The dicyclohexylcarbodiimide-sensitive ATPase complex from spinach chloroplast has been isolated. On sodium dodecyl sulfate gels, seven different polypeptides were seen. Five of these polypeptides coincided with the CP subunits, a 7,500-dalton peptide was identified as the proteolipid which interacts with $[^{14}C]$dicyclohexylcarbodiimide, and there was a 15,000-dalton hydrophobic polypeptide with unknown function. In two-dimensional gels, two additional peptides were resolved, one 17,500 daltons (co-migrating in sodium dodecyl sulfate gels with subunit 5) and one 13,500 daltons (co-migrating with subunit 5).

Reconstitution was obtained by freezing and thawing the complex with a crude mixture of phospholipids. After reconstitution the complex catalyzed $^{32}$Pi-ATP exchange (rates of 200 to 400 nmoles $\times$ mg $^{-1}$ $\times$ min $^{-1}$) and ATP formation during acid-to-base transition. These reactions were inhibited by dicyclohexylcarbodiimide and uncouplers. Uncouplers at low concentrations stimulated and at high concentrations inhibited the Mg$^{2+}$-ATPase activity. ATP hydrolysis and $^{32}$Pi-ATP exchange were catalyzed by the complex in the presence of either Mg$^{2+}$ or Mn$^{2+}$ but not with Ca$^{2+}$ or Co$^{2+}$.

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Purification and Reconstitution of the N,N'-Dicyclohexylcarbodiimide-sensitive ATPase Complex from Spinach Chloroplasts*

Uri Pick* and Efraim Racker
From the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

(Received for publication, August 22, 1978)

The dicyclohexylcarbodiimide-sensitive ATPase from spinach chloroplast has been isolated. On sodium dodecyl sulfate gels, seven different polypeptides were seen. Five of these polypeptides coincided with the CP subunits, a 7,500-dalton peptide was identified as the proteolipid which interacts with $[^{14}C]$dicyclohexylcarbodiimide, and there was a 15,000-dalton hydrophobic polypeptide with unknown function. In two-dimensional gels, two additional peptides were resolved, one 17,500 daltons (co-migrating in sodium dodecyl sulfate gels with subunit 5) and one 13,500 daltons (co-migrating with subunit 5).

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DCCD$^{1}$-sensitive ATPase complexes isolated from a variety of sources including mitochondria from bovine heart (1) or yeast (2), thermophilic bacteria (3) and chloroplasts (4), catalyze a DCCD- and uncoupler-sensitive $^{32}$Pi-ATP exchange after reconstitution into artificial phospholipid vesicles. These ATPase complexes all contain a water-soluble portion (CF$_i$), typically composed of five different polypeptides, which catalyzes a DCCD-insensitive hydrolysis of ATP, and a hydrophobic portion (F$_0$) which serves as a proton channel and confers DCCD sensitivity. The DCCD-binding component has been identified as a proteolipid of 7,000 to 11,000 daltons (5, 6) which mediates DCCD-sensitive proton movements in liposomes (6, 7). In thermophilic bacteria, one or two additional proteins have been described as part of F$_0$ (8). In mitochondria, two proteins (32,000 and 23,000 daltons) were present in the complex in addition to the oligomycin sensitivity-conferring protein and F$_0$. Similar proteins were described in yeast and Neurospora (cf. Ref. 9).

The chloroplast ATPase is latent in freshly isolated chloroplasts. However, $^{32}$Pi-ATP exchange and Mg$^{2+}$-ATPase activities can be triggered by light in the presence of sulfhydryl reagents or in the dark by incubation with 50 mM dithiothreitol (10-12).

The chloroplast DCCD-sensitive ATPase (DSA) was first solubilized with 2% cholate by Carmeli and Racker (13) and successfully reconstituted by removing cholate, thereby forming vesicles which catalyzed $^{32}$Pi-ATP exchange. The complex was further purified by Winget et al. (4) and incorporated into liposomes. It was reported to contain 13 different polypeptides including five CF$_{1}$ subunits and the DCCD-binding proteolipid.

We describe in this paper the purification of a chloroplast DSA complex which reveals seven different polypeptides on sodium dodecyl sulfate gels. Two additional polypeptides can be resolved in two-dimensional gels. The complex catalyzes a $^{32}$P-ATP exchange and phosphorylation of ADP driven by an acid-to-base transition.

EXPERIMENTAL PROCEDURES*

RESULTS AND DISCUSSION

Solubilization of DSA by a Combination of Cholate and Octylglucoside and Further Purification—The ATPase complex has been previously solubilized with 2% sodium cholate (4, 13). However, a combination of 0.5% sodium cholate with 30 mM octylglucoside, a mild neutral detergent (23), was more effective, yielding a 2- to 3-fold more active preparation and a 50 to 100% higher yield of protein. At higher detergent concentrations the activity in the extract decreased (Fig. 1). Most of the solubilized DSA complex was precipitated at 37.5 to 45% saturation of ammonium sulfate. This fraction had the highest $^{32}$P-ATP exchange activity and about 60% of the total protein. Later fractions had ATPase activity, probably due to free CF$_{1}$, but showed little or no $^{32}$P-ATP exchange activity after reconstitution.

The 37.5 to 45% fraction (5 mg) was mixed with 1 mg of Triton X-100 and layered onto a sucrose gradient which contained either 0.2% Triton X-100 (Fig. 2A) or 0.4% cholate (2A to V)

* Portions of this paper (including Materials and Methods, Figs. 1 to 11, and Tables I to V) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20014. Request Document No. 78M-1466, cite author(s), and include a check or money order for $2.70 per set of photocopies.

† Supported by the "Chaim Weizmann" Fellowship from the Weizmann Institute of Science, Rehovot, Israel.

The abbreviations used are: DCCD, N,N'-dicyclohexylcarbodiimide; DSA, DCCD-sensitive ATPase complex from spinach chloroplast; Tricine, N-tris(hydroxymethyl)methylglycine; RDP carboxylase, ribulose diphosphate carboxylase; MES, 2-(N-morpholino)ethanesulfonic acid; BSA, bovine serum albumin; S-13, 3-tert-butyl-5,2'-dichloro-4'-nitroacilinidyl; SF-6847, 3,5-di-tert-butyl-4'-hydroxybenzylidenemalonitrile; NGS, Amersham tissue-solubilizing mixture; ACS, Amersham scintillation solution; CD1A, (1,2-cyclohexylenedinitrilo)tetracetic acid.
(Fig. 2B). In both gradients, $^{32}$P-$\text{ATP}$ exchange activity was resolved from two pigmented fractions (Fractions 3 and 5) and from the major contaminant, RDP carboxylase (Fractions 11 and 9 in the Triton X-100 and sodium cholate-containing sucrose gradients, respectively). In Triton X-100, the DSA complex sedimented less rapidly than RDP carboxylase, whereas in cholate it sedimanted more rapidly. The specific weight of the hydrophobic complex is apparently influenced by the partial specific volume of the associated detergent (24). This value is significantly different for Triton X-100 (0.908) and sodium cholate (0.75). The DSA obtained in the presence of sodium cholate was better resolved from RDP carboxylase and had a higher specific activity in exchange (200 to 400 nmoles $\times$ mg$^{-1}$ $\times$ min$^{-1}$) than the complex obtained in the presence of Triton X-100 (100 to 150 nmoles $\times$ mg$^{-1}$ $\times$ min$^{-1}$). The DSA (cholate) lost 50% of its original activity after 4 days at 2°C, the DSA (Triton X-100) after 24 h. Both preparations stored frozen at $-20^\circ$C were stable for at least several weeks. The only advantage to the DSA (Triton X-100) preparation was that it was more concentrated than the DSA (cholate). DSA (cholate) was free of Triton X-100 as determined with [H]Triton X-100 added before layering the complex onto the sodium cholate sucrose gradient (data not shown).

The presence of phospholipids during sucrose gradient centrifugation considerably increased the specific activity of the isolated complex (Table I). The presence of phospholipids did not effect, however, the distribution of either the $^{32}$P-$\text{ATP}$ exchange activity or protein obtained from the sucrose gradient. Its main function appears to be stabilization of the complex. Table I also shows that 10 min incubation with 50 mM dithiothreitol after reconstitution of the complex into liposomes significantly stimulated the $^{32}$P-$\text{ATP}$ exchange. These results suggest that during the separation on the sucrose gradient the DSA partly reverts to its latent form with low activity and that the inactivation in the absence of phospholipids is unrelated to this process.

Table II gives a summary of the specific activity and yield of fractions. A purification of 5- to 8-fold was obtained and the recovery was about 35% of the original activity.

**Subunit Composition of DSA—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of DSA revealed seven different polypeptides (Fig. 3A).** Precipitation of the 37.5 to 45% fraction with antibodies specific to CF$_1$ yielded a polypeptide composition very similar to that of purified DSA (Fig. 3B). Determination of the apparent molecular weight of the polypeptides in the complex by polyacrylamide gel analysis gave the following values: 59,000 (a), 55,000 (β), 37,500 (γ), 17,500 (δ and a peptide in the complex), 15,500, 13,000 (for ε and a peptide in the complex), and 7,500 for the proteolipid.

Nelson et al. (6) extracted an 8,000-dalton polypeptide from chloroplasts which meditated DCCD-sensitive proton movements when incorporated into liposomes. Fig. 4 shows that following incubation of the DSA complex with [*C]*DCCD the radioactivity appears at the site of the 7,500-dalton polypeptide.

Small angle x-ray-scattering measurements carried out in collaboration with H. Paradis gave an estimated molecular weight of the complex of 435,000 ± 5,000. The protein appears to be less symmetrical than CF$_1$, with an axial ratio of 1.25 to 1.35 and the shape of an oblate ellipsoid of revolution.

**Stabilization of the DSA Complex by Detergents—** The DSA complex is rapidly inactivated and aggregates at room temperature. Fig. 5 shows that addition of either sodium cholate or octylglucoside, within a rather narrow range of concentrations, completely prevents this inactivation (for at least 5 h). However, the optimal detergent concentration varies with different preparations, ionic strength, protein concentration, etc., between 0.1 to 0.7% for cholate and 7 to 15 mM for octylglucoside. The detergents stabilize the activity probably by binding to hydrophobic regions of the complex, thereby preventing intermolecular hydrophobic interactions and aggregation.

**Detachment of CF$_1$ from DSA Complex and Subsequent Reconstitution—** CF$_1$, can be detached from the chloroplast membrane at very low ionic strength with 1 mM EJTA (25). Unlike the membrane-bound CF$_1$, which is mainly a Mg$^{2+}$-ATPase, the released CF$_1$ is a Ca$^{2+}$-ATPase catalyzing low rates of ATP hydrolysis in the presence of Mg$^{2+}$ (26). This change in specificity can be exploited to follow detachment of CF$_1$ from the purified DSA complex. Table III shows that EDTA or CDTA in the presence of 25 mM dithiothreitol were effective in stimulating Ca$^{2+}$-ATPase and partly decreased the Mg$^{2+}$-ATPase activity. The treatment resulted in a loss of $^{32}$P-$\text{ATP}$ exchange activity, which was partly restored by addition of CF$_1$ (Table IV). Exposure of DSA to 30 mM octylglucoside or 2% cholate, was more effective than EDTA treatment in depressing Mg$^{2+}$-ATPase and stimulating Ca$^{2+}$-ATPase, but recovery of exchange activity after addition of CF$_1$ was low (Tables III and IV).

A third procedure for CF$_1$ detachment consisted of exposing reconstituted DSA vesicles (cf. below) to 0.5 mM sodium bromoacetate for 30 min at 4°C in the presence of 10 mM dithiothreitol. This resulted in over 90% loss of exchange activity and recovery of 25% of the original activity on addition of CF$_1$. Although complete recovery of exchange activity after detachment of CF$_1$ by any method thus far used has not been achieved, it is not difficult to obtain virtually completely CF$_1$-dependent particles and observe over 10-fold stimulation of exchange activity on addition of the coupling factor (Table III).

**Reconstitution of DSA into Phospholipid Vesicles by Freezing and Thawing—** Winget et al. (4) used the cholate dilution technique (27) to reconstitute the DSA complex into phospholipid vesicles. However, this technique was not suitable for reconstitution of the DSA complex purified by sucrose gradient centrifugation because this method requires a concentrated enzyme solution during the incubation with the cholate-phospholipid mixture. Furthermore, Triton X-100 interferes with the reconstitution of the DSA complex. On the other hand, the freezing and thawing method could be performed with dilute solutions and it was not affected by up to 0.05% Triton X-100. Fig. 6A shows that reconstitution of the DSA complex with unwashed soybean phospholipids did not require sonication following freezing and thawing (17). In fact, about 50% of activity was lost after 2 min of sonication. Reconstitution was less effective with acetone-washed phospholipids (15) and was somewhat improved by 15 to 30 s sonication (Fig. 6A). Addition of 0.05% cholate to acetone-washed phospholipids during reconstitution (Fig. 6B) was more effective than sonication. It is likely, therefore, that the unwashed soybean phospholipids contain an acetone-soluble, detergent-like component which aids reconstitution during freezing and thawing.

Effective reconstitution of the DSA complex was completely dependent on the addition of phospholipids (Fig. 7). The dependency was not complete when the complex was isolated in the presence of phospholipids.

**Separation of Reconstituted DSA Vesicles on a Ficoll Gradient**

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Gradient—Reconstituted DSA consists of a heterogeneous population of vesicles which can be separated on a Ficoll gradient into two main populations (Fig. 8): a heavy fraction containing most of the $^{32}$P-ATP exchange activity and of the protein, but relatively little phospholipid (phospholipid:protein, about 3:1); and a lighter fraction with lower activity but containing most of the phospholipids (phospholipid:protein, about 15:1). Thirty seconds of sonication of the reconstituted vesicles had no effect on this distribution. However, we observed some variability with different preparations in the activity and protein recovered in the lighter fraction. Sometimes this fraction contained as little as 10%, sometimes as much as 50% of the total activity.

Stimulation of ATPase Activity by Uncoupler and Inhibition by DCCD—The response of the dithiothreitol-activated Mg$^{2+}$-ATPase of chloroplasts to uncouplers was reported to be biphasic: stimulation at low concentrations and inhibition at high concentrations (11). The inhibition of ATPase activity by uncouplers has only been observed with the chloroplast ATPase and it was suggested that a minimal threshold $\Delta \mathrm{pH}$ has to exist to keep the enzyme in its active state (28). Fig. 9 demonstrates a similar response of the reconstituted DSA complex. Whereas the $^{32}$P-ATP exchange was inhibited at all concentrations of uncouplers, a 2-fold stimulation of the Mg$^{2+}$-ATPase activity was observed at 3 $\mu$M SF-6847, but higher concentrations of uncouplers strongly inhibited the same phenomenon with other uncouplers (S-13, FCCP, nigericin + KCl), indicating that the inhibition of the ATPase activity is not likely caused by an uncoupler-specific interaction with the protein. DCCD (50 $\mu$M), which completely inhibited the $^{32}$P-ATP exchange, only partly inhibited the Mg$^{2+}$-ATPase (data not shown). This result suggests that part of the ATPase is not associated with the DCCD-sensitive component so that the complex is not properly incorporated into liposomes. Alternatively, the presence of residual detergent may be responsible for the lack of sensitivity of the ATPase to DCCD as has been previously observed for the mitochondrial ATPase complex (1).

Divalent Metal Ion Specificity—The ATPase activity of purified CF$\alpha$, from chloroplasts reveals a biphasic dependence on the concentration of divalent metal ions, an increase followed by a decrease in activity (29, 30). A two-point attachment hypothesis was suggested to explain these results, namely that the true substrate (which is an ATP:Mg complex) attaches at two points in the active center and that both free Mg$^{2+}$ and free ATP compete, thus inhibiting ATP hydrolysis (30).

Fig. 10A shows similar results with reconstituted DSA. In the presence of 5 mM ATP, optimal ATPase was obtained with 2 mM Mg$^{2+}$, 3 mM Mn$^{2+}$, or 5 mM Ca$^{2+}$, whereas higher concentrations inhibited. Fig. 10B shows that the $^{32}$P-ATP exchange was also dependent on Mg$^{2+}$ or Mn$^{2+}$, except that the apparent $K_m$ values were higher. Ca$^{2+}$ was ineffective in inhibiting the $^{32}$P-ATP exchange, only partly inhibited the Mg$^{2+}$-ATPase (data not shown). This result suggests that part the ATPase activity is Mg$^{2+}$ or Mn$^{2+}$, except that the apparent $K_m$ values were higher. Ca$^{2+}$ was ineffective in inhibiting the $^{32}$P-ATP exchange, only partly inhibited the Mg$^{2+}$-ATPase (data not shown). This result suggests that part the ATPase activity is Mg$^{2+}$ or Mn$^{2+}$, except that the apparent $K_m$ values were higher. Ca$^{2+}$ was ineffective in inhibiting the $^{32}$P-ATP exchange, only partly inhibited the Mg$^{2+}$-ATPase (data not shown). This result suggests that part of the ATPase is not associated with the DCCD-sensitive component so that the complex is not properly incorporated into liposomes. Alternatively, the presence of residual detergent may be responsible for the lack of sensitivity of the ATPase to DCCD as has been previously observed for the mitochondrial ATPase complex (1).

**Supplementary Material**

**Preparation and Reconstitution of the DCCD-Sensitive ATPase Complex from Spinachi Chloroplasts**

**Methods and Materials**

- Preparation of chloroplast membranes was carried out according to Vian et al. (1963, J. Biol. Chem. 237, 3747-3752) and the following modifications: the grinding medium contained 300 mM sucrose, 0.1 M NaCl, and 0.2 mg transferrin (pH 7.5) and the membranes were washed once in the original volume of 0.13 M NaCl and 20 mM Na-ATP (pH 8.0) before termination of the reaction. The membranes were then centrifuged at 15,000 x g for 20 min at 4°C before addition of 100 mM NaCl and 20 mM Na-ATP (pH 8.0) before assay.

- Purification of the complex was achieved according to Vian et al. (1963) using a 0.3-ml Teflon mill with 300 mM sucrose, 0.1 M NaCl, and 0.2 mg transferrin (pH 7.5) and the membranes were washed once in the original volume of 0.13 M NaCl and 20 mM Na-ATP (pH 8.0). Although the complex purified from the washed membranes was less active than the complex prepared according to Vian et al. (1963), it was not measured or assayed for ATPase activity.

- The chloroplast membranes were incubated for 30 min at 4°C with 100 mM NaCl and 20 mM Na-ATP before addition of 200 mM NaCl and 20 mM Na-ATP (pH 8.0) before assay.

- Purification of the complex was carried out as follows: the crude membranes (100 mg) were solubilized in 1 mg of octyl glucoside, mM by guest on September 22, 2017 http://www.jbc.org/ Downloaded from
**DCCD-sensitive ATPase from Chloroplasts**

Fig. 1. Phospholipid requirement for reconstitution. SSA complex (7.5 μg in 0.2 ml) incubated by nucleotide plus centrifugation in the presence of Triton X-100 with or without 0.15 mg phospholipids was reconstituted by dialysis and titration with the amounts of indicated phospholipids and assayed for ATPase exchange.

Fig. 2. Separation of recombinant SSA pellets on a Ficoll gradient. Vesicles reconstituted with SSA complex containing [32P]nucleotide were incubated on a Ficoll gradient and assayed for ATPase exchange, protein, and phospholipid as described under "Materials and Methods.

Fig. 3. The effect of SF-6847 on the ATPase activity of reconstituted SSA vesicles. Reconstituted SSA vesicles were isolated for 10 min at 4°C with SF-6847 and/or GTP in an assay mixture without SSA. The SSA was added just before the assay which was carried out as described under "Materials and Methods.

Fig. 4. Metal requirements for Mg^{2+}-ATP exchange and for ATPase exchange. ATPase activities were measured for Mg^{2+}-ATP exchange and Mg^{2+}-Pi exchange and the corresponding Mg^{2+} plus metal ion activities at the specified concentrations. Ca^{2+}+Mg^{2+} was used for the ATPase assay.

Fig. 5. Nucleotide specificity for ATP, incorporated in the exchange reactions catalyzed by SSA vesicles. Mg^{2+}-nucleotide exchange was assayed at the indicated equilibrant concentrations of Mg^{2+} and nucleotide.
TABLE I

Effect of phospholipids during the sucrose gradient separation of DCCD-sensitive ATPase from Chloroplasts

<table>
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TABLE II

Multiplication of the DCCD complex from chloroplasts

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TABLE III

Phosphorylation driven by artificial pH gradients

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TABLE IV

DCCD-sensitive ATPase exchange in detaching and reattaching Mg

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(b) ATP formation by a pH gradient

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(3) Stimulators of ATP formation by ammonium and inhibition by uncoupler or uncoupler

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U Pick and E Racker


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