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ASSOCIATION OF A THIOREDOXIN-LIKE PROTEIN WITH CHLOROPLAST COUPLING FACTOR (CF_1)

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Summary: The δ subunit isolated from chloroplast coupling factor (CF_1) preparations partially replaced thioredoxin in the dithiothreitol-linked activation of chloroplast fructose 1,6-bisphosphatase. The δ subunit fraction also stimulated the dithiothreitol-dependent ATPase of heated CF_1 in a manner analogous to that observed with each of the three thioredoxins isolated from spinach leaves (thioredoxins *f*, *m*, and *c*). The δ subunit used in most of these experiments was obtained from CF_1 that had been isolated by a newly devised procedure based on acid precipitation.

We previously reported (1) activation of the ATPase associated with chloroplast coupling factor (CF_1)‡ by thioredoxin that had been reduced by the sulfhydryl reagent dithiothreitol (DTT). In view of the basal DTT-dependent ATPase activity that is observed in the absence of added thioredoxin, those results raised the possibility that CF_1 itself may contain a component protein that acts like thioredoxin. We have addressed ourselves to that question, and we now report evidence that CF_1 contains a thioredoxin-like protein as one of its subunits.

When preparations of CF_1 were fractionated by defined procedures, thioredoxin activity was recovered in the fraction enriched in the δ subunit--the 20,000-dalton component that is envisioned to be essential for the attachment of CF_1 to the chloroplast membrane (2,3). The isolated δ subunit could partially replace authentic thioredoxin in the DTT-linked activation of both chloroplast fructose 1,6-bisphosphatase and CF_1 ATPase.

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‡Abbreviations: CF_1, chloroplast coupling factor; DTT, dithiothreitol; SDS, sodium dodecyl sulfate

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METHODS

Preparation of chloroplasts and extraction of CF_1

The temperature for preparation of chloroplasts and for extraction of CF_1 was 4°C. Chloroplasts were prepared (4) from 5 kg of spinach leaves that had been homogenized for 1 min in 300-g lots in a Waring Blender (2-qt capacity, Model HBG-100). The extraction solution (600 ml) contained 0.3 M sucrose, 0.025 M Tris-HCl buffer (pH 7.9), and 210 mg of isoascorbic acid. Whole chloroplasts were collected by centrifugation (3000 x g, 1 min) and then broken osmotically by suspension in 50 mM Tris-HCl buffer (pH 7.9). Chloroplast membranes, collected by centrifugation (10,000 x g, 10 min), were washed once by resuspension in a solution of 50 mM Tris-HCl buffer (pH 7.9) and were then again collected by centrifugation (10,000 x g, 10 min). CF_1 was extracted by resuspending the washed membrane pellet (chlorophyll concentration, 2 mg/ml) in a solution containing 0.25 M sucrose, 5 mM DTT, 2 mM ATP, 1 mM EDTA, and 10 mM Tris-HCl buffer (pH 7.9) (final volume, ~800 ml; ref. 5). One-half volume of chloroform was added, and the mixture was centrifuged (1000 x g, 2 min) to break the emulsion. The organic layer was discarded, and the aqueous layer was clarified by centrifugation (20,000 x g, 25 min). The precipitate was discarded and the gold-colored supernatant fraction was used as a source of CF_1, which was then purified at room temperature by procedures described below. In some experiments, the sucrose method of Strotmann et al. (6) and the EDTA method of Lien and Racker (7) was used to extract CF_1 from chloroplast membranes.

Purification of CF_1 by isoelectric focusing

CF_1, 50 ml of a solution containing about 1.5 mg/ml, was added to an ampholine:50% sucrose solution (pH range 3.5 - 10) to a final volume of 215 ml (Ampholine 8100 Instruction Manual, LKB Instruments, Bromma, Sweden). The solution was combined with 215 ml of 5% sucrose:ampholine solution in a gradient mixer to form a 5-50% sucrose gradient (w:v) in an LKB-8100-2, 440-ml capacity isoelectric focusing column. The column was subjected to a current of 8-9 watts for 48 hr, during which time a dense white protein band, containing CF_1, formed at pH 4.7-4.9, just above a diffuse green contaminant band at pH 4.5-4.7. The power was turned off and 2-ml fractions were collected. The fractions from pH 4.7-4.9 were combined and dialyzed with one buffer change against 200 vol of 50 mM Tris-HCl buffer (pH 7.9). During dialysis, the sucrose and ampholine were largely removed and the CF_1 was redissolved. After dialysis, the precipitate was removed by centrifugation (10,000 x g, 10 min). The supernatant fraction was used as a source of CF_1-ATPase activity. Based on its behavior in native polyacrylamide gel electrophoresis, this fraction contained 98% pure CF_1, excluding the low-molecular-weight protein band attributable to ampholine that remained.

It was found that fractions from the isoelectric focusing column must be dialyzed sufficiently to remove the bulk of the ampholine prior to the ATPase assay. The ampholine reacts with the Fiske-SubbaRow reagent (see assay below) to give a dense white solid which renders the colorimetric determination of free P_i ineffective. Even after extensive dialysis, past the point where dialysis affects the P_i determination, a significant amount of ampholine remains associated with CF_1 as demonstrated by gel electrophoresis.

In further work with the isoelectric focusing column procedure, we attempted to achieve improved resolution by using a shallower pH gradient, i.e., pH 4-6. This modification, however, proved to be ineffective. The pH of the ampholine solution that is used in the pH 4-6 range was pH 4.9 when the components were initially mixed. This pH was sufficiently acidic to precipitate a large amount of CF_1 before electrophoresis was begun.

Purification of CF_1 by acid precipitation

Once we observed that the ATPase associated with CF_1 survives isoelectric focusing, we devised an acid precipitation method for preparing large amounts

of CF_1. Acetic acid, 0.1 M, was added dropwise to the chloroform-extracted CF_1 to give a pH of 5.2. HCl, 0.5 M, was then added to give a final pH of 4.7. The suspension was centrifuged (10,000 x g, 10 min), the light green supernatant fraction was discarded, and the pellet containing CF_1 was resuspended in 10 mM Tricine-HCl buffer, pH 8, to 2-3 mg protein per ml. Insoluble material was removed by centrifugation (10,000 x g, 10 min). The colorless supernatant fraction contained CF_1 that was found to be 90-95% pure by SDS polyacrylamide gel electrophoresis (2) (Fig. 1, left trace).

Isolation of δ and ε subunits from purified CF_1

CF_1 obtained by the acid precipitation procedure described above was extracted with 50% pyridine to effect dissociation of its subunits (8). The δ and ε subunits so obtained were separated and purified by DEAE-cellulose column chromatography with a solution containing 7 M urea and 20 mM Tricine (pH 8.0) as described by Younis et al. (5). Fractions containing the ε subunit (eluted with the urea-Tricine buffer supplemented with 25 mM NaCl) or the δ subunit (eluted with the urea-Tricine buffer supplemented with 100 mM NaCl) were dialyzed overnight against 50 vol of a solution that contained 20 mM Tricine-HCl buffer (pH 8) and 1 M urea. This step was followed by sequential dialysis, first for 6 hr against a solution of 20 mM Tricine-HCl buffer (pH 8) and 0.2 M urea and then against 20 mM Tricine-HCl buffer (pH 8) and 0.02 M urea. The δ and ε subunits were finally dialyzed for 24 hr against a solution of Tricine-HCl (pH 8) and 0.001 M urea. At this point the urea was sufficiently dilute so that there was no interference in the thioredoxin assays. The ε subunit fraction contained 6 mg of protein (in 5 ml), and the δ subunit fraction contained 2.5 mg of protein (in 6 ml) when 180 mg of CF_1 was used as starting material. The subunit fractions were stored at 4°C until assay in the CF_1-ATPase and Fru-P_2ase systems. The δ subunit obtained by this procedure was judged on the basis of native and SDS polyacrylamide gel electrophoresis to be about 80% pure (Fig. 1, right trace).

Other methods

Chlorophyll was determined spectrophotometrically after extraction in 80% acetone (9). Protein concentration was measured by the phenol method (10). Previously published procedures were used for the purification of spinach chloroplast Fru-P_2ase (11) and thioredoxins *m*, *f*, and *c* (12,13). Polyacrylamide gel electrophoresis was carried out in native gels by the Davis procedure (14) and in SDS gels by the Laemmli procedure (15). Assays previously described were used for CF_1-ATPase (1) and for thioredoxin with chloroplast Fru-P_2ase (13).

RESULTS AND DISCUSSION

Thioredoxin activity of CF_1 subunits

Our first attempts to demonstrate thioredoxin activity associated with CF_1 met with no success. Neither crude nor purified preparations of the enzyme showed thioredoxin activity, as measured by the ability to activate Fru-P_2ase in the presence of DTT. However, when the enzyme was resolved into its component subunits, thioredoxin activity was unmasked and shown to reside with the δ subunit fraction (Table I). On a protein basis, the freshly isolated δ subunit showed about one-third the activity of chloroplast thioredoxin *f*, the thioredoxin that activates Fru-P_2ase and other enzymes of the reductive pentose

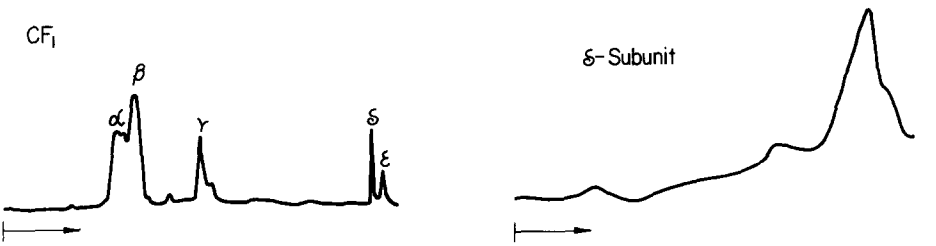


Figure 1. Densitometric trace of CF_1 isolated by the acid precipitation method and of its δ subunit component. CF_1 (7.5 g) and δ subunit (40 µg) were subjected to electrophoresis in SDS slab and tube gels (10%) respectively.

Table I

THIOREDOXIN ACTIVITY OF CF_1 AND ITS SUBUNITS
(Fru-P_2ase assay)

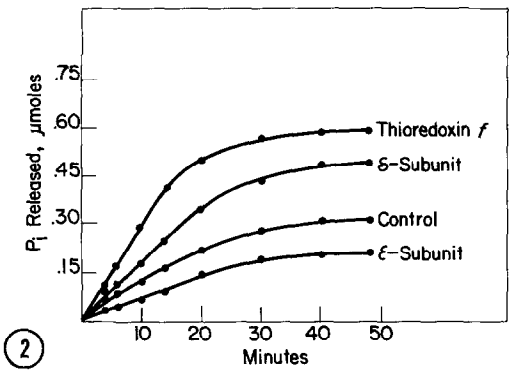
Fraction Added*	P_i Released (µmol)
Complete CF_1	0.28
α + β + γ	0.30
δ + ε	0.36
δ	0.40
ε	0.25
None	0.31

*In parallel experiments, 40 µg chloroplast thioredoxin *f* activity showed 0.58 µmol of P_i released.

The complete system contained 40 µg of the indicated fraction, 15 µg of Fru-P_2ase, and the following (µmol): Tris-HCl buffer (pH 7.9), 50; $MgSO_4$, 0.5; DTT, 2.5; fructose 1,6-bisphosphate, 3.0. Final volume, 0.5 ml. After the addition of all components except fructose bisphosphate, the reaction mixture was preincubated for 5 min, fructose bisphosphate was added, and the reaction was continued for 25 min. Temperature, 25°C. The reaction was stopped by the addition of 2 ml of the mixture used for P_i analysis (11). The subunit fractions were prepared from a CF_1 preparation obtained by the acid-precipitation method.

phosphate cycle of CO_2 assimilation (12,13) (Fig. 2). As is the case for thioredoxin (16,17), the activation of Fru-P_2ase by the δ subunit was effected only by DTT and not by other reduced compounds, viz., reduced glutathione and 2-mercaptoethanol (data not shown).

As also is evident from Table I and Fig. 2, the 15,000-dalton ε subunit of CF_1 (2,3) suppressed chloroplast Fru-P_2ase activity. The inhibitory effect of the ε subunit was observed irrespective of thioredoxin *f* when Fru-P_2ase was



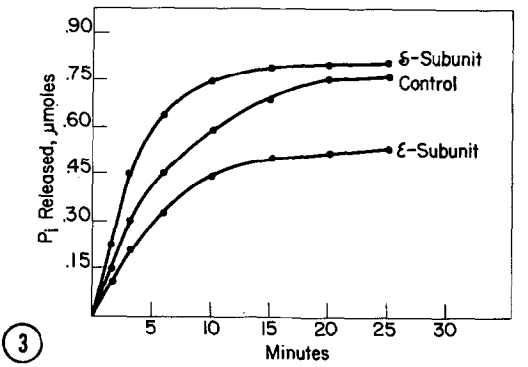


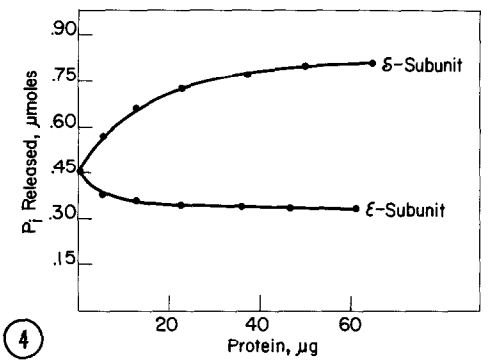
Figure 2. Effect of δ and ε subunits of CF_1 on thioredoxin-linked Fru-P_2ase. Except for use of 40 µg of the indicated thioredoxin or CF_1 subunit, experimental conditions were as described in Table I.

Figure 3. Effect of time on the δ or ε subunit-induced change in ATPase activity of heated CF_1. The complete system contained 40 µg of the indicated subunit, 25 µg of heat-activated CF_1, and the following (µmol): Tricine-NaOH buffer (pH 8), 50; $CaCl_2$, 5.0; ATP (pH 7.6), 6; DTT, 2.5. Final volume, 0.5 ml; temperature, 25°C. The reaction was stopped by the addition of 2 ml of the mixture used for Pi analysis. Prior to assay, CF_1 was activated in 13 mM ATP for 4 min at 60°C.

assayed in the presence of DTT. However, inhibition by the δ subunit was not observed when Fru-P_2ase activity was measured (11) in the absence of DTT and in the presence of a saturating concentration of Mg^{++} (data not shown). The δ subunit was reported earlier to inhibit the ATPase associated with CF_1 (8).

Effect of δ and ε subunits on CF_1-ATPase activity

The ability of the δ subunit to replace thioredoxin *f* in the Fru-P_2ase assay raised the question whether the δ subunit can also replace thioredoxin in the DTT-linked activation of the parent CF_1-ATPase. The evidence in Fig. 3 shows this to be the case, particularly during the linear phase of the reaction. The δ subunit-induced enhancement in ATPase activity was observed over a wide range of δ subunit concentrations (Fig. 4). At maximal enhancement, the δ subunit showed about double the activity of thioredoxin *f*, half the activity of thioredoxin *m*, and about the same activity as nonchloroplast thioredoxin *c* (cf. Figs. 4 and 5). As noted above with Fru-P_2ase, the activity of the ATPase by the δ subunit was specific for DTT and was not observed with reduced glutathione or 2-



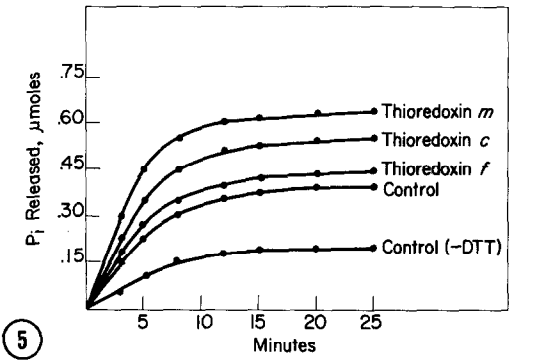


Figure 4. Effect of [δ] and [ε] subunits on ATPase activity of heated CF_1. Except for the addition of the δ and ε subunits of CF_1, as indicated, and a reaction time of 7.5 min, experimental conditions were as described in the legend to Figure 3.

Figure 5. Effect of leaf thioredoxins on ATPase activity of heated CF_1. Except for the addition of thioredoxin as indicated, experimental conditions were as described for Figure 2.

mercaptoethanol. As expected, the ε subunit consistently inhibited the ATPase activity of heated CF_1 (Fig. 4).

The stimulation of CF_1-ATPase by the δ subunit raises the possibility that the δ subunit is itself one of the chloroplast thioredoxins described earlier (12). Present evidence, however, speaks against this possibility. First, there are differences in molecular weight (2,3,12). The molecular weight of the δ subunit (20,000) is appreciably greater than that of either chloroplast thioredoxin *f* (16,000) or thioredoxin *m* (9,000). In addition, there are differences in activity. Unlike chloroplast thioredoxins *f* and *m* (12), the δ subunit showed no thioredoxin activity in the NADP-malate dehydrogenase assay. The δ subunit was also ineffective in the light-dependent activation of Fru-P_2ase in which DTT is replaced by ferredoxin-thioredoxin reductase and photoreduced ferredoxin (17).

CONCLUDING REMARKS

The evidence reported above indicates that, when separated from its parent CF_1 protein complex, the δ subunit is partially able to replace thioredoxin in the DTT-linked activation of chloroplast enzymes. The specificity of such activation for DTT (or related disulfides) raises the question whether the δ

subunit participates *in situ* in the recently reported dithiol-dependent reversal of triphenyltin-induced inhibition of proton transfer in chloroplast membranes (18). The answer to this question, as well as to the broader question of whether thioredoxin (or its phosphorylated derivative, ref. 20) functions in energy transduction, awaits further experimental work.

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