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Reconstitution of photophosphorylation with coupling factor 1 ATPases from the thermophilic bacterium PS3 and lettuce chloroplasts

D. Bar-Zvi *, M. Yoshida ** and N. Shavit ***

Department of Biology, Ben Gurion University of the Negev, P.O. Box 653, Beer Sheva 84105 (Israel)

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(1) EDTA-resolved particles (partially depleted of CF_1) from lettuce chloroplast thylakoids were reconstituted with CF_1 from lettuce and with TF_1 from the thermophilic bacterium PS3. The binding affinity of both F_1 proteins for the membranal CF_0 is similar. (2) The phosphorylation activities of the EDTA-resolved particles after reconstitution with either the tentoxin-sensitive CF_1 or the tentoxin-resistant TF_1 are equally inhibited by tentoxin. The same degree of reconstitution of photophosphorylation is observed either with the native TF_1, the DCCD-modified TF_1 or the DCCD-modified CF_1. Particles reconstituted with native CF_1 always catalyzed a higher rate of ATP formation. (3) NaBr-resolved particles (CF_1-depleted) bind TF_1 with the reconstitution of the light-dependent H^+ uptake. However, no significant enhancement of ATP formation accompanies the binding of TF_1 to the membranal CF_0. (4) These findings suggest a close relationship between the F_0-F_1 complex of the prokaryotic thermophilic bacterium PS3 and that from lettuce, a eukaryotic organism. It is also apparent that the rebinding of added TF_1 or CF_1 to CF_1-depleted particles reforms ATP synthetase complexes that are not fully catalytically functional. In particular, the TF_1 incorporated into the membrane appears to have only a structural role of sealing the membrane and preventing protons from leaking out but does not participate in the catalytic process itself. The endogenous CF_1 that is present in the native undissociated ATP synthetase complex and still remains in these CF_1-partially depleted membranes is the catalytically active enzyme species.

Introduction

It is well accepted, that in the process of energy transduction, energy from oxidation-reduction reactions is transmitted to the ATP synthetase, a reversible proton-translocating ATPase complex associated with the energy-transducing membrane. This protein complex, isolated from mitochondria, bacteria and chloroplast membranes, consists of two main portions, F_1 and F_0 [1–4].

The F_1 portion is an extrinsic membrane-bound

* Present address: Department of Cellular and Developmental Biology, The Biological Laboratories, Harvard University, Cambridge, MA 02138, U.S.A.
** Department of Biochemistry, Jichi Medical School, Minamikawachi-machi, Tochigi-ken 329-04, Japan
*** To whom correspondence should be addressed.

Abbreviations: CF_1, F_1, TF_1, coupling factors one from chloroplasts, mitochondria and the thermophilic bacterium PS3, respectively; CF_0, chloroplast F_0, DCCD, dicyclohexylcarbodiimide; Chl, chlorophyll; Mops, 4-morpholinepropanesulphonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

complex containing five different types of subunits: α, β, γ, δ and ε in order of decreasing molecular weight. Membrane-bound F_1 catalyzes ATP synthesis, hydrolysis and the partial reactions (e.g., P_i-ATP exchange), while the solubilized F_1 complex catalyzes only ATP hydrolysis. Rebinding of F_1 to membranes that have been depleted of F_1 restores their capacity to form ATP. Besides the catalytic sites, the catalytic F_1 portion also contains several nucleotide binding sites in the α and β subunits. More recently, it has been shown that soluble F_1-ATPases catalyze phosphorylation of both medium and bound ADP to form ATP tightly bound to the enzyme [5,6]. However, the relation of this reaction to the steady-state phosphorylation catalyzed by the membrane-bound ATP synthetase is not yet clear. The F_0 portion is an intrinsic membrane-bound complex that contains at least three highly hydrophobic subunits. This complex has been extensively investigated, and one of its subunits is a proteolipid that binds DCCD and participates in translocation of protons across the membrane (for a review see Ref. 7).

Studies on the structure and function of the ATP synthetases from different sources indicate that the enzymes from prokaryotic and eukaryotic cells are very similar [1–4,7,8]. The two large subunits from *Escherichia coli* F_1 and bovine heart mitochondrial F_1 are highly homologous in their amino acid sequences [9]. The comparison between the β subunits of *E. coli* F_1, bovine heart mitochondrial F_1 and spinach or maize CF_1 shows a high degree of conservation of certain sequences, in particular those around a glutamic acid residue [9–11]. Immunological cross reactivity between β subunits of F_1 from different sources has also been reported [8]. Sequences of the DCCD-binding proteolipid from many systems show a short central homologous region containing an aspartyl or glutamyl residue that binds DCCD [7].

Differences in the structure and other properties of ATP synthetases have been useful in the study of their function. The mitochondrial ATPase contains a regulator type of protein that is not found in chloroplasts and functions as an ATPase inhibitor [4]. The chloroplast F_1-ATPase is a latent enzyme that, in contrast with other ATPases, requires activation [1]. Inhibitor studies with several ATPases indicate that the sensitivity to certain compounds depends on the protein in question, i.e., the mitochondrial ATPase but no other ATPases are inhibited by oligomycin [3].

Another approach involves the formation of hybrid complexes between the isolated subunits from different proteins and comparison of their properties with those of the 'parental' complexes. Selman and Durbin [12] reported that CF_1 from different plants fulfills both a structural and a catalytic role after rebinding to spinach EDTA-resolved particles. However, Schatz et al. [13] have suggested that the yeast mitochondrial F_1 that rebinds to MF_1-depleted membranes has only a structural role, since yeast MF_1 reconstitutes oxidative phosphorylation of mitochondrial F_1-partially depleted but not that of F_1-fully-depleted bovine heart submitochondrial particles. Takeda et al. [14] have prepared hybrid complexes from the isolated α, β and γ subunits of TF_1 and *E. coli* F_1. Although termostable active complexes were not obtained they have nevertheless obtained hybrid complexes of TF_1 and *E. coli* F_1 subunits with intermediate halophilicity.

In this report we demonstrate that TF_1 from the thermophilic bacterium PS3 binds to the lettuce chloroplast F_0. The hybrid formed between TF_1 from a prokaryotic and F_0 from a eukaryotic species seals leaks produced in the thylakoid membrane by the removal of CF_1. However, the reattached TF_1 does not function catalytically, and the reconstituted system has no greater capacity to synthesize ATP than that with reattached CF_1. The possibility that the CF_1 added to CF_1-depleted membranes has mainly a structural role is discussed.

Materials and Methods

$^{32}P_i$ was obtained from the Nuclear Research Center-Negev, Israel. [γ-^{32}P]ATP was prepared as described [15]. Hexokinase, type F-300, inhibitors and unlabeled nucleotides were obtained from Sigma Chemical Co.

Preparation of enzymes

CF_1 was isolated from lettuce thylakoid membranes by EDTA extraction, purified on DEAE-Sephadex A-50, and stored at 4°C as a suspension in 50% ammonium sulfate as described [16]. Before

use, the CF_1 suspension was centrifuged for 5 min at $12000 \times g$ at room temperature. The pellet was resuspended in 10 mM Tricine-NaOH (pH 8.0) (in a volume equal to the pellet's original volume) and then desalted on a Sephadex G-25 centrifuge column [17] preequilibrated with the same buffer.

TF_1 was isolated from the thermophilic bacterium PS3, purified, dialyzed against H_2O and stored after lyophilization at $-20°C$ as described [18]. Just before use, a sample was dissolved in 10 mM Tricine-NaOH (pH 8.0) buffer.

ATPase assay

The ATPase activity of the soluble CF_1 was assayed by incubation of 25 μg of the activated enzyme for 15 min at 37°C in a reaction mix (1 ml) containing 50 mM Tricine-NaOH (pH 8.0)/5 mM $CaCl_2$/5 mM [γ-^{32}P]ATP (containing $5 \cdot 10^5$ cpm/μmol). The reaction was ended by the addition of 0.4 ml of 5% ammonium molybdate, 1 mM P_i solution in 4 M HCl. The phosphomolybdate complex was extracted with an isobutanol-xylene mix as described [19].

The ATPase activity of TF_1 was assayed at 60°C in a reaction mix (1 ml) containing 50 mM glycine (pH 9.4 at 20°C, but 8.6 at 60°C), 5 mM $MgCl_2$, 5 mM [γ-^{32}P]ATP and 3–5 μg TF_1. The reaction was terminated after 5–10 min and $^{32}P_i$ was determined as described previously. In some cases, the hydrolytic activity of TF_1 was assayed as described for the CF_1-ATPase assay, but with $MgCl_2$ or $CaCl_2$ as cofactor.

Reconstitution of photophosphorylation

EDTA-resolved thylakoid particles were obtained with the following modifications according to the procedure of Shoshan and Shavit [20]. Uncoupling was achieved by suspending the thylakoid membranes for 3 min in 1 mM EDTA and 1 mM Tricine-NaOH (pH 8.0) at a chlorophyll concentration of 50–100 μg/ml. The EDTA resolved particles (partially depleted of CF_1) were collected by centrifugation for 2 min at $16000 \times g$, resuspended in a solution (STN) containing 0.4 M sucrose/20 mM Tris-HCl (pH 8.0)/10 mM NaCl, and used immediately. For reconstitution freshly prepared EDTA-resolved particles (containing 5–15 μg chlorophyll) were incubated with the appropriate coupling factor in a final volume of 0.3 ml containing 10 mM Tricine-NaOH (pH 8.0) and 10 mM $MgCl_2$. After 1 h at 4°C, the mixes were brought to 23°C, 0.7 ml of a reaction mix for ATP synthesis was added, and the samples were illuminated with saturating white light at 23°C for 2 min. The assay mix (1.0 ml) comprised the following components: 50 mM Tricine-NaOH/20 mM NaCl/5 mM $MgCl_2$/5 mM (containing $0.5–1 \cdot 10^6$ cpm/μmol) [^{32}P]P_i (pH 8.0)/0.033 mM phenazine methosulfate/20 mM glucose/20 units hexokinase. The reaction was terminated by the addition of trichloroacetic acid (3% (w/v) final concentration) immediately after the light was turned off. $^{32}P_i$ incorporated was determined after extraction with the isobutanol-xylene mix [19].

NaBr-resolved thylakoids were prepared as described [21]. Reconstitution of ATP formation was as described for EDTA-resolved particles, except for the addition of 0.2 mg/ml bovine serum albumin. ATP formation was assayed as described above, except that the $^{32}P_i$ concentration and its specific radioactivity were 3 mM and $1.5–3 \cdot 10^6$ cpm/μmol, respectively.

H^+ uptake was assayed as described [22]. Reaction mixtures contained in a volume of 2.5 ml: 50 mM NaCl/5 μM pyocyanine/0.3 ml of the reconstitution mix containing 30–40 μg of chlorophyll. The pH was adjusted to 6.2 with HCl before illumination. Protein was determined according to Bradford [23] using bovine serum albumin as a protein standard. Chlorophyll was determined according to Arnon [24].

Results

Reconstitution of photophosphorylation with EDTA-resolved particles

Addition of CF_1 to EDTA-resolved thylakoid membranes in a medium of relatively high ionic strength restores the phosphorylative ability of the particles (Fig. 1). Adding TF_1 to these particles also results in the reconstitution of ATP formation. The maximum degree of reconstitution obtained with TF_1 is somewhat less than that with CF_1, but the degree of reconstitution similarly depends on the concentrations of each coupling factor, which suggests that the binding affinity of both CF_1 and TF_1 to the CF_0 in the uncoupled

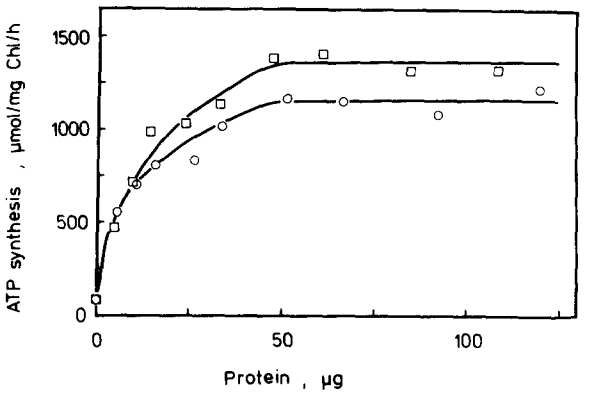

Fig. 1. Reconstitution of ATP synthesis of EDTA-resolved particles by CF_1 and TF_1. CF_1 (□) or TF_1 (○) were added to EDTA-resolved particles containing 10.7 µg chlorophyll. Reconstitution and assay of ATP synthesis were as described. Activity of control thylakoids was 2017 µmol ATP/mg chlorophyll/h.

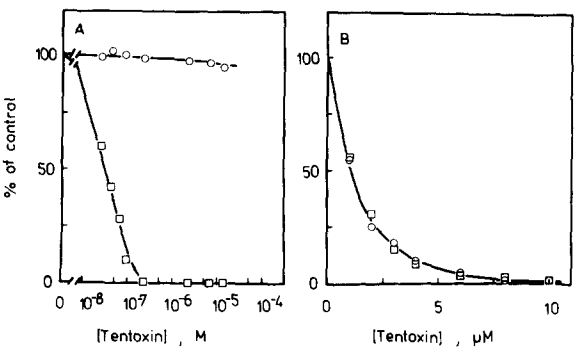

Fig. 2. Sensitivity of EDTA-resolved particles reconstituted with CF_1 or TF_1 to tentoxin. (A) CF_1 (□) or TF_1 (○) (11.5 µg) were incubated for 1 h at 23°C in a mix (0.9 ml) containing 50 mM Tricine-NaOH (pH 8.0) and tentoxin as indicated. ATP hydrolysis was assayed at 37°C. Reactions were started by the addition of 5 µmol [γ-^{32}P]ATP with 5 µmol $CaCl_2$ (□) or $MgCl_2$ (○). (B) EDTA-resolved particles containing 75.8 µg chlorophyll were incubated with 310 µg of CF_1 (□) or TF_1 (○) in a reconstitution mix (1.5 ml) as described. Samples (0.1 ml) of the reconstituted thylakoids were mixed with the phosphorylation reaction mix containing tentoxin, as indicated. Samples were incubated in the dark at 23°C for 1 min and then assayed for ATP formation. Activities were 1490, 400, 1408 and 1151 µmol ATP/mg chlorophyll/h for control thylakoids, EDTA-resolved particles, thylakoids reconstituted with CF_1 or TF_1, respectively.

thylakoids is similar. Since EDTA extraction removes only 30–50 percent of the CF_1 present in the thylakoid membranes [20,25,26], the restoration of photophosphorylation by TF_1 could be attributed to sealing of leaks made in the membranes during uncoupling, while the CF_1 molecules that still remain on the uncoupled membranes act as the catalytic species. To distinguish between a structural and a catalytic role for the coupling factor added to CF_1-depleted membranes, we tested the sensitivity of the ATP synthetic activity to tentoxin, in EDTA-resolved thylakoid membranes reconstituted with either CF_1 or TF_1. This cyclic tetrapeptide is a species-specific energy-transfer inhibitor [12,27], and reactions catalyzed by TF_1 should be less sensitive to the inhibitor than those catalyzed by CF_1. Fig. 2A shows that CF_1 is indeed very sensitive, 50% inhibition of ATP hydrolysis being obtained at 30 nM tentoxin, while the TF_1-ATPase, under the same conditions, is almost insensitive. The TF_1-ATPase is also insensitive to tentoxin even if assayed at its optimum pH (8.6) and temperature (60°C). However, when assayed at 37°C as a Ca^{2+}-ATPase, 10 µM of tentoxin gave about 25% inhibition (not shown). Fig. 2B shows that the photophosphorylation activity of the EDTA-resolved thylakoids after recoupling with either the tentoxin-sensitive CF_1 or the tentoxin-resistant TF_1 are equally inhibited by tentoxin. Binding of TF_1 to the thylakoid membranes does not enhance the sensitivity of this ATPase to tentoxin (not shown). This suggests that TF_1 may only seal leaks for H^+ and other ions made during uncoupling. This is also supported by the finding that the same degree of reconstitution is achieved by adding either the native TF_1 or the inactivated TF_1 obtained by pretreating it with DCCD under conditions causing almost complete loss of its ATPase activity (Table I). In contrast, the DCCD-modified CF_1 is less effective than the native CF_1 in the reconstitution of photophosphorylation. Moreover, the degree of reconstitution obtained with either DCCD-modified CF_1, the native or DCCD-modified TF_1 are the same (Table I).

Reconstitution of ATP formation with NaBr-resolved thylakoids

NaBr particles have been shown to be devoid of CF_1. Compared with EDTA-extracted thylakoids they constitute a more appropriate membrane

TABLE I

RECONSTITUTION OF ATP SYNTHESIS OF EDTA-RESOLVED PARTICLES WITH CF_1 OR TF_1 MODIFIED BY DCCD

CF_1 and TF_1 were modified with DCCD at 37°C. The incubation mixture contained in a volume of 0.5 ml at pH 7.0 as follows: Mops-NaOH (50 mM)/EDTA (1 mM)/ADP (2 mM)/DCCD (0.3 mM) and 0.33 mg CF_1 or 0.54 mg TF_1. After 50 min the reacted mixture was passed through a Sephadex G-25 centrifuge column equilibrated with 50 mM Tricine-NaOH (pH 8.0). Modification by DCCD results in the inactivation of 98 and 95% of the CF_1 and TF_1 hydrolytic activity, respectively. The coupling factors were added to the EDTA-resolved particles (10.9 μg chlorophyll) as indicated. Reconstitution conditions and activity assay were as described in Materials and Methods. The activities of EDTA-resolved and control particles were 445 and 2180 μmol/mg chlorophyll/h, respectively.

Type of protein added	Photophosphorylative activity of EDTA-resolved particles reconstituted with coupling factor (μmol per mg chlorophyll/h)	
	Native	DCCD-modified
50 μg CF_1	1523	1261
101 μg CF_1	1663	1321
58 μg TF_1	1321	1251
117 μg TF_1	1260	1266

material for reconstitution studies. Adding CF_1 to NaBr-resolved thylakoid membranes results in the restoration of rather low rates of ATP formation

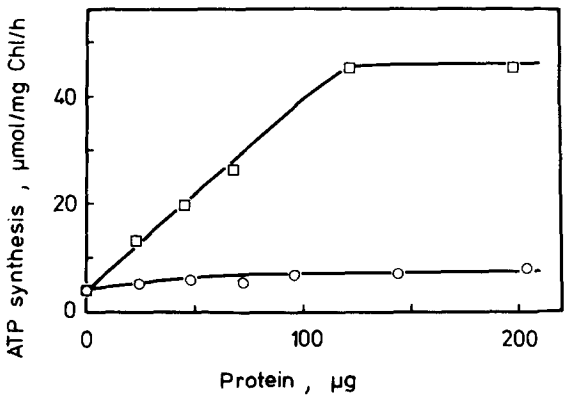

Fig. 3. Reconstitution of ATP synthesis of NaBr-resolved particles with CF_1 and TF_1, CF_1 (□) or TF_1 (○) as indicated were added to NaBr-resolved particles containing 53.6 μg chlorophyll.

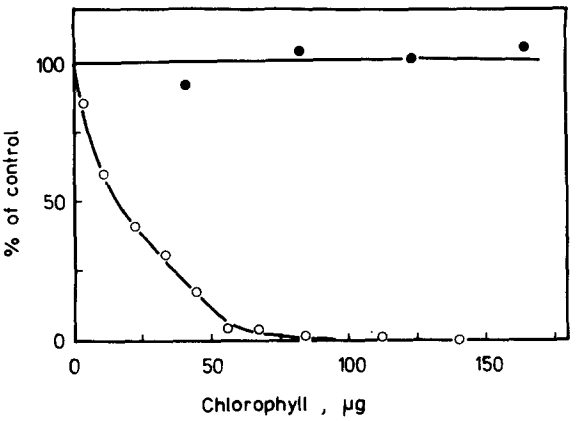

Fig. 4. Binding of TF_1 by NaBr-resolved particles. TF_1 samples (9 μg) were incubated in microcentrifuge tubes with the indicated amounts of control (●) or NaBr-resolved particles (○) suspended in the reconstitution mix. After 1 h at 4°C, the samples were centrifuged for 5 min at 12000 × *g*. Samples (0.1 ml) of the supernatants were assayed for ATPase.

(Fig. 3). However, even these low rates were not significantly enhanced by reconstitution with TF_1 (Fig. 3). The NaBr-resolved particles do, however, bind TF_1 (Fig. 4). These findings suggest that, although binding of either CF_1 or TF_1 occurs, a catalytically fully active F_0-F_1 complex is not formed. Under the conditions used, NaBr particles containing 65 μg chlorophyll were needed to bind 9 μg of TF_1. Binding of TF_1 to NaBr-resolved thylakoids also results in the reconstitution of the light-dependent H^+ uptake. The extent of recon-

TABLE II

H^+ UPTAKE BY NaBr-RESOLVED PARTICLES RECONSTITUTED WITH CF_1 AND TF_1

NaBr-resolved particles (50.3 μg chlorophyll) were reconstituted as indicated.

Reconstituted system	H^+ uptake (μmol/mg chlorophyll)	ATP synthesis (μmol per mg chlorophyll/h)
NaBr-resolved particles	0.06	6.1
+216 μg CF_1	0.13	19.9
+370 μg CF_1	0.19	24.8
+212 μg TF_1	0.18	8.0
+371 μg TF_1	0.19	7.6
Control chloroplasts	0.50	1000.0

stitution is quite similar to that obtained by adding CF_1 (Table II). This again indicates that both coupling factors repair leaky proton channels in the uncoupled particles equally well, but the catalytic function of ATP formation is not restored by simple rebinding of the protein.

Discussion

Binding of the F_1 protein complex to the F_0 moiety in the membrane requires recognition of the F_1 protein by the binding site on the membranal F_0. However, even though binding is rather specific, it does not a priori promise the formation of a catalytically active complex. As demonstrated above, TF_1 is recognized by the binding site on CF_0 and when bound, seals the membrane and prevents proton leakage. This suggests that at least a partly functional CF_0-TF_1 complex has been formed and that both TF_1 and CF_1 have homologous structures that enable recognition and attachment to occur. It is known that the δ and the ε subunits of the bacterial F_1's are needed for the binding of the F_1 moiety to the membranal F_0 [1–4,28]. Functional similarities between subunits from different F_1's might also be expected in view of the homology found in some of the amino acid sequences, as those of the ε subunits of *E. coli* [9] and CF_1 [10]. Similarities between amino acid sequences of F_1 and F_0 subunits from different sources were also reported [7,9–11].

Three methods are usually employed to uncouple chloroplast membranes before rebinding CF_1. Treatment with EDTA or with other low ionic strength media results in uncoupling and partial removal of CF_1 and gives a rather high degree of reconstitution by rebinding of CF_1 [20,25,26,29]. The other two methods involve almost complete removal of CF_1 by treatment of the membranes with NaBr [21] or silicotungstate [30]. However, the reconstituted rates of ATP formation after rebinding of CF_1 to these membranes are very low [28,30]. Since ATP formation is reconstituted by binding of TF_1 only to the EDTA-resolved particles which are only partially depleted of CF_1 but not to the NaBr particles, it seems that TF_1 can substitute for the extracted CF_1 only in its structural capacity but not in its catalytic role. It would seem that by rebinding of the added CF_1 or TF_1 plugs up leaks and enables the CF_1 still remaining in the membrane to act catalytically. The restoration of ATP synthesis by EDTA-resolved-particles by adding low concentrations of DCCD [29] or chlorotributyltin [31] is probably also the result of sealing leaks in the membrane and allowing the catalytic activity of the remaining CF_1 in situ to be expressed.

We concur with Shoshan and Selman [32] that the inactivated DCCD-modified CF_1 has a lower efficiency from that of the native CF_1 in the reconstitution of phosphorylation of EDTA-resolved particles (Table I). The difference between the activities of both types of preparations allows an estimate of the catalytic role of the added native coupling factor [32]. In our case, about 20% of the activity in the restored membranes appears to be due to the catalytic role of the added CF_1. Since native and DCCD-modified TF_1 are indistinguishable in their reconstitutive ability it therefore follows that TF_1 is not involved in the catalytic process proper. This interpretation is also supported by the similar sensitivity to tentoxin of the reactions catalyzed by the EDTA particles reconstituted, with either CF_1 or TF_1. ATP hydrolysis of CF_1 isolated from different plants and the photophosphorylation activity of the corresponding membrane systems have similar sensitivities towards the inhibitor tentoxin [12]. Since ATP hydrolysis by both the soluble and chloroplast membrane-bound TF_1 is tentoxin-resistant (Fig. 2), we assume that ATP formation with TF_1 is also a tentoxin-resistant reaction.

The phosphorylation rate of membranes reconstituted with CF_1 was only by 10–25% higher than with TF_1 (Fig. 1, Table I) which indicates that neither added membrane-bound -TF_1 nor -CF_1 do play a major catalytic role in ATP formation in the reconstituted membranes. The added coupling protein fulfills mainly a structural role and probably serves to seal ion leaks in the CF_1-extracted membranes without producing a catalytically active CF_0-CF_1 complex upon binding to the CF_0 moiety. However, Selman and Durbin [12] showed that only 25% of the ATP synthesis is resistant to tentoxin when EDTA-resolved particles from lettuce (tentoxin-sensitive) were reconstituted with CF_1 from tentoxin-resistant *Nicotiana tabacum*. The rate of ATP formation that is restored after EDTA

or silicotungstate uncoupling depends on the CF_1 remaining bound in the membrane [26,30]. Moreover, binding of CF_1 to NaBr- or silicotungstate-resolved particles results in very low rates of ATP formation, suggesting that the contribution of added CF_1 to the catalytic process is rather small. On the other hand, release of tightly bound ADP from CF_1 recoupled to thylakoid membranes [25] suggests that the rebound CF_1 undergoes conformational changes upon energization of the membranes. Energy dependent conformational changes of CF_1 labeled with fluorescamine [33] and eosine isothiocyanate [34] which was rebound to partially depleted-CF_1 thylakoids, were also reported. However, these findings cannot be taken as convincing proof for the catalytic function of the rebound CF_1, since the relevance of the release of tighly bound nucleotides or of the reported conformational changes, to the catalytic process, is not yet clear.

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