 units/ml), 0.2 M glucose, 0.02 M Na+/tricine (pH 8) and 0.7 mM Na+/citrate were then added and the volume adjusted to 0.9 ml by the addition of distilled water. The reaction mixture was illuminated with yellow light (>500 nm) at an intensity of 8×10^5 ergs/cm^2-sec for 30 min at 20 °C. The reaction was terminated with 5% trichloroacetic acid.

contaminants from electron transport chain components. However, polypeptides 2 and 3 probably still contain some α and β subunits of CF_1. Similar preparations have been obtained from beef heart mitochondria (CF_o) by ammonium sulfate fractionation of similarly depleted particles (TU-particles) in the presence of 2% cholate (11), and from a thermophilic bacterium (TF_o) by extraction of the

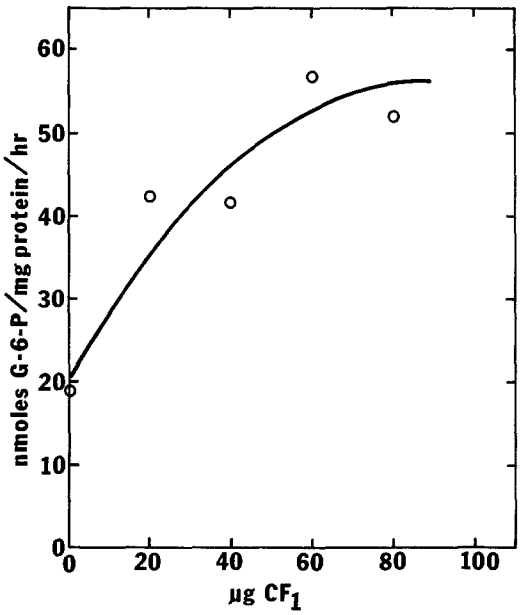

Figure 2: Dependence of ATP formation on added CF_1, in F_o /bacteriorhodopsin proteoliposomes. Reconstitution of vesicles and reaction conditions were as described in the legend to table II.

washed membranes with 2% Triton X-100 and 0.2 M Na_2SO_4 (12). Furthermore, the F_o polypeptides numbered 5 through 10 in Fig. 1 have counterparts of the same molecular weight in the preparations from bovine heart (13, 14, 15) and a thermophilic bacterium (12). The ATPase complex from chloroplasts is similar in other respects to the comparable complex from mitochondria and bacteria (6). A DCCD-binding proteolipid has been found in each of the ATPase complexes. The F_o fraction contains this proteolipid as shown by the binding of 0.24 nmole DCCD per mg after a 2 hour incubation (see ref. 2 for details of the procedure).

ATP formation catalyzed by reconstitution of F_o-CF_1 in a light dependent proton pump: Incorporation of proton-pumping bacteriorhodopsin protein together with CF_1 in F_o liposomes

yielded vesicles which catalyzed a light-dependent, uncoupler- and DCCD-sensitive ATP formation (Table II). Titration of F_o - bacteriorhodopsin liposomes with CF_1 showed that ATP formation was proportional to the amount of CF_1 added to the vesicles (Figure 2). Similar experiments have been done by others using bovine heart oligomycin-sensitive ATPase (7), DCCD-sensitive ATPase from a thermophilic bacterium (17) and DCCD-sensitive ATPase from chloroplasts (2).

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