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Partial deglycosylation of chloroplast coupling factor 1 (CF_1) prevents the reconstitution of photophosphorylation

(glycosidase treatment/ATPase/glycoenzyme/ATP synthesis/chloroplasts)

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ABSTRACT **Treatment of spinach chloroplast coupling factor 1 (CF_1) with a mixture of glycosidases resulted in the removal of ≈75% of the carbohydrate associated with the enzyme. The ATPase of CF_1 was not activated by this treatment nor were its heat-activated or methanol-dependent ATPase activities inhibited. The deglycosylated enzyme, however, was unable to catalyze photophosphorylation when recoupled with CF_1-depleted thylakoids. The glycosidase-treated protein competed with native CF_1 for specific binding sites on the depleted membranes and was able to reconstitute proton uptake in uncoupled thylakoids. The inhibition of photophosphorylation could not be overcome with added δ subunit. We conclude that deglycosylated CF_1 was unaffected in its ability to bind to the membrane sector of the chloroplast proton-pumping ATPase (CF_0) but was altered in some property essential for photophosphorylation but not ATPase activity.**

Chloroplast coupling factor 1 (CF_1) is the extrinsic portion of the reversible ATP-synthetase (proton-pumping ATPase) complex of the thylakoid membranes. The solubilized protein is a latent ATPase and consists of five different subunits (1, 2).

The possible carbohydrate content of spinach CF_1 has been a matter of controversy. Andreu *et al.* (3, 4) first reported bound carbohydrate, consisting of a complex set of nine different sugars and amounting to 4.5% carbohydrate (wt/wt of protein). They also reported (3, 4) carbohydrate associated with a bacterial coupling factor and preliminary data on carbohydrate of beef heart mitochondrial F_1.

In conflict with these reports, it has been reported that no carbohydrate was present on CF_1 (5), on bacterial F_1 (6), or on F_1 from beef heart mitochondria (7). The possibility was raised therefore that the bound sugars reported by Andreu *et al.* had resulted from contaminating materials.

Tyler and Webb (8), however, showed that F_1 from liver mitochondria did contain orcinol-reactive material equivalent to 1–2% carbohydrate (8). Also, two independent groups found (9, 10) that the presence of concanavalin A significantly alters physical and enzymatic properties of F_1 from yeast mitochondria. Thus, it may be that the carbohydrate content of coupling factors differs greatly depending on the source of the protein.

Recently, we have reevaluated the carbohydrate content of spinach CF_1 (ref. 11; unpublished data) and found sugars to be present at <1% (wt/wt of protein). Also, the saccharide composition was somewhat different (11) than that reported by Andreu *et al.* (3). The present study was initiated to determine if enzymatic removal of bound carbohydrate would affect the catalytic activity of CF_1 or its relation to membrane components.

MATERIALS AND METHODS

Preparation of Protein. Spinach CF_1 was prepared by the method of Binder *et al.* (12). Before use, both CF_1 and glycosidases were dialyzed against 32 mM citric acid/136 mM Na_2HPO_4 (McIlvaine's buffer), pH 6.3/1 mM ATP/3 mM NaN_3/1 mM phenylmethylsulfonyl fluoride/0.5% (vol/vol) ethanol (carried over from phenylmethylsulfonyl fluoride)/40 mM 6-aminocaproic acid. Glycosidases were purchased from Sigma and were used without further purification.

Incubation of CF_1 with Glycosidases and Repurification of CF_1. Approximately 5 mg of pure CF_1 was incubated with 2.5 units of α-galactosidase (green coffee bean), 100 units of β-galactosidase (*Escherichia coli*), 30 units of α-glucosidase (brewers' yeast), 5 units of β-glucosidase (almond), and 2.5 units of β-*N*-actylglucosaminidase (jack bean). The control contained no glycosidases. To each sample was added $MgCl_2$, 2-mercaptoethanol, and *p*-aminobenzamidine(·2HCl) to final concentrations of 1 mM, 1% (vol/vol), and 6 mM, respectively. Incubations at 37°C for 72 hr caused extensive precipitation with poor yields of CF_1 after repurification. Incubation at 25°C for 4–5 days allowed significantly better recovery of protein and was the method used in this study.

After incubation with glycosidases, CF_1 was repurified by sucrose density gradient centrifugation as in ref. 12, except that the gradients contained 100 mM NaCl and 3 mM NaN_3 as well as 20 mM Tris·HCl (pH 7.8), and 2 mM EDTA. The entire content of each sample tube was applied to one 40-ml gradient tube and centrifuged (12) for 4 hr. Purity of CF_1 was monitored by electrophoresis of the gradient fractions in 12.5% polyacrylamide gels containing 0.1% $NaDodSO_4$ and the buffer system of Laemmli (13), followed by staining with Coomassie blue.

Preparation of NaBr Particles. Spinach thylakoids, virtually depleted of CF_1, were prepared by treatment with 2 M NaBr according to Nelson and Eytan (14). Chlorophyll (Chl) was measured by the method of Arnon (15). These particles possessed almost no methanol-dependent ATPase activity (16) and lacked visible bands corresponding to the α and β subunits of CF_1 when the membranes were electrophoresed in polyacrylamide gels containing $NaDodSO_4$.

Recoupling NaBr Particles and Assay of Photophosphorylation. NaBr particles equal to 25 μg of Chl were mixed with an equal volume of 10 mM Tricine/NaOH, pH 8.0/1 mM ATP/0.1 mM dithiothreitol containing pure CF_1. Finally, $MgCl_2$ was added to a final concentration of 1.5 mM and the samples (100 μl final volume) were incubated on ice for 10 min. Photophosphorylation was initiated by the addition of 0.9 ml of a complete reaction mixture and by illumination.

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Abbreviations: CF_1, chloroplast coupling factor 1; CF_0, membrane sector of the chloroplast proton-pumping ATPase; Chl, chlorophyll.

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The phosphorylation reaction mixture contained 50 mM Tricine/NaOH (pH 8.5), 50 mM sorbitol, 50 mM choline chloride, 5 mM $MgCl_2$, 2 mM ADP, 5 mM Na_2HPO_4, 50 μM phenazine methosulfate, and 0.5 mg of bovine serum albumin per ml. The mixture contained $^{32}PO_4$ at a specific activity of 10^4–10^6 cpm/μmol. Samples were illuminated for 2 min at 25°C, with white light at 800–1000 μEinsteins $m^{-2}\cdot sec^{-1}$. The reaction was terminated by adding an equal volume (1 ml) of 0.66 M HCl/72 mM triethylamine/24 mM ammonium molybdate/1% (vol/vol) saturated bromine water (17). After 10 min at room temperature the PO_4–molybdate–triethylamine complex was removed by centrifugation. An aliquot of the supernatant was mixed with an equal volume of a "color stop" solution (18) to prevent further complex formation and then was assayed for Cerenkov radiation. Controls contained all reaction components but were not illuminated.

Measurement of Proton Uptake by NaBr Particles. Alkalinization of the medium pH by illuminated NaBr particles was measured with a combination glass electrode. NaBr particles (50 μg of Chl per ml) were illuminated in a reaction mixture at 25°C containing 0.5 mM Tricine/NaOH (pH 6.4), 100 mM KCl, 5 mM $MgCl_2$, and 50 μM pyocyanin. The amount of proton uptake was calibrated by the titration of the reaction mixture with HCl. All light-induced pH changes were reversed when light was removed and inhibited by the inclusion of 10 μM gramicidin.

Assay of ATPase Activity. ATPase activity of purified CF_1 after heat-activation (19) or in the presence of methanol (20) was assayed by measurement (21) of released inorganic phosphate. ATPase associated with membranes was also assayed in the presence of methanol (16).

RESULTS

Extent of Deglycosylation and Properties of Treated CF_1. Treatment of CF_1 with glycosidases for 98 hr at 25°C resulted in the removal of ≈73% of the carbohydrate from the protein, including 100% of the bound galactose and 83% of the apparently associated glucose, as measured by acid hydrolysis and gas/liquid chromatography (11). Most of the previously detected (11) arabinose and glucosamine remained associated with the repurified glycosidase-treated CF_1.

No obvious changes in the physical properties of CF_1 were observed after deglycosylation. Both control and treated samples behaved similarly when dialyzed, desalted by gel filtration, or subjected to DEAE-cellulose chromatography. When repurified by ultracentrifugation, both treated and control CF_1 migrated to the same level in the sucrose density gradients. The subunit composition of the glycosidase-treated protein was also unaffected if protease inhibitors (phenylmethylsulfonyl fluoride, 6-aminocaproic acid, *p*-aminobenzamidine) were included in the incubation medium with glycosidases. The α and β subunits of the treated protein, though, migrated to a slightly higher apparent molecular weight value when electrophoresed in $NaDodSO_4$/polyacrylamide gels.

Treatment of CF_1 with glycosidases did not activate the ATPase of CF_1, in contrast to the effects of protease treatment (22, 23). Neither the V_{max} nor the K_m for ATP (≈1 mM) of the heat-activated ATPase was altered after treatment (data not shown). Similarly, no change in the methanol-dependent ATPase activity of the deglycosylated enzyme could be detected.

Reconstitution of Photophosphorylation in NaBr Particles. NaBr particles equal to 25 μg of Chl were recoupled with various amounts of CF_1, followed by assay of phenazine methosulfate-catalyzed photophosphorylation. The glycosidase-treated CF_1 was unable to reconstitute phosphorylation at any concentration of protein employed (Fig. 1). Similar results were obtained by using two other preparations of glycosidase-treated CF_1 in separate experiments. The NaBr particles were competent in binding CF_1, since control protein significantly recoupled phosphorylation when present in amounts in excess of that of Chl.

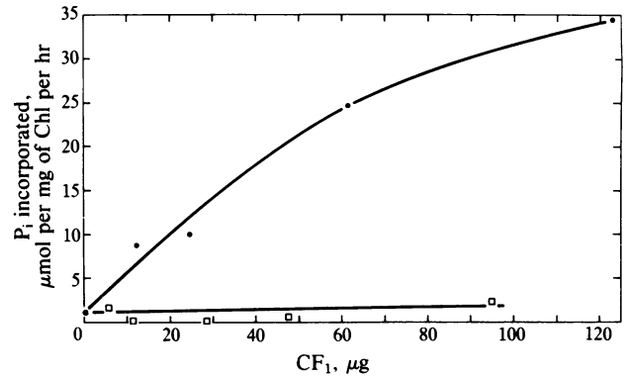

FIG. 1. Reconstitution of photophosphorylation by control (●) and deglycosylated (□) CF_1. NaBr particles equal to 25 μg of Chl in 200 mM sucrose/10 mM Tricine/NaOH, pH 8.0/1 mM ATP/2.5 mM dithiothreitol were incubated at 0°C with varying amounts of CF_1 as shown, in the presence of 1.5 mM $MgCl_2$ for 15 min. Photophosphorylation then was measured.

The failure to restore photophosphorylation might have been due to an inability of glycosidase-treated CF_1 to bind to stripped thylakoids. To examine this, after incubation of CF_1 with NaBr particles, the latter were centrifuged, then washed once more, and assayed for methanol-activated ATPase as an indicator for rebound CF_1. Both control and deglycosylated CF_1, by this criterion, had become attached to the NaBr particles (Table 1). Although deglycosylated CF_1 was bound at a 26% lower level than control CF_1, this difference could not have caused the complete lack of recoupling seen in Fig. 1.

The possibility that deglycosylated CF_1 bound nonspecifically to thylakoids at sites other than the membrane sector of the thylakoid ATPase (CF_0) was examined by incubating NaBr particles with a mixture of both treated and control CF_1. The presence of deglycosylated CF_1 prevented recoupling by native CF_1 until the concentration of untreated protein exceeded that of treated CF_1 (Fig. 2). These data suggested that both treated and control CF_1 competed relatively equally for the same CF_0 binding sites with the deglycosylated enzyme forming an inactive complex.

In previous work (24), CF_1 deficient in the δ subunit was shown to bind to NaBr particles but not catalyze photophosphorylation. When supplemented with purified δ subunit the δ-deficient CF_1 recovered the ability to recouple phosphorylation (24). In the present work, added δ subunit (prepared as in ref. 24) did not restore the ability of deglycosylated CF_1 to reconstitute phosphorylation by NaBr particles nor did the added subunit enhance the activity associated with control CF_1 (Table 2). The biological activity of the isolated δ subunit we used was demonstrated by its ability to stimulate

Table 1. Rebinding of deglycosylated CF_1 to NaBr particles

Sample	Methanol-dependent ATPase activity, μmol of P_i per mg of Chl per hr
Control thylakoids	296
NaBr particles	5
+ Control CF_1	65
+ Deglycosylated CF_1	48

NaBr particles (or thylakoids) equal to 25 μg of Chl were incubated in the presence or absence of 15 μg of control or deglycosylated CF_1 as described in the legend to Fig. 1. Data presented here are the average of duplicate samples.

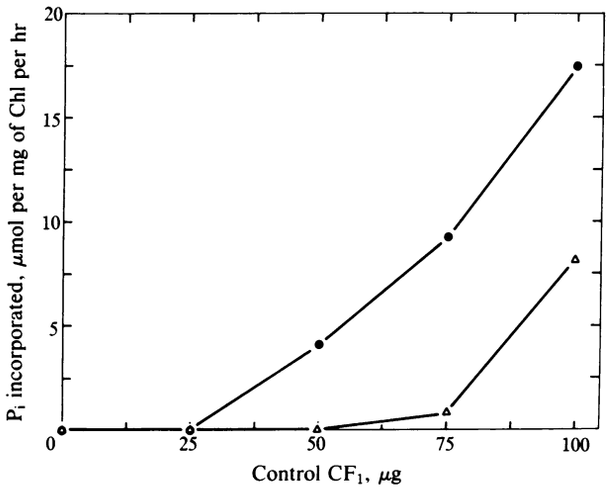

FIG. 2. Competition between control (●) and deglycosylated (△) CF_1 in reconstitution of photophosphorylation. NaBr particles (25 μg of Chl) were recoupled with varying amounts of control CF_1 in the presence or absence of 50 μg of glycosidase-treated CF_1, followed by measurement of photophosphorylation. Conditions for recoupling were the same as in Fig. 1.

recoupling by a δ-deficient (octylglucoside treated) CF_1 (24) (Table 2). The inhibition of photophosphorylation associated with deglycosylated CF_1 is therefore distinct from the effect associated with the δ-depleted enzyme.

Reconstitution of Proton Uptake in NaBr Particles. Previously (24), octylglucoside-treated CF_1, which could bind to CF_1-depleted membranes, could not reconstitute the ability of thylakoids to raise the pH of the medium when illuminated, unless δ subunit was added. In similar experiments, we have found that both control and deglycosylated CF_1, recoupled to NaBr particles, allowed approximately the same amount of light-dependent proton uptake (Table 3). Similar degrees of pyridine stimulation were observed in both treated and control samples. *N,N'*-Dicyclohexylcarbodiimide was also able to partially restore proton uptake (Table 3) due to its ability to block the proton channel through CF_0 (25). The finding that extensively deglycosylated CF_1 blocked the flow of protons through CF_0 as well as did control CF_1 indicated that the treated protein possessed sufficient δ subunit to bind appropriately to the correct functional site. Its failure to catalyze photophosphorylation must be ascribed to other causes.

DISCUSSION

Extensive incubation of CF_1 with a mixture of specific glycosidases removed ≈70% of its bound carbohydrate, as was found previously with other proteins (26, 27). Denaturation of the protein was usually necessary for more extensive deglycosylation to occur (28). Since the carbohydrate–protein bonds would not have been hydrolyzed by the enzymes employed, and some of the saccharides might have been inaccessible to attack by exoglycosidases, more extensive deglycosylation of CF_1 might not have been possible by using this procedure. Also, the lack of an active arabinosidase may have limited the degree of deglycosylation. The finding that all of the galactose moieties were removed by glycosidase treatment suggested that these sugars were located in the periphery of the bound oligosaccharide(s). Glucosamine was more resistant to glycosidic removal and is most likely to be located in a less accessible position, probably linked directly to the protein.

The ATPase activity of CF_1 was neither induced nor inhibited by glycosidase treatment, indicating that the carbohydrate removed was not involved in catalysis or suppression of catalytic activity in the native enzyme. Similar results have been obtained with other glycoenzymes. Partial deglycosylation of bovine deoxyribonuclease A and ribonuclease B produced no changes in the enzymatic activity or specificity of the enzymes (29). Enzymatic removal of 90% of the carbohydrate of yeast invertase (27) or 75% of the carbohydrate of pancreatic ribonuclease (26) similarly did not inhibit their activities. The carbohydrate did, though, impart stability to the glycosylated enzyme under mildly denaturing conditions (27) and, in another case, changed the tertiary structure of the protein in the region of a specific tyrosine residue (26), although the proximity of this amino acid to the glycosylation site(s) was not determined.

The inability of partially deglycosylated CF_1 to catalyze photophosphorylation (Fig. 1) was unexpected. The treated enzyme was able to bind to stripped thylakoid membranes as much as did the control enzyme (Table 1), restored proton impermeability to the membranes as much as did the control enzyme (Table 3), and competed with the control enzyme for effective restoration of ATP synthesis (Fig. 2). The lack of activity of the treated enzyme was not caused by loss of the δ subunit, since adding back this subunit did not stimulate photophosphorylation, whereas it did stimulate activity by another enzyme preparation lacking the δ subunit (Table 2). Although it cannot be ruled out completely, we feel that a proteolytic cut is probably not responsible for this effect of glycosidases because of the absence of any lowering of the subunit molecular weight values on $NaDodSO_4$ electrophoretic gels and because of the absence of an activating effect, typical of all proteases so far tested, on the latent ATPase of CF_1. The most likely explanation is that the loss of phosphorylation ability results directly from loss of the sugar residues.

Table 2. Effect of added δ subunit on recoupling of deglycosylated CF_1 to NaBr particles

CF_1 added	Rate of photophosphorylation, μmol of ATP formed per mg of Chl per hr	
	Control	δ subunit added
Control	21	16
Deglycosylated	1.0	1.7
Octylglucoside treated	1.5	9

CF_1 (50 μg) in 10 mM Tricine/NaOH, pH 8.0/1 mM ATP/0.1 mM dithiothreitol was incubated for 10 min at room temperature with 7 μg of purified δ subunit. The samples were added to NaBr particles (25 μg of Chl), recoupled, and assayed for photophosphorylation activity.

Table 3. Restoration of proton accumulation in NaBr particles by deglycosylated CF_1

Sample	Extent of H^+ accumulation, nEq/mg of Chl	
	Control	1 mM pyridine added
Control thylakoids	614	—
NaBr particles	26	36
+ Control CF_1	57	98
+ Deglycosylated CF_1	61	86
+ 125 μM DCCD	252	—

NaBr particles (80 μg of Chl) were recoupled with 100 μg of control or deglycosylated CF_1. The control thylakoids were taken from the twice-washed thylakoid suspension before NaBr treatment. *N,N'*-Dicyclohexylcarbodiimide (DCCD), in ethanol, was added to the NaBr particles 2 min before illumination in the specified sample. The data presented here are the average of three trials with the same recoupled sample.

These findings are unique among reports on the deglycosylation of glycoenzymes, where deglycosylation usually yields fully active molecules (26–29). In nonenzymatic glycoproteins, the inhibition of biological activities caused by deglycosylation is attributed to the reduced binding of the glycoconjugate to membrane or soluble protein receptors (30–33). The preliminary report that treatment of algal autolysine with α-glucosidase inhibits its cell wall hydrolytic activity (34) may represent another situation in which enzyme-bound carbohydrate influences catalytic activity, although in this case the nature of the inhibition was not well characterized. As of yet, the specific role of CF_1-bound carbohydrate in photophosphorylation is also unknown. An essential role for the sugars in catalysis is not expected since the ATPase activity of CF_1 was not inhibited by deglycosylation. Also, F_1 of beef heart mitochondria does not contain carbohydrate (7), indicating that the role of bound carbohydrate in phosphorylation may be specific for the thylakoid reaction.

The sugars associated with CF_1 may be involved in some type of interaction between CF_1 and membrane proteins or membrane glycolipids or may be needed for proper interactions between subunits. The function of human pituitary hormones is dependent on the presence of an intact oligosaccharide on the hormones (30). In a recent report, though, the binding of one hormone to the receptor was not prevented by deglycosylation, but its ability to stimulate adenylate cyclase activity was significantly diminished (32). In that case, it was proposed that the sugars were required for "coupling" of the receptor and adenylate cyclase, although the mechanism of coupling was not investigated. The carbohydrate of CF_1 may similarly possess a role in a hypothetical secondary interaction of CF_1 with the membrane, since the primary binding to CF_0 is not inhibited.

Alternatively, the carbohydrate associated with CF_1 may be needed for, or may stabilize, one or more (possibly transient) conformational states essential for ATP synthesis but not hydrolysis. The ATPase of CF_1 is activated by drastic methods (20–35% organic solvents, octylglucoside, partial proteolysis) that must cause major nonspecific changes in conformation (20–23, 35). Therefore, it would not be surprising for the ATPase to be resistant to modest conformational effects of deglycosylation. Photophosphorylation is known to be accompanied by one or more reversible conformational changes in CF_1 (36) and some of the intermediate configurations could be dependent on the presence of the oligosaccharides. Under these circumstances deglycosylation might be expected to inhibit photophosphorylation, but not ATP hydrolysis, by CF_1.

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