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QUANTITATIVE DETERMINATION OF COUPLING FACTOR CF_1 OF CHLOROPLASTS

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SUMMARY

Repeated washes of isolated chloroplasts with dilute sodium pyrophosphate solution results in the removal of carboxydismutase and some minor proteins from the thylakoid membranes. A subsequent treatment of the membranes with hypertonic sucrose solution yields pure coupling factor CF_1 in the supernatant. Purity of the protein was demonstrated by disc electrophoresis.

The amount of CF_1 protein liberated was quantitatively determined. The percentage of CF_1 removed by this treatment was calculated from the Ca^{2+}-dependent ATPase activity retained at the thylakoid membranes. From these data the total CF_1 content of chloroplasts was calculated. An average value of 0.42 mg CF_1 protein/mg chlorophyll was obtained. Based on a molecular weight for CF_1 of 326000 (see Farron, F. (1970) *Biochemistry* 9, 3823–3828), a ratio of 1 mole CF_1 per 860 moles chlorophyll was computed.

INTRODUCTION

The concentrations of various components of the electron transport chain of chloroplasts have been determined in several laboratories. With the exception of plastoquinone, most of the known electron carriers, including P700[1,2], cytochrome f[3,4], plastocyanin[5,6], and ferredoxin[7], seem to occur at comparable concentrations of one molecule per 400 to 500 chlorophyll molecules. From several points of view it would also be interesting to know the molar concentrations of enzymes participating in the mechanism of energy conversion.

In 1961 Jagendorf and Smith[8] observed that treatment of isolated spinach chloroplasts with EDTA uncoupled photophosphorylation. These experiments led to the discovery of a coupling factor CF_1 by Avron[9,10]. A protein released from the thylakoid surface by EDTA was able to overcome uncoupling of EDTA-treated membranes when recombined in the presence of excess metal ions. CF_1 was extensively studied by Racker and his collaborators[11–14] and by several other workers[15–19]. The protein possesses a latent Ca^{2+}-dependent ATPase activity which can be unmasked by trypsin or heat treatment[11]. CF_1 was shown to catalyze light-triggered ATP hydrolysis of chloroplasts[13]. Moreover, it is most probably involved in photophosphorylation as a catalyst for the reversal of ATPase reaction[12].

Methods of purification of spinach coupling factor have been described by several authors[11,14,16–18,20]. All these procedures employ the usual purification steps, *e.g.* ammonium sulfate fractionation and chromatography. The preparation of pure CF_1 described in the present paper avoids these steps. After a few washes of chloroplasts with dilute salt medium, pure coupling factor is obtained by extraction of the membranes with sucrose solution. This method can be applied to the quantitative determination of CF_1 protein.

The results indicate about a 2-fold lower concentration of CF_1 compared to most of the electron carriers. This is in contradiction to earlier calculations based on evaluations of electron micrographs of negatively stained thylakoid surfaces[21].

EXPERIMENTAL

Spinach plants (var. "Vital") were grown in a greenhouse or in a room with the temperature controlled between 10 and 15 °C and a light (8000 lux) to dark period of 12–12 h. Chloroplasts were prepared from freshly harvested washed and chilled leaves by homogenization in a blendor, with a medium containing 300 mM sucrose and 10 mM sodium pyrophosphate, pH 7.4 (ref. 22). After squeezing through several layers of muslin and cotton, the chloroplasts were precipitated by centrifugation at $2000 \times g$. Usually the chloroplasts were washed once with isolation medium.

Protein fractions were qualitatively analyzed by disc electrophoresis on acrylamide gels using the method of Davis[23]. The gels were stained with amido black and scanned with an Eppendorf photometer and suitable equipment.

Quantitative determination of protein in chloroplast extracts was performed using the biuret method according to Beisenherz *et al.*[24]. Usually, 5 ml extract were precipitated by 5 ml 50% trichloroacetic acid and the precipitate was redissolved in 2.5 ml biuret reagent. This method of protein determination was found to be the most reliable and reproducible of the commonly used protein assays, although it is limited in sensitivity.

Ca^{2+}-dependent ATPase activity of supernatant fractions was measured following activation of the enzyme by trypsin[11]. 1 ml of CF_1-containing solution was incubated at room temperature with 1 ml trypsin solution containing 50 μg bovine pancreas trypsin (Merck), 50 μmoles Tricine buffer (pH 8.0), 0.5 μmole EDTA, and 0.1 μmole ATP. After 5 min digestion, the reaction was interrupted by the addition of 0.5 ml trypsin inhibitor solution containing 300 μg soy bean trypsin inhibitor (Merck) in distilled water.

Activation of thylakoid membrane fractions for the Ca^{2+}-dependent ATPase reaction was performed as follows: 0.5 ml chloroplasts was incubated with 0.5 ml trypsin solution which contained 0.25 mg trypsin, 25 μmoles Tricine buffer, 2.5 μmoles EDTA, and 0.5 μmole ATP. Digestion was stopped by addition of 0.5 ml trypsin inhibitor containing 0.75 mg trypsin inhibitor.

ATPase reaction was performed at 36 °C in a water bath[11]. 0.5 ml of the activated enzyme was added to 0.5 ml incubation medium containing 40 μmoles Tricine buffer (pH 8.0), 5 μmoles ATP, and 5 μmoles $CaCl_2$. After 6 min the reaction was stopped by addition of 0.2 ml 3 M $HClO_4$ and centrifuged. Inorganic phosphate contents of the supernatants were measured by photometry of the reduced phosphomolybdate complex[25].

Ribulose-1,5-diphosphate carboxylase (carboxydismutase) activity was measured by following the production of 3-phosphoglycerate from ribulose 1,5-diphosphate and bicarbonate. The incubation medium contained in 0.5 ml: 20 μmoles Tricine buffer (pH 8.0), 40 μmoles $NaHCO_3$ (pH adjusted to 8.0 with CO_2), 10 μmoles $MgCl_2$, 5 μmoles glutathione (GSH), 0.1 μmole EDTA, and 0.4 μmole ribulose 1,5-diphosphate (Sigma). 0.5 ml of this solution was incubated at 20 °C with 0.5 ml enzyme solution. The reaction was interrupted by the addition of 50 μl 1 M HCl and heating in a boiling water bath for 1 min. After cooling in an ice bath and centrifugation, 0.3 ml of the neutralized supernatant was used for enzymatic determination of 3-phosphoglycerate. The cuvette contained in 1 ml final volume: 50 μmoles Tricine buffer (pH 7.8), 2.5 μmoles GSH, 4 μmoles hydrazine, 7.5 μmoles ATP, and 0.2 μmole NADH. The reaction was started by adding 10 μl phosphoglycerate kinase and 20 μl glyceraldehyde-3-phosphate dehydrogenase (Boehringer). The decrease of NADH concentration was followed at 366 nm using an Eppendorf photometer.

RESULTS

The protein pattern of water extracts of spinach thylakoids was analyzed by Karu and Moudrianakis[17] using the technique of disc electrophoresis on acrylamide gels. The authors found that the two main bands contained carboxydismutase and coupling factor CF_1, respectively. We studied the conditions which control the release of these two proteins. As can be seen from Fig. 1, the removal of carboxydismutase was predominantly affected by the osmolarity of the extraction medium. High concentrations of sucrose (or salt) largely abolish extraction of carboxydismutase; on the

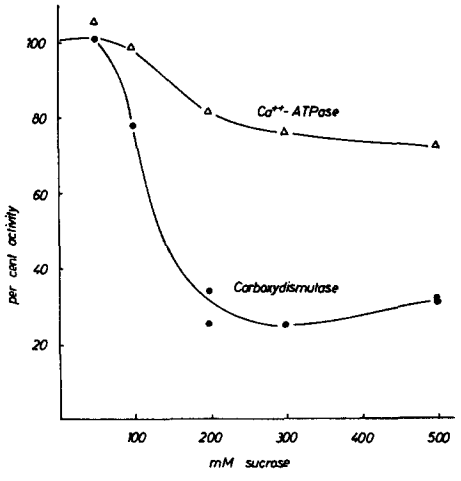

Fig. 1. Release of coupling factor and carboxydismutase from chloroplasts treated with sucrose solutions of different concentration. Washed chloroplasts were resuspended in sucrose media of the concentrations indicated (containing 2 mM Tricine buffer, pH 7.8). After centrifugation at 50000 × *g* for 20 min, the supernatants were adjusted to equal sucrose concentrations of 250 mM. Enzyme activities of the supernatants were measured as described under Experimental. Control rates (=enzyme activities of the extracts obtained with 0 mM sucrose, corresponding to 100%): 2.54 μmoles CO_2/mg chlorophyll per h, and 74.9 μmoles P_i/mg chlorophyll per h, respectively.

other hand, most of CF_1 protein (as measured by Ca^{2+}-dependent ATPase activity following trypsin treatment) was removed even by 0.5 M sucrose solution. However, low concentrations of salt prevented resolution of CF_1, even under hypotonic conditions (Fig. 2). About 0.5 mM salt of a divalent cation or 10-fold higher concentrations of a monovalent cation were sufficient to retain CF_1 completely. These results correspond to earlier observations[8,10]. The removal of carboxydismutase was not affected by the presence or absence of salt at concentrations which do not change the osmotic value of the extraction medium appreciably (Fig. 2).

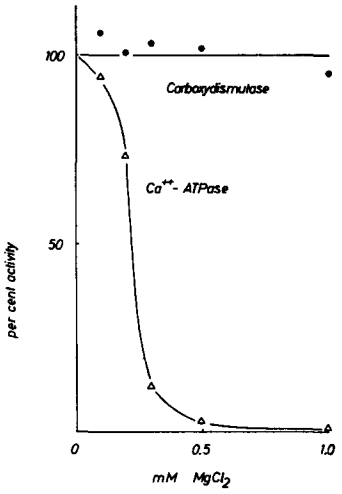

Fig. 2. Release of coupling factor and carboxydismutase in the presence of different Mg^{2+} concentrations. Washed chloroplasts were treated with 70 mM sucrose, containing 2 mM Tricine buffer, pH 7.8, and the $MgCl_2$ concentrations indicated. After centrifugation, the supernatants were adjusted to the same Mg^{2+} concentration of 1 mM. Control rates (=enzyme activities of the extracts obtained with 0 mM Mg^{2+}, corresponding to 100%): 3.09 μmoles CO_2/mg chlorophyll per h, and 38.6 μmoles P_i/mg chlorophyll per h, respectively.

From the results presented in Figs 1 and 2, the following predictions can be made. Repeated washes of the thylakoid membranes with dilute salt media will remove carboxydismutase, but retain coupling factor. A subsequent treatment with a medium of high sucrose concentration would liberate CF_1 but retain residual caboxydismutase. If the solubilization properties of the minor membrane proteins resemble those of carboxydismutase, pure CF_1 would be obtained in the supernatant during the latter extraction.

The prediction was fully confirmed by experimental results. High amounts of carboxydismutase protein and activity were released by the first washing with 10 mM sodium pyrophosphate solution (Fig. 3). As expected, no CF_1 band was detected in the protein pattern of the supernatant. After two subsequent washes with pyrophosphate solution, no further protein was released into supernatant. However, a subsequent treatment of the membranes with salt-free 0.3 M sucrose medium resulted in the removal of coupling factor, which was indentified by electrophoresis (Fig. 3) and by its Ca^{2+}-dependent ATPase activity. The recoupling activity of the protein is shown in Table I.

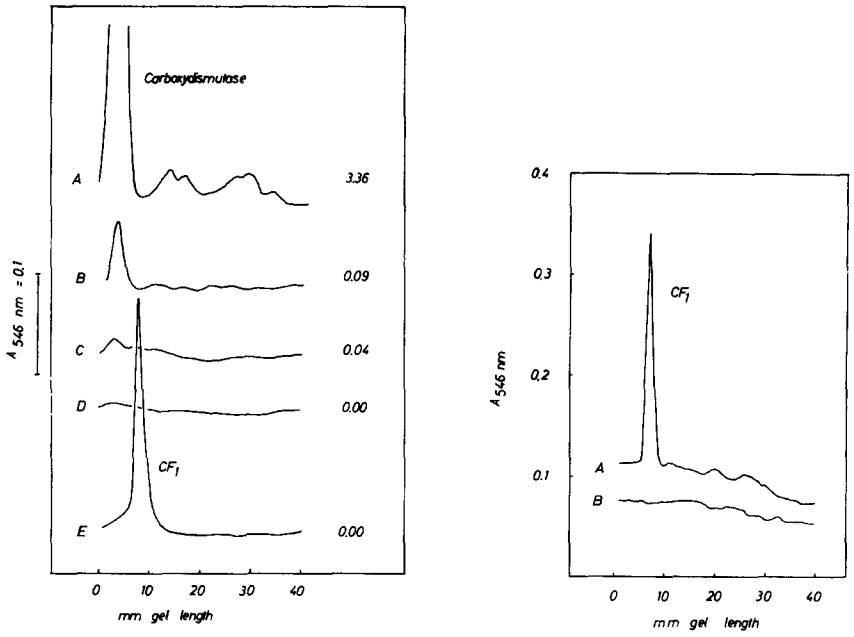

Fig. 3. Densitograms of protein extracts from chloroplasts. Washed chloroplasts were treated 4 times with 10 mM sodium pyrophosphate (pH adjusted to 7.4). Subsequently, they were resuspended in 300 mM sucrose (containing 2 mM Tricine buffer, pH 7.8). The supernatants were cleared by high-speed centrifugation and analyzed by electrophoresis. The densitograms were normalized to equal chlorophyll concentrations during the washes. The numbers on the right indicate the carboxydismutase activities of the corresponding supernatants. A–D, successive pyrophosphate washes; E, sucrose treatment.

Fig. 4. Densitograms of proteins extracted from chloroplasts with 300 mM sucrose (containing 2 mM Tricine buffer, pH 7.8) in the absence (A) and presence (B) of 1 mM $CaCl_2$. The chloroplasts were previously washed 4 times with 10 mM sodium pyrophosphate, pH 7.4.

In another experiment, after 4 pyrophosphate washes the suspension was divided into two portions. After centrifugation one pellet was resuspended in 0.3 M sucrose medium, the other one in a medium which additionally contained 1 mM $CaCl_2$. The supernatants were qualitatively analyzed by electrophoresis (Fig. 4). A large CF_1 band was detected in the salt-free supernatant corresponding to the results shown in Fig. 3. However, no protein bands occur in the Ca^{2+}-containing supernatant. The protein content of the supernatants was quantitatively determined. The control was found to contain less than 7% of the protein of the Ca^{2+}-free supernatant (Table II). Assuming 7% residual protein contamination present in the CF_1 containing supernatant, the actual amount of coupling factor protein released by salt-free washing was 0.203 mg/mg chlorophyll.

CF_1 is not completely removed by this treatment. The percentage of coupling factor liberated can be determined by the percentage of Ca^{2+}-ATPase retained in the membrane fraction. For this purpose the two membrane fractions were subjected to trypsin digestion and their ability to hydrolyze ATP was followed. Since CF_1 was completely retained in Ca^{2+}-treated chloroplasts (*cf.* Fig. 4), ATPase activity of this

TABLE I

RECOUPLING ACTIVITY OF CF_1 PREPARATION

Chloroplasts were isolated in 0.3 M sucrose, containing 10 mM Tricine buffer and 10 mM NaCl. After washing in isolation medium, the chloroplasts were resuspended in 70 mM sucrose + 2 mM Tricine, pH 7.8, +1 mM $MgCl_2$ (control chloroplasts) or in a medium which contained, instead of $MgCl_2$, either 0.2 mM or 1 mM EDTA. After centrifugation, the pellets were resuspended in isolation medium and adjusted to a chlorophyll content of 1.25 mg/ml. 0.2 ml of each suspension was then incubated for 30 min at room temperature with 0.05 ml 50 mM $MgCl_2$ and 0.2 ml CF_1 preparation in 0.3 M sucrose medium. Phenazine methosulfate-cyclic photophosphorylation was followed by incorporation of ^{32}P-labeled inorganic phosphate[31]. The assay medium contained 50 μmoles Tricine buffer, pH 8.0, 100 μmoles NaCl, 10 μmoles $MgCl_2$, 10 μmoles inorganic labeled phosphate, 2 μmoles ADP, 0.04 μmole phenazine methosulfate, and chloroplasts corresponding to 50 μg chlorophyll in a total volume of 2 ml.

	μmoles P_i incorporated/mg chlorophyll per h
Control chloroplasts	255.6
Treated with 0.2 mM EDTA	88.0
Recoupled with 53.5 μg CF_1/mg chlorophyll	106.4
Recoupled with 107.0 μg CF_1/mg chlorophyll	174.0
Treated with 1.0 mM EDTA	4.8
Recoupled with 53.5 μg CF_1/mg chlorophyll	8.6
Recoupled with 107.0 μg CF_1/mg chlorophyll	16.4

TABLE II

QUANTITATIVE DETERMINATION OF CF_1 PROTEIN OF CHLOROPLASTS

For experimental details see under Results.

	Chloroplast treatment	
	$-Ca^{2+}$	*$+Ca^{2+}$*
I. Protein content of the supernatants (mg protein/mg chlorophyll)	0.218	0.015
II. Ca^{2+}-ATPase activity of the membranes (μmoles P_i/mg chlorophyll per h)		
Final chlorophyll concentration during trypsin activation:		
0.250 mg/ml	36.5 (=52.4%)	69.6
0.100 mg/ml	72.8 (=50.1%)	145.2
0.050 mg/ml	111.9 (=50.1%)	223.1
III. Total CF_1 protein of the membranes, computed from I and II (mg protein/mg chlorophyll)	0.413	

membrane fraction corresponds to 100% coupling factor. As can be seen from Table II, about 50% of the control activity was lost by washing the membranes with salt-free medium.

From the data presented in Table II, an exact computation of total CF_1 protein of chloroplasts is possible. In 5 independent experiments values between 0.409 and 0.435 mg protein/mg chlorophyll were obtained.

DISCUSSION

Carboxydismutase and coupling factor CF_1 have been shown to be particulate structures of the thylakoid membrane surface, visible in the electron microscope upon negative staining and freeze-etching[26,27]. The two proteins are similar in structure; however, they differ in their size and molecular weight[26]. The removed particles can be separated by gradient centrifugation[26].

CF_1 and carboxydismutase are entirely different with respect to their binding properties. As demonstrated by Figs 1 and 2, carboxydismutase is retained at the membranes if the tonicity of the medium is high; on the other hand, CF_1 remains attached in the presence of rather low concentrations of salts, independent of the osmolarity of the medium. The latter fact is well known from earlier studies[8,10]. The CF_1-membrane bond may be mediated by divalent cations; capture of divalent cations by chelating agents results in removal of CF_1. However, in the presence of sufficient amounts of monovalent cations, the liberation of the protein is prevented. Because of the presence of monovalent counter-cations, even EDTA fails to remove coupling factor when applied at higher concentrations[8,12].

In our experiments we used pyrophosphate as a divalent cation complexing agent. 10 mM pyrophosphate solution contains 40 mM sodium counter-ion which is sufficient to abolish the effect of pyrophosphate on CF_1 liberation. Only dilution of Na^+ by a subsequent treatment with salt-free sucrose medium results in the removal of CF_1 protein.

This protein fraction consists of pure coupling factor when carboxydismutase and the minor membrane proteins were previously removed by 4 pyrophosphate washes. Thus a quantitative determination of CF_1 protein was easily obtained by measurement of protein content of the extract.

The molar concentration of coupling factor related to chlorophyll can be calculated from the molecular weight of CF_1. The values determined by several authors vary between 250000 (ref. 16) and 350000 (ref. 26). Probably the most careful determination was performed by Farron[20], who obtained a molecular weight of 326000. Using this value, a molar ratio of 1 CF_1 per 830 to 890 chlorophylls is computed from our results.

Murakami[21] calculated the ratio CF_1 to chlorophyll from the number of CF_1 particles present per area of negatively stained thylakoid surfaces. Based on Wolken's[28] calculation of chlorophyll concentration per membrane area, Murakami[21] obtained about 1 particle per 100 chlorophyll molecules. This value is of considerable discrepancy with our results, based on biochemical determination.

Mainly two reasons may be responsible for this inconsistency. (i) There may be uncertainties in the morphological identification of the particles, in the calculation of chlorophyll concentration of the membrane areas, and in the determination of CF_1 molecular weight. However, the deviations are too large to be explained by such errors alone. (ii) CF_1 particles may not be randomly distributed over the whole membrane surface but be restricted to certain regions. One might speculate that grana membranes

are lacking CF_1 particles where they are in contact with each other, and occupied by CF_1 only where they are freely exposed to the stroma space. Presumably only the latter membrane areas are visible in negatively stained preparations. Thus a high concentration of CF_1 would be obtained when related to the chlorophyll content of the occupied area, but a much lower concentration when related to total chlorophyll.

This speculation is in accordance with the results of Arntzen *et al.*[29] and Hauska and Sane[30], who found CF_1 to be enriched in isolated stroma lamellae compared to grana. Since the stroma membranes are single vesicles, the relative size of the stroma exposed surface is larger, and thus the concentration of CF_1 would be higher in those lamellae compared to grana.

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