# SYNTHESIS OF COUPLING FACTOR $CF_1$ PROTEIN BY ISOLATED SPINACH CHLOROPLASTS

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## 1. Introduction

It has been demonstrated in several laboratories that isolated chloroplasts incorporate labeled amino acids into at least nine discrete polypeptides, distinguishable by SDS-polyacrylamide gel electrophoresis, and are therefore capable of synthesizing certain of their own proteins [1–5]. The labeled polypeptides were found to be distributed among the soluble proteins, the thylakoid membrane fractions [1,2,3,5], and the envelope membranes [4,5]. Blair and Ellis [1] have identified the major labeled soluble polypeptide with the large subunit of ribulose diphosphate carboxylase. The identities of the other labeled polypeptides remain unknown.

We have examined the possible synthesis of chloroplast coupling factor $CF_1$ by spinach chloroplasts through the use of a procedure developed by Strotmann et al. [6] for the preferential extraction of coupling factor from spinach chloroplasts. Our results show that, contrary to previous indications [2], the two largest subunits of coupling factor $CF_1$ and possibly the smallest inhibitory subunit are made in this organelle.

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## 2. Methods

### 2.1. *Extraction of coupling factor $CF_1$*

Chloroplasts from greenhouse spinach (*Spinacea olereacas*, L.) were isolated be centrifugation in density gradients of silica sol as described before [7].

Coupling factor was extracted from the chloroplasts following the method of Strotmann et al. [6]. The purified chloroplasts were first washed three times with 10 mM sodium pyrophosphate at pH 7.4. The pelleted membranes were subsequently washed with 300 mM sucrose containing 2 mM Tricine buffer at pH 7.8. All the extracts were clarified by centrifugation at 50 000 rev/min for 1 h (Damon/IEC, A-321 rotor). Washing with the sucrose solution extracted mainly coupling factor, whereas the extracts made with dilute sodium pyrophosphate solution contained mostly ribulose diphosphate carboxylase along with some minor proteins [6].

### 2.2. *Other methods*

The conditions for the incorporation of amino acids into isolated chloroplasts and for the electrophoretic analyses on SDS-polyacrylamide gels were as described previously [5,8]. The labeled amino acids were either [$^{35}$S]methionine (spec. act. 280 Ci/mmole) or [$^{3}$H]leucine (spec. act. 83 Ci/mmole) obtained from New England Nuclear, Boston, Mass.

For electrophoresis on SDS-polyacrylamide gels, aliquots of the extracts were either first concentrated by precipitation with 10% (w/v) trichloracetic acid or directly diluted (1 : 1) with the sample buffer for electrophoresis.

The conditions for the electrophoretic separation

of proteins on gels not containing SDS were similar, except that the separation gel contained 5% polyacrylamide. Aliquants of the extracts, containing 10% (w/v) glycerol were layered directly on the gels.

## 3. Results

Our electrophoretic analyses of chloroplast extracts on non-SDS-polyacrylamide gels confirm the observations of Strotmann et al. [6] that dilute salt and sucrose solutions extract quite different sets of proteins. The first sodium pyrophosphate extract and the sucrose extract each contained one distinctive major protein component (designated I and II in fig.1a, b) which Strotmann and his co-workers had previously shown to be ribulose diphosphate carboxylase and coupling factor $CF_1$, respectively. That the proteins in the two kinds of extracts are nonidentical is indicated by their different mobilities on the gels and the resolution between them where the two extracts were co-electrophoresed (fig.1c).

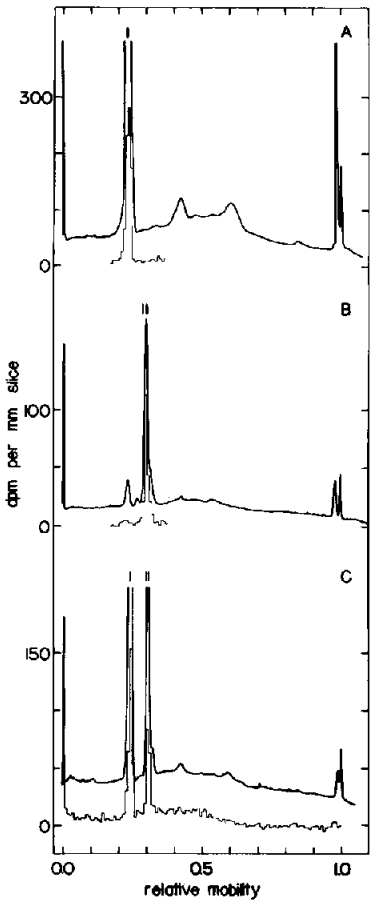


Fig.1. Electrophoretic patterns of extracts from chloroplasts incubated with [$^{35}$S]methionine on 5% polyacrylamide gels not containing SDS. The absorbancy profile of the stained gels scanned at 530 or 563 nm is shown by the solid trace. The histogram under each absorbancy tracing is the radioactivity measured on each 1 mm gel slice. (A) First sodium pyrophosphate extract. (B) Sucrose extract. (C) Mixture of sodium pyrophosphate and sucrose extracts.

When the chloroplasts are incubated in the light with [$^{35}$S]methionine, both the carboxylase and the coupling factor proteins become distinctly labeled, as shown by the radioactivity profiles in fig.1. The patterns of radioactivity in figs.1a and 1b also indicate very little, if any, cross-contamination between the two proteins in the extracts. Although the synthesis in vitro of the large subunit of ribulose diphosphate carboxylase has been shown previously [1,3,5], this is the first report on the synthesis of coupling factor $CF_1$ in isolated chloroplasts.

The coupling factor $CF_1$ from spinach chloroplasts has been shown to consist of 5 subunits designated $\alpha, \beta, \gamma, \delta$, and $\epsilon$, with mol. wts. of 59 000, 56 000, 37 000, 17 500 and 13 000, respectively [9,10]. Since we found the native protein to be labeled, it was of great interest to determine whether all or only some of the subunits of the coupling factor were made in the chloroplast. We therefore examined the polypeptide and radioactivity patterns of the extracts after electrophoresis on SDS-polyacrylamide gels.

The polypeptide profile from the sucrose extract in SDS gels showed 2 major slow-moving components along with smaller amounts of other polypeptides (fig.2a). The profile is in general quite similar to the electrophoretic patterns reported for the subunits of purified coupling factor $CF_1$ from spinach [11]. The $\alpha, \beta$ and $\gamma$ subunits are recognizable from the relative amounts of these polypeptides as well as their relative mobilities, although our calculated molecular weights for these three polypeptides (based on the mobilities of standard proteins on 12.5% gels) are somewhat lower than the reported values. The minor protein peaks in the region of the gels corresponding to the mol. wts. of approx. 17 000–12 000 are real and become quite distinct in gels which had been loaded with more protein (cf. fig.3). Two of these peaks

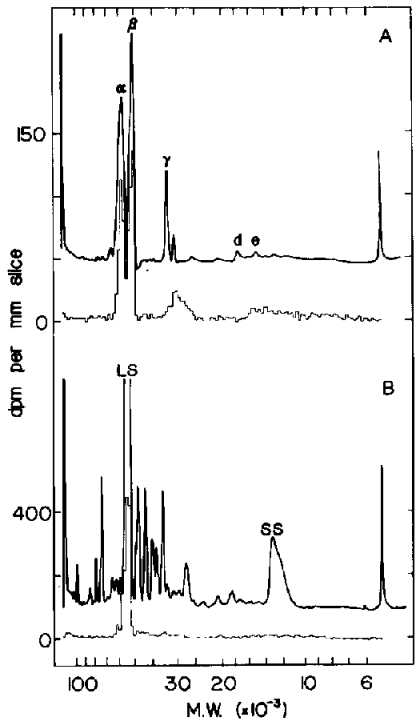


Fig.2. SDS-polyacrylamide gel electrophoretic patterns of extracts from chloroplasts which had been incubated with [$^{35}$S]methionine. Gels were 12.5% w/v in acrylamide. The mol. wts. were estimated from the mobilities of standard proteins. (A) Sucrose extract showing subunits of coupling factor $CF_1$. (B) First sodium pyrophosphate extract. LS and SS refer to the positions of the large and small subunits, respectively, of ribulose diphosphate carboxylase.

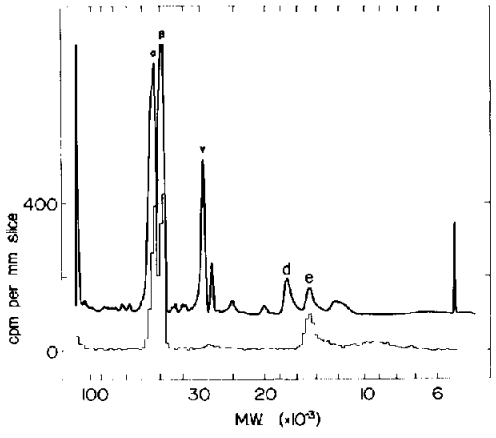


Fig.3. SDS-polyacrylamide gel electrophoresis of the sucrose extract obtained from chloroplasts incubated with [$^{3}$H] leucine.

designated d and e may correspond to the $\delta$ and $\epsilon$ subunits of the coupling factor.

The radioactivity profile in fig.2a shows that the two largest subunits, $\alpha$ and $\beta$, are also the major labeled components in the extracts containing coupling factor. No radioactivity is seen in the region of the gel corresponding to the $\gamma$ subunit. Instead, a small zone of radioactivity coincides with the small polypeptide migrating slightly faster than the $\gamma$ subunit. To insure better correspondence between the polypeptide and radioactivity profiles, some of the stained bands were marked with fine bristles before the gels were sliced.

In contrast to the sucrose extract, the SDS-polyacrylamide gels of the first sodium pyrophosphate extract showed only one large peak of radioactivity, coincident with the position of the large subunit of ribulose diphosphate carboxylase (fig.2b). Both the polypeptide profile and the pattern of radioactivity were very similar to those we obtained for the soluble protein fraction of spinach chloroplasts [5].

So far, we have shown that the two largest subunits, $\alpha$ and $\beta$, but not the $\gamma$ subunit of coupling factor $CF_1$ become labeled when the chloroplasts are incubated with [$^{35}$S]methionine. We wondered if the small subunits might also be labeled but escape detection due to their presence in relatively minor amounts. In gels which had been loaded with more material, we observed a barely perceptible peak of radioactivity coincident with the position of peak e in fig.2a. Since the content of leucine is 4 to 5 times that of methionine in the $\alpha$, $\beta$, and $\gamma$ subunits and 13 times in the smallest subunit [10,11], we sought to improve sensitivity be testing the incorporation of leucine into the polypeptides in the extracts of coupling factor.

Isolated chloroplasts were therefore incubated with [$^{3}$H]leucine, extracted as before, and the sucrose extract was again analyzed by electrophoresis on SDS-polyacrylamide gels. The resulting polypeptide profile in fig.3 is qualitatively identical to that in fig.2a, but the small minor peaks are now more distinct. As with the labeling with [$^{35}$S]methionine, the major components labeled with leucine are the $\alpha$ and $\beta$ subunits. This time, however, there is a very distinct peak of radioactivity which coincides exactly with the position of peak e. We suspect that this polypeptide might correspond to the smallest

subunit of coupling factor $CF_1$; however, our best estimate of its mol. wt. is about 13 700, based on co-electrophoresis with ribonuclease. Curiously, the small peak of radioactivity migrating just ahead of the $\gamma$ subunit in the [$^{35}$S]methionine-labeled polypeptides (cf. fig.2a) is barely perceptible with [$^{3}$H] leucine.

## 4. Discussion

Our results indicate that spinach chloroplasts are capable of synthesizing certain portions of coupling factor $CF_1$. This conclusion had been excluded by Eaglesham and Ellis [2] in their work with pea chloroplasts because of the absence of any effects of EDTA on the labeling pattern of the thylakoid membranes. In our experiments the two largest subunits of the coupling factor, and possibly the smallest subunit as well, are synthesized by isolated chloroplasts. The possible identity of the minor labeled polypeptide e with the smallest subunit of $CF_1$ will have to be confirmed, using purified preparations of the enzyme.

In any case, it is evident that only certain subunits of $CF_1$ protein are made in the chloroplast, while others are made in the cytoplasm. This is analogous to the situation with ribulose diphosphate carboxylase, where only the large subunit of this enzyme is made in the chloroplast. Thus far, these are the best characterized among the chloroplast proteins that have been shown to be synthesized in this organelle. In both cases the enzymes are composed of two or more subunits and in both cases subunits are synthesized in separate loci. We wonder if this may be a general phenomenon for all proteins synthesized in the chloroplast. The case of the synthesis of chloroplast ribosomal proteins in *Chlamydomonas* may be analogous, with some coded by nuclear DNA and others by DNA of the chloroplast [12].

A pattern of cooperation between cytoplasmic and organellar informational systems is clearly demonstrated in the synthesis of cytochrome oxidase, cytochrome *b*, and oligomycin-sensitive ATPase complex in mitochondria (cf. review, [13]).

The oligomycin-sensitive ATPase of mitochondria is of special interest to us as a functional and structural analog of the chloroplast coupling factor [14]. The mitochondrial enzyme also consists of 5 subunits with mol. wts. similar to those of $CF_1$; however, in contrast to the chloroplast enzyme, all 5 subunits of the mitochondrial ATPase are synthesized in the cytoplasm, along with the protein that confers oligomycin sensitivity. The four hydrophobic proteins associated with the ATPase and believed to function in the binding of the enzyme to the mitochondrial membrane are thought to be made in the organelle.

There is one curious discrepancy between our results and those of Blair and Ellis [1], which concerns the electrophoretic behavior of ribulose diphosphate carboxylase on non-SDS-polyacrylamide gels. Blair and Ellis electrophoresed soluble proteins from pea chloroplasts under non-dissociating conditions and found a single radioactive peak that migrated slightly faster than native carboxylase. Consequently, they inferred that the newly synthesized large subunit does not exchange with preformed carboxylase but occurs as an aggregate, and that isolated chloroplasts contain little or no pool of small subunits. Our results with spinach chloroplasts show an exact coincidence of radioactivity and protein both in the case of carboxylase and of coupling factor (cf. fig.1). The question of the existence of pools of cytoplasmically derived protein subunits in chloroplasts is central to our understanding of the regulation of protein synthesis in these organelles and deserves the closest examination.

## Acknowledgements

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