

Chloroplast molecular chaperone-assisted refolding and reconstitution of an active multisubunit coupling factor CF₁ core

(ATPase/assembly)

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ABSTRACT The chloroplast coupling factor 1 (CF₁) is composed of five kinds of subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. Reconstitution of a catalytically active $\alpha_3\beta_3\gamma$ core from urea-denatured subunits at a physiological pH is reported here. A restoration of approximately 90% of the CF₁ ATPase activity has been observed. The reconstitution was achieved by using subunits overexpressed in *Escherichia coli*, purified, and combined in the presence of MgATP, K⁺, and a mixture of several chloroplast molecular chaperones at pH 7.5. The combination of chaperonin 60 and chaperonin 24 failed to reconstitute the active CF₁ core, as did the GroEL/GroES pair (*E. coli* chaperonin 60/10 homologues). Characteristics of the reconstituted ATPase were very close to those of the native complex, including methanol-reversible inhibition by the purified ϵ subunit of CF₁ and sensitivity to inhibition by azide and by tentoxin. In reconstitution with a mixture of tentoxin-resistant and -sensitive β subunits, the extent of inhibition by tentoxin depended on the proportion of sensitive subunits in the reconstitution mixture. Finally, a model for the assembly of the CF₁ core $\alpha_3\beta_3\gamma$ structure is proposed.

Chloroplast coupling factor 1 (CF₁), like the other F-type ATPases, is composed of five kinds of subunits designated α , β , γ , δ , and ϵ in a 3:3:1:1:1 stoichiometry. It has been known for at least 10 years (1) that removal of the δ and ϵ subunits leaves a fully active core of $\alpha_3\beta_3\gamma$. Recent preparations of an $\alpha\beta$ complex have ATPase rates 2 orders of magnitude lower (2, 3).

Analysis of the biochemical functions of several F₁ ATPases has been greatly aided by the ability to reconstitute an active enzyme from separated subunits. This has been possible with F₁ from a thermophilic bacterium (4) and from *Escherichia coli* (5), but not in the case of CF₁ from higher plants. However, individual subunits have been removed and restored. The ATPase core, $\alpha_3\beta_3\gamma$, can bind to thylakoid membranes given high levels of Mg²⁺ (1), but restoring photophosphorylation needs the addition of purified δ and ϵ subunits (6). The isolated β subunit of spinach (7), lettuce (8), or tobacco (8) was able to reconstitute ATPase of the β -subunit deficient F₁ of *Rhodospirillum rubrum*. This ability was suggested to depend upon a small amount of contaminating α subunit, which was postulated to act with a "molecular chaperone-like function" (8).

The term "molecular chaperone" has been applied to proteins that assist in the folding of other proteins and assembly into oligomeric complexes when necessary. Identification and function of molecular chaperones are reviewed by Hendrick and Hartl (9) and by Gatenby and Viitanen (10). In higher plant chloroplasts, chaperonin 60 (cpn60) (11, 12), and its cochaperonin, cpn24 (13), as well as 70-kDa heat shock proteins (hsp70s) (14–16) have been isolated and studied. However, 90-kDa heat shock proteins and homo-

logues to *E. coli* DnaJ and GrpE have not yet been identified in chloroplasts.

Unlike the cpn60 homolog in *E. coli* designated GroEL, the chloroplast cpn60 contains two kinds of subunits, which share about 50% sequence homology (12). The stoichiometry of its α and β subunits has been suggested to be 1:1 (10). Electron microscopic imaging revealed a structure very similar to that of GroEL: a stacked double-ring with a sevenfold rotational symmetry and a central cavity where unfolded proteins probably bind (17).

The chloroplast cochaperonin (cpn24) is a homologue of cpn10s, but is different from any cpn10 described previously, including the *E. coli* homologue designated GroES. It contains two distinct cpn10-like structures fused in tandem (13), each of which possesses several highly conserved amino acid residues that are encoded in many other GroES genes. This chloroplast protein can complement the normal functions of *E. coli*-GroES deficient strains (A. A. Gatenby, personal communication).

Most studies on molecular chaperone-assisted protein folding concerned the refolding and reactivating of monomeric proteins and homogenous protein oligomers. A functional refolding and reactivation of an oligomeric protein complex from individual heterogeneous subunits has not yet been reported to our knowledge.

We demonstrate here that, using purified overexpressed individual subunits, the CF₁ core structure $\alpha_3\beta_3\gamma$ can be functionally reconstituted in the presence of chloroplast molecular chaperones, including cpn60, cpn24, and hsp70. The reconstituted CF₁ core is similar to the native core in every aspect examined. A model for the chloroplast molecular chaperone-assisted refolding and reconstitution is also discussed.

MATERIALS AND METHODS

Chemicals. All chemicals were purchased from Sigma unless otherwise specified in the text.

Gene Sources, Overexpression, and Purification of the Tobacco CF₁ Core Subunits. The *atpA* gene of *Nicotiana tabacum* was obtained from J. D. Palmer (Indiana University, Bloomington). The tentoxin-resistant *atpB* gene of *Nicotiana tabacum* was a gift from X. Shinozaki (Institute of Physical Chemistry Research, Tsukuba Science City, Japan) (18). The tentoxin-sensitive *atpB* gene of *Nicotiana plumbaginifolia* was provided by M. Edelman (Weizmann Institute of Science, Rehovot, Israel) (19). The *atpC* gene of *Nicotiana tabacum* was given by J. C. Gray (Cambridge University, England) (20). The *atpA* gene was cloned in the

Abbreviations: CF₁, coupling factor 1 of chloroplasts; cpn, chaperonin; hsp70, 70-kDa heat shock protein.

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pGGC-DT7GroE expression vector, whereas the *atpB* and *atpC* genes were cloned into the pET11c expression vector (Novagen). All constructs were introduced into *E. coli* expression strain BL21(DE3), cells were grown in M9NZ medium, and gene expression was induced with isopropyl β -D-thiogalactoside (IPTG). All subunits were produced in inclusion bodies in *E. coli*. These were individually purified and dissolved in 6 M urea (Research Organics)/25 mM Tris chloride, pH 8.0/0.1 mM dithiothreitol at room temperature for 1 hr before being used in reconstitution. The details of construction, overexpression, and purification of the subunits will be reported elsewhere.

Purification of Molecular Chaperones. The expression construct pT7GroE was obtained from A. A. Gatenby (DuPont), and the over-expressed GroEL and GroES proteins were purified by a procedure to be described elsewhere (G.G.C., unpublished data). A mixture of spinach chloroplast molecular chaperones was obtained by the method of Yuan *et al.* (21). The cpn60 component (present as a tetradecamer) was retained by a filter with 300-kDa molecular mass cut-off (Filtron Technology, Northborough, MA), permitting its separation from the rest of the molecular chaperones. The spinach cpn24 by itself was prepared from a construct provided by A. A. Gatenby. It was introduced into *E. coli* strain JM105, induced by isopropyl β -D-thiogalactoside, and purified (G.G.C., unpublished data).

Purification of the Native Spinach CF₁ Core and the ϵ Subunit. The CF₁ core, lacking δ and ϵ , was prepared as described by Xiao and McCarty (22). The ϵ -subunit fraction was concentrated with a Macrosep filter (Filtron Technology) having a 3-kDa molecular mass cut-off. The CF₁ core fraction was retained by successive filtration through a membrane with a 30-kDa cut-off (Amicon) followed by a Macrosep filter with a 100-kDa cut-off. The two fractions were then dialyzed against 500 ml of 25 mM Tris chloride (pH 8.0) and evaluated by SDS 15% PAGE.

Refolding and Reconstitution Conditions. The incubation mixture for coupled refolding and reconstitution contained 0.2 μ M of the purified chloroplast molecular chaperones, 0.087 μ M of the denatured α subunit, 0.087 μ M of the denatured β subunit, 0.026 μ M of the denatured γ subunit, 50 mM KCl, 5 mM MgCl₂, 2 mM ATP, 0.1 mM dithiothreitol, 5% (vol/vol) glycerol, and 25 mM Tris chloride (pH 7.5) at room temperature (\approx 23°C). The denatured proteins were diluted rapidly into this complex mixture. The concentration of the chloroplast molecular chaperones was estimated by immunoblot (Western blot) analysis of the amount of cpn60 in the mixture with an antibody specific for the pea cpn60 (provided by H. Roy, Rensselaer Polytechnic Institute, Troy, NY). On the same Western Blot, a purified spinach cpn60 concentration series was used as the concentration standard. The molar ratio of the CF₁ core subunits was approximately α : β : γ = 3:3:1, and the molar ratio of total CF₁ core subunits/chloroplast molecular chaperones was about 1:1. Typically, the refolding and reconstitution was conducted at room temperature for 1 hr.

ATPase Assay of the Reconstituted CF₁ Core. After the coupled refolding and reconstitution reaction, aggregates generated during the incubation were removed by centrifugation in a microfuge. The supernatant was then assayed for ATPase by release of ³²P-labeled inorganic phosphate from [γ -³²P]ATP (Amersham). The reaction medium contained 100 mM 3-[[Tris(hydroxymethyl)methyl]amino]propanesulfonic acid (Taps) (adjusted with KOH to pH 8.8 at room temperature), 50 mM Na₂SO₃, 20% (vol/vol) methanol, and 2 mM [γ -³²P]ATP. Released phosphate was separated from residual [γ -³²P]ATP as described by Harris *et al.* (23). ATPase rates were calculated based on the amount of core subunit protein added initially as indicated in the figures. Background activ-

ity due to chloroplast molecular chaperones alone (about 15% to 20% of the total) was subtracted from the observed rates.

Purification of Tentoxin. Tentoxin was purified from the fungus *Alternaria alternata* as described by Liebermann and Oertel (24) and Liebermann *et al.* (25). The purity of the preparation was not determined, and a considerable part might have been dihydrotentoxin, which is very similar to tentoxin but is not active in inhibiting CF₁ ATPase. In the study of cross-reconstitution, a nominal concentration series from 0 to 50 μ M was used in the ATPase assay.

RESULTS

The Refolding and Functional Reconstitution of the CF₁ Core. The CF₁ core reconstituted with the described procedure exhibited rates of ATP hydrolysis between 10 and 20 μ mol/mg of protein per min (Fig. 1). These are minimal values because they refer to the mg of initial denatured core subunits in the reconstitution mixture and do not take into account protein lost in the aggregates that had been removed. Even so, these rates are approximately 90% of those found with native spinach CF₁ core ATPase (see below). With the proviso that the specific activity of native tobacco CF₁ core is similar to that of the one from spinach, a very effective reconstitution of enzymatic activity has been achieved.

The time course during the reconstitution procedure (Fig. 1) showed a maximal activity by 75 min of incubation with the chaperones. Sulfite had been found to stimulate ATPase of chloroplast cpn60 and of CF₁ (unpublished data); therefore, we checked for a possible role in reconstitution. There was a distinct stimulation of the initial rate (first 30 min or less) by 50 mM sulfite. Precautions were not taken against nonenzymatic oxidation of the sulfite in this study, so further work is needed to see how large the maximal stimulation might be.

Mg²⁺, ATP, and K⁺ are absolute requirements for most commonly used molecular chaperone-assisted protein refolding/reconstitution systems (9, 10). Although we had observed that the chloroplast cpn60 can use Ca²⁺ as well as Mg²⁺ to support ATP hydrolysis (G.G.C., unpublished data), it could not substitute for the 5 mM MgCl₂ in reconstitution (26).

Successful Reconstitution Requires Two Chaperonins and Other Proteins. The chloroplast cpn60 and cpn24 were purified and used by themselves for reconstitution. They were not able to facilitate reconstitution of the active CF₁ core (data not shown). Only the total mixture of chloroplast

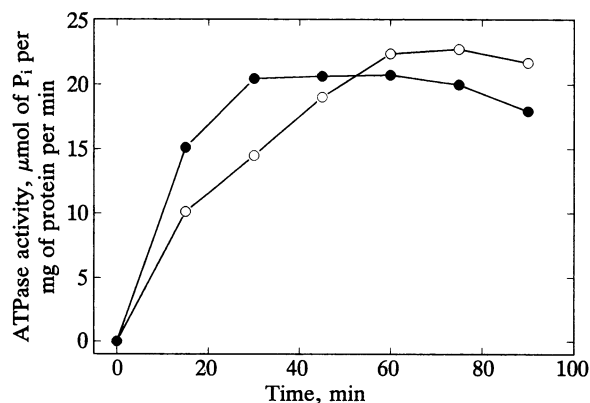


FIG. 1. Time course of the reconstitution \pm 50 mM sulfite. Reconstitution was performed by using purified chloroplast molecular chaperones and the CF₁ core subunits: α and β at 0.0855 μ M and γ at 0.0285 μ M. The reactions were conducted for different lengths of time as indicated and then stopped by rapid chilling in an ethanol/ice mixture. After aggregates were removed, the soluble fractions were assayed for the ATPase. ○, No sulfite; ●, with 50 mM sodium sulfite.

molecular chaperones supported the reconstitution. This preparation contained SDS/PAGE bands at 72 kDa (probably the hsp70 component), 58.6 kDa (cpn60), 53 kDa, 42 kDa, and 24 kDa (cpn24).

The stromal chaperone mixture lacking cpn60, prepared by filtration through a 300-kDa cut-off filter, was not active in reconstitution. Its activity could be restored by fresh cpn60, but not by the *E. coli* GroEL (26). From these two experiments, it appears that reconstitution requires chloroplast cpn60 together with further components of the mixture. A requirement for cpn24 in reconstitution seems likely but has not been proven yet.

The purified *E. coli* chaperonins GroEL and GroES were tested for reconstitution of active CF₁ core. However, they were completely inactive. Binding studies were performed (26) in which, in the absence of ATP, the complexes were incubated individually with the denatured α , β , and γ subunits. Then, aggregates were removed by centrifugation, and the soluble fractions were filtered through 300-kDa cut-off filters to allow noncomplexed subunits to be discarded. The retentates were analyzed by SDS/PAGE, followed by Western blotting with antibodies against the three subunits from spinach CF₁. These experiments showed that the *E. coli* cpn60, GroEL, bound to the β subunit as well as the chloroplast cpn60 did and to the α subunit very weakly, whereas it failed to bind to the γ subunit at all. The chloroplast cpn60 could efficiently bind to all three CF₁ subunits.

Reconstitution of Active CF₁ Core Requires Interaction Between the α Subunit and the β Subunit During Refolding. The refolding/reconstitution pathway was investigated in part by using either individual subunits or different combinations of two at a time in initial mixtures with the chloroplast molecular chaperones. After their refolding, they were combined together and assayed for reconstituted ATPase activities (Table 1). No activity was observed when individual subunits were folded with the help of the chaperones and then combined or when most of the two-subunit combinations were folded together followed by addition of the prefolded third subunit. ATPase activity that was close to that seen upon reconstitution with all three subunits was detectable only when α and β subunits were prefolded together and then combined with the prefolded γ subunit. Therefore, we suggest either that an interaction between the α subunit and the β subunit is required during refolding or that the α subunit is not stable by itself.

Biochemical Characteristics of the Reconstituted ATPase. The ϵ subunit is known as an inhibitor of CF₁, whose effect is largely annulled by 20% methanol. Accordingly, the ϵ subunit at 0.2 μ M was incubated for 30 min with both the reconstituted $\alpha_3\beta_3\gamma$ complex and the native CF₁ core at room temperature (Fig. 2). The ϵ subunit by itself has no ATPase

Table 1. Reconstitution of the CF₁ cores with different subunit combinations in the presence of chloroplast molecular chaperones

| First-incubation subunit combinations | ATPase activity, μ mol of P _i per mg of protein per min |
|---------------------------------------|--|
| $[\alpha_3], [\beta_3], [\gamma]$ | 0 |
| $[\alpha_3\gamma], [\beta_3]$ | 0 |
| $[\beta_3\gamma], [\alpha_3]$ | 0 |
| $[\alpha_3\beta_3], [\gamma]$ | 14.8 |
| $[\alpha_3\beta_3\gamma]$ | 14.4 |

The CF₁ core subunits (in the left column) were combined as indicated by Greek letters in each set of brackets and incubated with the chloroplast molecular chaperones in the reconstitution system at room temperature for 1 hr. Then all of the subunits on the same row were mixed and further incubated for 0.5 hr. After aggregates (mainly the α and γ subunits and hsp70) were removed, the soluble fractions were assayed for ATPase activity. The subscript 1 indicates 26 nM and 3 indicates 87 nM subunit concentration.

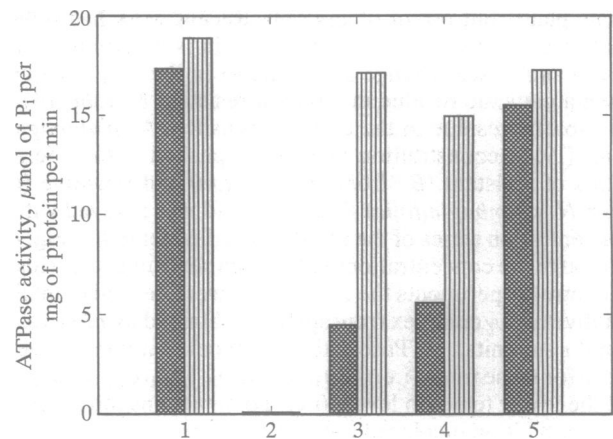


Fig. 2. Effects of methanol and the ϵ subunit on reconstituted CF₁ core ATPase activity. The reconstitution was carried out as described in the previous figure, and the mixture was divided into aliquots with respect to the CF₁ core. The ϵ subunit (0.2 μ M) was added and incubated with both the native and the reconstituted CF₁ cores, both at 0.0285 μ M as in Fig. 1, at room temperature prior to the ATPase assay as indicated. ATPase assays were conducted both in the presence (vertical-line bars) and in the absence (cross-hatched bars) of 20% methanol, while all reactions contained 50 mM sodium sulfite. Bars: 1, native CF₁ core; 2, ϵ by itself; 3, native CF₁ core plus ϵ ; 4, reconstituted CF₁ core plus ϵ ; 5, reconstituted CF₁ core.

activity and served as a control for background ATPase. These mixtures were also assayed \pm 20% methanol (Fig. 2). The ϵ subunit inhibited ATPase activity of the native spinach CF₁ core about 75% and that of the reconstituted tobacco CF₁ core 65%. This inhibition was almost completely reversed by 20% methanol. With both CF₁ cores, a slight methanol stimulation was observed even in the absence of the ϵ subunit.

Azide is a powerful inhibitor of mitochondrial (27) and chloroplast (28) F₁ ATPases. The reconstituted CF₁ core was assayed by using azide between 0 and 1 mM (Fig. 3) and was compared to the native CF₁ core. The extent of inhibition of these two preparations was very close; in both cases, 50% inhibition occurred at about 0.1 mM, and 90% occurred by 0.5 mM. Complete inhibition was not observed with either preparation.

Tentoxin Sensitivity of the Reconstituted CF₁ Cores. The fungal toxin, tentoxin, is a very powerful inhibitor of CF₁ of

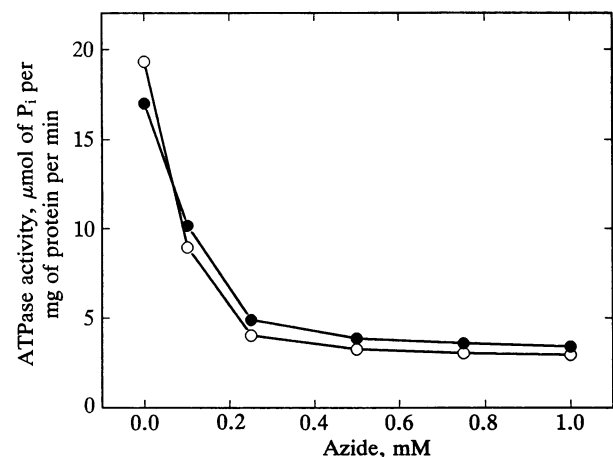


Fig. 3. Azide inhibition of the reconstituted CF₁ core. After the reconstitution, aliquots of the soluble fraction containing 0.0285 μ M CF₁ core were assayed with various sodium azide concentrations. ○, Native (unreconstituted) spinach CF₁ core control; ●, reconstituted tobacco CF₁ core.

some plants but not of others (29). Recent work has shown that resistance or sensitivity to tentoxin is determined by a single amino acid change at residue 83 in the CF₁ β subunit from a glutamic residue in tentoxin-resistant *N. tabacum* to an aspartic residue in the tentoxin-sensitive *N. plumbaginifolia* (19). Reconstitution was accomplished with differing ratios of resistant (β_r , from *N. tabacum*) and sensitive (β_s , from *N. plumbaginifolia*) β subunits and was assayed with a concentration series of the partially purified CF₁ (Fig. 4). Although the concentrations of tentoxin seem unusually high, in control experiments the same levels were needed to inhibit a native $\alpha_3\beta_3\gamma$ complex from spinach (obtained by removal of δ and ϵ subunits). ATPase activity without tentoxin was very close for these four—i.e., (β_s)₃, (β_s)₂(β_r)₁, (β_s)₁(β_r)₂, and (β_r)₃. At the lower tentoxin levels (i.e., nominal concentration, 10 μ M), inhibition increased proportionally to the number of β_s subunits in the reconstitution mix. At higher tentoxin levels, however, sensitivity with two β_s subunits was surprisingly high—nearly equal to that with three β_s subunits. Because of extremely high sequence identity between these two kinds of β subunits, it is reasonable to assume that they assemble randomly into the CF₁ core and that their ratio in the final complex is the same as in the reconstitution mixture. Thus, the greater the number of β_s subunits in the complex, the greater the sensitivity to tentoxin.

DISCUSSION

Subunits of CF₁ from *Nicotiana* species, overexpressed in *E. coli*, were used to reconstitute a fully active catalytic core enzyme. Reconstitution was possible only with the help of a mixture of purified spinach chloroplast molecular chaperones. It was interesting that reconstitution was not possible with just cpn60 and cpn24 from this mixture, even though we demonstrated that cpn60 is a crucial component in the chloroplast molecular chaperones. Apparently, this pair of chaperonins must be supplemented with one or more other components, possibly hsp70. Precedent for the required participation of two different chaperone systems is found in data on the refolding of mitochondrial rhodanese (30). The active rhodanese could be refolded and released under the conditions used only if both the *E. coli* DnaK molecular chaperone system (DnaK/DnaJ/GrpE) and the chaperonins (GroEL/GroES) were present.

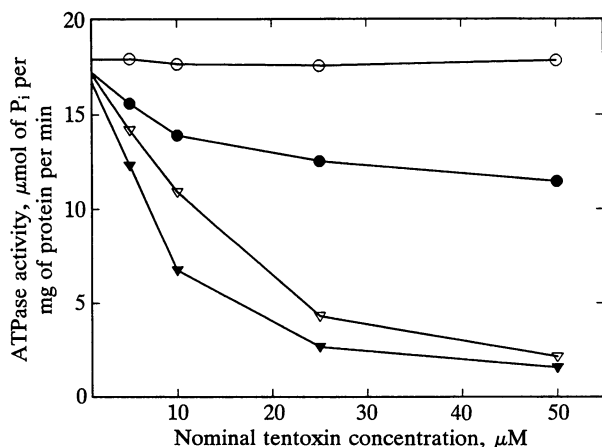


FIG. 4. Cross-reconstitution of the CF₁ cores. Reconstitution was performed with various proportions of the tentoxin-sensitive and -resistant β subunits as indicated in the text. ATPase assays were performed with different nominal concentrations of tentoxin. ○, All three tentoxin-resistant β subunits; ●, two tentoxin-resistant β subunits and one tentoxin-sensitive β subunit; ▽, one tentoxin-resistant β subunit and two tentoxin-sensitive β subunits; ▼, all three tentoxin-sensitive β subunits.

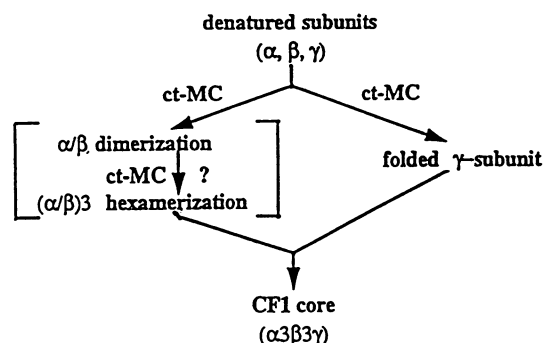


FIG. 5. Model for the chloroplast molecular chaperone (ct-MC)-assisted CF₁ core refolding and reconstitution.

One earlier report (31) indicated that the active CF₁ core could be assembled *in vitro* from individual fast protein liquid chromatography-purified subunits and without the use of molecular chaperones. Initially we attempted to repeat that work but could not separate the subunits on columns as they had reported. As an alternative, we isolated the spinach CF₁ core subunits from an SDS/polyacrylamide gel, transferred them into urea, dialyzed the three together to remove the chaotrope gradually (over 72 hr), and found a low ATPase activity (0.07 nmol of P_i released per mg of CF₁ core per min—only 1% of that reported in ref. 31). By contrast, when these subunits were put into our chloroplast molecular chaperone-assisted reconstitution system, nearly 50% of the native spinach CF₁ core ATPase activity was restored (unpublished observation). Thus, the previous failure was not totally due to the quality of the isolated subunits. Attempts at a more rapid nonenzymatic reconstitution at pH 7.5 failed totally.

We rather expected that the *E. coli* chaperonins GroEL + GroES would be able to reconstitute CF₁, as they do for many other denatured proteins (see refs. 9 and 10 for reviews). That they did not could possibly be ascribed to the absence of DnaJ, DnaK, and GrpE in these experiments. However, the failure of GroEL to replace cpn60 in the chloroplast chaperone mixture depleted of this component indicates the likelihood that CF₁ reconstitution is specific for the chloroplast chaperonins. Our work supports the hypothesis that some chaperonins have specialized functions (32). An earlier example is the failure of assembly of higher plant L₈S₈ ribulose-1,5-bisphosphate carboxylase (RuBisCo) when expressed in *E. coli* (33–35) contrasted with the successful assembly of cyanobacterial L₈S₈ RuBisCo under the guidance of endogenous molecular chaperones. In the present case, we conclude that the chloroplast molecular chaperones are uniquely required for refolding and reconstitution of the catalytically functional CF₁ core.

The chloroplast molecular chaperone-assisted cross-reconstitution of a catalytically active CF₁ core represents a practical approach to investigate the structure–function relationship of the CF₁ subunits. With this system one could investigate functional effects of site-directed mutants or chemical modification of specific subunits. The demonstration of a correlation between tentoxin sensitivity and the proportion of tentoxin-sensitive β subunits illustrates one way to find the site of action of an inhibitor.

It was not expected that (at higher tentoxin concentrations) two sensitive subunits conferred as much sensitivity as did three (see Fig. 4). One possible way to explain this would be if the binding of tentoxin causes a conformational change in the tentoxin-sensitive β subunit, which in turn affects a neighboring tentoxin-resistant β subunit, the net result being overall sensitivity to tentoxin.

The pathway for assembly of the CF₁ complex may be inferred in part from the results presented here. With com-

binations of different subunits treated with chaperones prior to mixing of all three together, active enzyme was only obtained when α and β were prefolded together (Table 1). Based on this result, a model for reconstitution can be proposed (Fig. 5) in which the α subunit and the β subunit are folded and assembled into a dimer, assisted by chloroplast molecular chaperones. Three such dimers would further develop into a hexamer. Finally, the folded γ subunit joins onto the hexamer to form the catalytically active CF₁ core. Individually folded α or β subunits are incapable of proper dimer formation, but the individually folded γ subunit is capable of further interactions. Further, when the α subunit is already folded, it cannot help the folding of the β subunit and vice versa. In other words, their folding pathway is irreversible.

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