A NEW COUPLING FACTOR FOR PHOTOPHOSPHORYLATION*

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Several coupling factors have been isolated from mitochondria (Racker, 1967) which are required for oxidative phosphorylation in resolved submitochondrial particles. Phosphorylation in chloroplasts, like that in mitochondria, can be resolved and reconstituted (Avron, 1963; Vambutas and Racker, 1965), however, only one coupling factor, CF_1[1] which in several respects is similar to mitochondrial F_1 (McCarty and Racker, 1966) has so far been isolated.

The purpose of this communication is to report the discovery of a second coupling factor in a protein fraction obtained by alkaline extraction of spinach chloroplasts. This factor (CF_2) restored photophosphorylation in deficient chloroplast particles in the presence of added CF_1 and also interacted with CF_1 in solution.

Methods

Spinach chloroplasts and EDTA-treated chloroplasts were prepared as described previously (McCarty and Racker, 1967). Subchloroplast particles were prepared from freshly made chloroplasts by exposure of the chloroplasts to sonic oscillation according to McCarty (1968). Trypsin-treated subchloroplast particles were prepared before each assay of photophosphorylation as

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[1]Abbreviations: CF_1, CF_2 - chloroplast coupling factor 1 and 2, respectively; F_1, F_4 - mitochondrial coupling factor 1 and 4, respectively; SCP - subchloroplast particles; T-SCP - trypsin-treated subchloroplast particles; Pi - inorganic phosphate; DTT - dithiothreitol; DTT-ATPase - ATPase of CF_1 activated by DTT.

follows: SCP (600 μg chlorophyll) suspended in 0.1 ml of 0.4 M sucrose, 20 mM Tricine-NaOH, pH 8.0, and 10 mM NaCl were incubated in 25 mM Tricine-NaOH pH 8.0 with 180 μg trypsin (Worthington) in a total volume of 1.0 ml at 20° for 2 min. Trypsin digestion was terminated with 1 mg soybean trypsin inhibitor and the reaction mixture was transferred to ice. After addition of 10 mg of defatted bovine serum albumin, 1/10 of the mixture was assayed for photophosphorylation. "Control particles" were similarly prepared, except that trypsin inhibitor was added prior to trypsin. It should be emphasized that the amount of trypsin required to give optimal results had to be titrated with each batch of chloroplasts. Too little trypsin gave incomplete resolution, too much gave low rates of phosphorylation.

CF_1 was prepared as described (Vambutas and Racker, 1965), except that a step of purification on a DEAE- Sephadex column was included (Bennun and Racker, in preparation) and the protamine step was deleted. CF_2 was prepared from either fresh or frozen (-65°C) spinach chloroplasts as follows: at ice temperature, 6 ml of 3 M KCl, 0.5 ml of 0.2 M EDTA pH 8.0 and 20 ml of 1.8 M NH_4OH were added with constant stirring to 50 ml of chloroplasts (3 mg chlorophyll per ml) suspended in 0.4 M sucrose, 20 mM Tricine-NaOH pH 8.0 and 10 mM NaCl. The mixture (pH 11.4) was centrifuged for 60 min at 104,000 xg and the pH of the supernatant was carefully brought to 8.0 with 2 N acetic acid. The precipitate was removed by centrifugation as before. The extract was then either dialyzed against 10 liters of 10 mM Tricine-NaOH, pH 7.1 containing 1 mM EDTA for 2 hours, or passed through BioGel P-100 column equilibrated with 50 mM Tricine-NaOH buffer pH 7.1 containing 20 mM NaCl and 0.5 mM DTT. The crude extract consisted of about 15% of the protein in the starting particles.

Chlorophyll was determined spectrophotometrically (Arnon 1949). Protein was assayed by a colorimetric procedure (Sutherland, et. al., 1949).

Results

It was shown previously (McCarty and Racker, 1968) that activation of latent ATPase activity of CF_1 can be achieved with DTT without destruction of coupling factor activity. It was also shown that when this preparation was allowed to interact with chloroplasts that were extracted with dilute EDTA (Jagendorf and Smith, 1962), a masking of ATPase activity took place. It can be seen from Table I that exposure of EDTA-extracted chloroplasts to trypsin eliminated the ability of the chloroplasts to inhibit the ATPase activity of DTT-CF_1. The factor responsible for this inhibition was isolated from chloroplasts by a procedure used for the extraction of F_4 from mitochondria

TABLE I

INHIBITION OF DTT-ACTIVATED ATPase BY EDTA-TREATED CHLOROPLASTS AND BY CF_2

The reaction mixture for the preparation of DTT-ATPase contained in a final volume of 1.0 ml: 650 µg of CF_1, 5 µmoles of ATP, 1 µmole of EDTA, 50 µmoles of Tris-HCl pH 8.0 and 50 µmoles of DTT. The mixture was incubated at 20° for 3 hours. For purpose of assay, the ingredients listed in the table were added to 10 µg of DTT-ATPase, followed by 2.5 µmoles of $CaCl_2$ and 25 µmoles of Tris-HCl pH 8.0 in a total volume of 0.5 ml. After 15 min at 20°, 0.5 ml of a solution which contained 25 µmoles Tris-HCl pH 8.0, 2.5 µmoles of $CaCl_2$ and 5 µmoles of ATP was added and the tubes were incubated for 6 min at 37°. The reaction was terminated with 1.0 ml of ice-cold 0.5 N trichloracetic acid and Pi was determined as described (Taussky and Shorr, 1953). Trypsin-treatment of EDTA-chloroplasts was carried out at 25° for 60 min (200 µg trypsin per 1 mg of particle protein). For trypsin digestion of CF_2, 350 µg trypsin per 1 mg of CF_2 was used at 20° for 15 min. In both cases digestion was terminated by addition of trypsin inhibitor in 3 fold excess. Trypsin-treated EDTA chloroplasts were centrifuged at 105,000 xg for 30 min and the pellet suspended in the original medium to which 50 µg of trypsin inhibitor per ml was added. These particles had no ATPase activity.

Addition to DTT-activated ATPase	Ca^{++}-ATPase activity	
	mµmoles Pi formed per min	% inhibition
Experiment 1		
None	89	
EDTA chloroplasts (300 µg protein)	46	49
Trypsin-treated EDTA chloroplasts	82	8
Experiment 2		
None	85	
CF_2 (120 µg protein)	40	53
Trypsin-treated CF_2	85	0

(Conover et. al., 1963). As shown in Experiment 2 of Table I this fraction (CF_2) inhibited the activity of DTT-activated ATPase. Exposure of CF_2 to trypsin eliminated all masking activity of the protein.

It became apparent from these studies that there is a trypsin-sensitive component in the chloroplast membrane which appears to be responsible for inhibition of the DTT-activated hydrolysis of ATP by CF_1.

In order to explore the role of this factor in photophosphorylation,

TABLE II

STIMULATION OF PHOTOPHOSPHORYLATION IN TRYPSIN - TREATED SUBCHLOROPLAST PARTICLES BY CF_1 AND CF_2

Trypsin-treated subchloroplast particles (T-SCP), prepared as described in Methods, were incubated in an ice bath with the following substances added in the order given: CF_2 and CF_1 as indicated in the table, 5 μmoles of $MgCl_2$ and water to 0.5 ml. After 10 min, 0.5 ml of a mixture containing 40 μmoles of Tricine-NaOH, pH 8.0, 50 μmoles of NaCl, 1 μmole of potassium ferricyanide (experiment 1) or 0.05 μmole of pyocyanine (experiment 2), 2 μmoles of ADP and 2 μmoles of potassium phosphate, pH 8.0, containing 5×10^5 cpm/μmole ^{32}Pi was added. The tubes were flushed with N_2, tightly stoppered and illuminated 2 minutes with white light (2.4×10^6 ergs/cm^2/sec). Ferricyanide reduction was assayed by a direct spectrophotometric method (Jagendorf and Smith, 1962) and ^{32}Pi esterification as described previously (McCarty and Racker, 1967).

Conditions	$Fe(CN)_6^{-3}$ reduced	Pi esterified
	μmoles/mg chlorophyll/hr	
Experiment 1 (Ferricyanide)		
Control particles	585	95.0
T-SCP	315	5.8
+ 30 μg CF_1	310	6.3
+ 50 μg CF_1	315	6.0
+ 80 μg CF_2	330	6.3
+ 30 μg CF_1 + 80 μg CF_2	325	14.3
+ 50 μg CF_1 + 80 μg CF_2	325	14.1
Experiment 2 (Pyocyanine)		
Control particles		254.0
T-SCP		21.4
+ 30 μg CF_1		20.8
+ 50 μg CF_1		19.2
+ 80 μg CF_2		21.2
+ 30 μg CF_1 + 80 μg CF_2		70.5
+ 50 μg CF_1 + 80 μg CF_2		66.5

subchloroplast particles were exposed to trypsin. As shown in Table II these particles catalyzed low rates of photophosphorylation with either ferricyanide as electron acceptor or in the presence of pyocyanine. On addition of both CF_1 and CF_2, there was a partial restoration of photophosphorylation. CF_2 had

little effect on the rate of ferricyanide reduction. Rates up to 70 μmoles Pi esterified per mg chlorophyll per hour (about 25% of the rates in control particles) were observed in the presence of pyocyanine (Experiment 2). With some batches of T-SCP stimulation by CF_2 was seen even without addition of CF_1.

No ATPase activity could be detected in CF_2 either before or after trypsin digestion. CF_2 was not dialyzable and was excluded by BioGel P-100 column. Coupling activity was lost after exposure of CF_2 to 70° for 2 min or to trypsin. It is concluded that CF_2 is a protein and preliminary work on its purification is in line with this conclusion.

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