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LIGHT/DARK LABELING DIFFERENCES IN CHLOROPLAST MEMBRANE POLYPEPTIDES ASSOCIATED WITH CHLOROPLAST COUPLING FACTOR 0

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Summary

The fluorogenic reagent fluorescamine has been used to determine the labeling patterns of Type C spinach chloroplast membrane polypeptides. Membrane polypeptides labeled with fluorescamine were detected by scanning high resolution sodium dodecyl sulfate polyacrylamide gradient slab gels for fluorescence emission.

Three membrane polypeptides show a decrease in the extent of labeling when chloroplast membranes are labeled in the light compared to when they are labeled in the dark. These polypeptides have apparent molecular weights of 32 000, 23 000 and 15 000.

The decrease in labeling observed in the light is abolished or reduced by treatments which inactivate the light-generated transmembrane pH gradient. CF_1-depleted chloroplasts show neither a light-activated pH gradient nor a light/dark difference in labeling of these three polypeptides. Both a light-activated pH gradient and light/dark differences in labeling are observed in CF_1-depleted chloroplasts which have been treated with *N,N'*-dicyclohexylcarbodiimide.

The same ammonium sulfate fractions of a 2% sodium cholate extract, which are believed to be enriched in the membrane-bound sector of the chloroplast ATPase (CF_o) are also found to be enriched in the 32 000, 23 000 and 15 000 molecular weight polypeptides. The three polypeptides are believed to be components of CF_o, and the light/dark labeling differences may indicate conformational changes within CF_o. Such conformational changes may reflect a mechanism which couples light-generated proton gradients to ATP synthesis.

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Abbreviations: CF_1, chloroplast coupling factor 1; CF_o, hydrophobic portion from chloroplast ATPase; DCCD, *N,N'*-dicyclohexylcarbodiimide; DCMU, 3-(3,5-dichlorophenyl)-1,1-dimethylurea; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; SDS, sodium dodecyl sulfate.

Introduction

The complex responsible for ATP synthesis in chloroplast membranes consists of a soluble portion, CF_1, which in solution can act as an ATPase [1,2] and an intrinsic membrane portion, CF_o *, the membrane binding site of CF_1 which apparently contains the membrane proton pore [3]. Removal of CF_1 from chloroplast membranes abolishes the light-generated pH gradient [4]. This gradient can be restored by incubating CF_1-depleted membranes with DCCD [4].

Although the subunit composition and many of the functional properties of CF_1 are known (for a recent review see ref. 5), the nature of CF_o is less well characterized. The extraction of chloroplasts with sodium cholate by Younis and Winget [6] yields a fraction containing several polypeptides which, when reconstituted in liposomes together with bacteriorhodopsin and purified CF_1, catalyze a light-dependent, *N,N'*-dicyclohexylcarbodiimide (DCCD)-sensitive ATP synthesis. This particular fraction thus apparently contains the component(s) constituting CF_o. The exact interaction between CF_o and CF_1 which couples a light-driven pH gradient to ATP synthesis is not known. Light-induced changes in labeling patterns [7—11] and nucleotide binding affinities [12—16] have, however, been interpreted as evidence that CF_1 can undergo conformational changes in response to membrane energization. Evidence implying that CF_o also undergoes conformational changes has not, to our knowledge, been reported. During the course of investigations of labeling chloroplast membranes in the light and in the dark with the fluorogenic reagent fluorescamine [17], we have observed light/dark labeling differences in the labeling of three membrane polypeptides having molecular weights of approximately 32 000, 23 000 and 15 000. These polypeptides are identical with three major polypeptides of the sodium cholate extract which reconstitutes light-sensitive ATP synthesis. The differences in labeling which we describe here are associated with the chloroplast membrane proton gradient, and they may reflect conformational changes in proteins that participate in light-induced proton movement.

Methods

Chloroplast isolation. Type C [18] chloroplast membranes, those lacking the outer limiting membrane, were prepared from spinach by the method of Avron [19]. Final suspension was either in a medium consisting of 0.4 M sucrose/0.05 M Tricine (pH 8.0)/0.01 M KCl/2 mM $MgCl_2$, or in either of two low-salt media: 5 mM phosphate or 5 mM Tricine, pH 8.0. The chloroplasts were suspended to a final chlorophyll concentration of 100 μg/ml. Chlorophyll determinations were performed according to a modification [20] of the method of MacKinney [21].

Fluorescamine labeling and sample preparation. The labeling procedure consisted of adding, while vortexing, enough 0.4% (w/v) fluorescamine (Pierce)

* We consider 'CF_o' to consist of the membrane sector of the DCCD-sensitive ATPase complex which not only includes the actual DCCD-sensitive site, but also the CF_1 binding site.

in acetone to a suspension of chloroplasts at 20—22°C to achieve a final acetone concentration of 0.5% (v/v). Chloroplasts to be labeled in the light were illuminated with white light ($1 \cdot 10^6$ erg/cm$^2 \cdot$ s^{-1}) at least 5 s before the addition of fluorescamine. For labeling in the dark, chloroplasts were kept in the dark for at least 5 min before addition of the labeling reagent. The labeled chloroplast membranes were centrifuged for 10 min at 10 000 × *g*. The resulting pellet was solubilized to a final protein concentration of 2 mg/ml in a pH 8.4 buffer consisting of 6 M urea/0.5% SDS/0.01 M EDTA/0.1 M Tris. Protein concentrations were determined by the method of Schaffner and Weissmann [22]. Prior to electrophoresis, mercaptoethanol was added to 1% (v/v) to the solubilized membranes; this solution was heated for 15 s in boiling water before an aliquot was loaded onto the gel.

Cholate extraction. Chloroplasts isolated and labeled in the dark or in the light, as described above, were extracted with sodium cholate according to a modification of the procedure of Younis and Winget [6]: instead of a 1 h, 229 000 × *g* centrifugation of the initial 2% cholate extract, a 20 min, 20 000 × *g* centrifugation was used. Fractions precipitating between 33—39% and 39—49% saturation (at 20°C) in ammonium sulfate were centrifuged for 10 min at 20 000 × *g* and solubilized for electrophoresis.

Electrophoresis. Linear 7.5—15% polyacrylamide gradient slab gels (14 × 18 × 0.07 cm) topped with a 1.5 cm, 5% polyacrylamide stacking gel were made according to the method of Laemmli [23]. Solubilized samples containing 25—60 μg of protein were loaded into each well, and electrophoresis proceeded overnight at 5—8°C with a constant current setting between 7.5 and 11 mA. Gels were stained with Coomassie Blue and destained according to Fairbanks et al. [24].

Fluorescence detection. Different lanes of the processed gel were sliced with the aid of a circular pizza cutter. Each gel slice was trimmed to 13 cm to fit a rectangular glass cuvette, and the slices were scanned for fluorescence using a fluorescence gel scanner constructed in part from the gel transport and monochrometer units of a Zeiss M4QIII gel scanning spectrophotometer. Excitation light from a 100 W tungsten projection lamp powered by an Aminco regulated DC power supply was modulated at 300 Hz with a chopping wheel. A Corning 7-51 filter placed at the exit slit of the monochrometer blocked unwanted stray light from passing through the detection system. Broad band emission originating from the gel passed through a Corning 4-64 and Wratten No. 4 filter and was detected by a photomultiplier tube (EMI 9558 operated at 900 V). The modulated component of the detected signal was amplified using a Princeton Applied Research HR-8 Lock-in amplifier. Recordings were made with an Esterline Angus Speed Servo Recorder.

Results

The distribution of Type C spinach chloroplast membrane polypeptides separated by SDS polyacrylamide gel electrophoresis and stained with Coomassie Blue is shown in Fig. 1a. The transmission scan at 550 nm is shown in Fig. 1b. Polypeptide bands are numbered sequentially starting at the high molecular weight end of the gel. This distribution is similar in appearance to

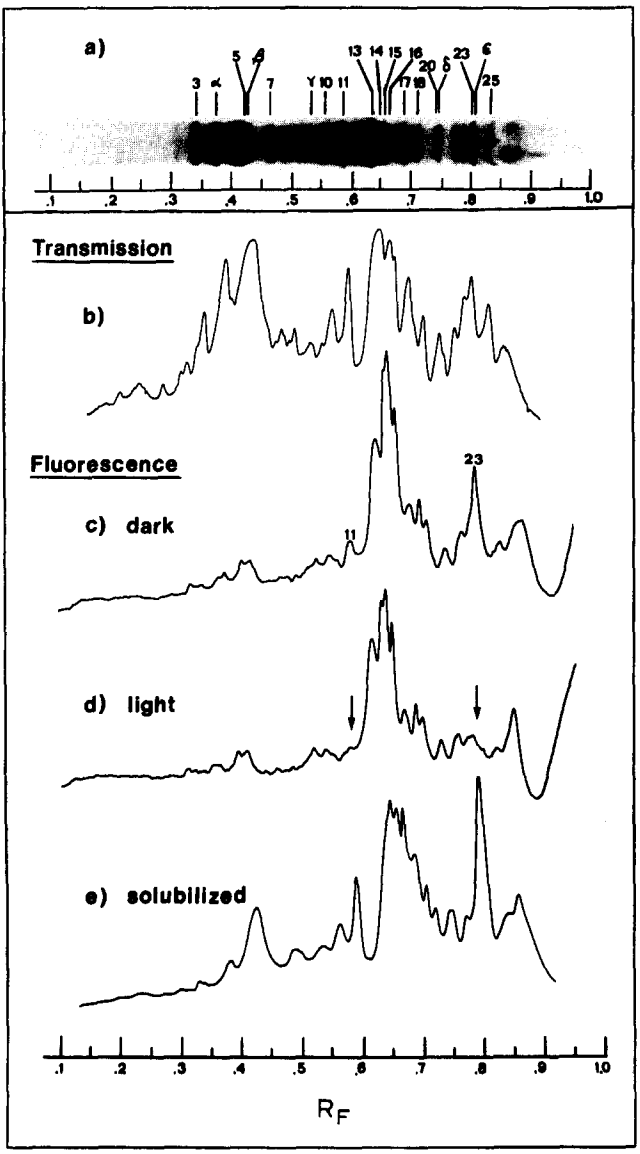

Fig. 1. (a) Coomassie staining pattern of chloroplast membrane polypeptides separated by SDS polyacrylamide electrophoresis. Major polypeptide bands have been numbered in order of decreasing molecular weight. The positions of the five subunits of CF_1 have been identified as α, β, γ, δ, and ϵ and correspond to molecular weights of 62 000, 57 000, 38 000, 18 000 and 14 000 [29], respectively. (b) Transmission scan at 550 nm of the gel slice in (a). (c) Fluorescence scan of an unstained gel slice showing the fluorescence labeling pattern of polypeptides originating from chloroplast membranes labeled with fluorescamine in the dark. (d) Same as (c), but with fluorescent labeling pattern of polypeptides originating from illuminated chloroplasts. Arrows mark the positions where bands 11 and 23 would appear. (e) Fluorescence profile of chloroplast membrane polypeptides labeled with fluorescamine after solubilization of the membranes in 1% SDS at pH 8.0. The horizontal dimension, R_F, for (a)—(e) is measured as the relative retention factor of polypeptides along the length of the gel slice relative to the buffer front. The gel slices have been truncated to fit a 13 cm scanning cuvette. The vertical axis in (b) measures relative transmission at 550 nm, and vertical axes in (c)—(d) measure fluorescence intensity in arbitrary units. The methodology for membrane preparation, labeling, solubilization and gel electrophoresis and scanning is given in Methods.

that reported by Chua et al. [25] and Henriques et al. [26]. In order to determine which polypeptides of Type C chloroplast membrane are accessible to and can react with fluorescamine, membranes were incubated with the reagent either in the light or in the dark, pelleted, solubilized in SDS, separated by SDS polyacrylamide gel electrophoresis, and scanned for fluorescence as described in Methods.

Light/dark fluorescamine labeling patterns. Fig. 1c shows a fluorescence profile of chloroplast membrane polypeptides labeled after an aliquot of fluorescamine was added to a suspension of chloroplasts in the dark, while Fig. 1d is the pattern obtained when fluorescamine was added in the light. Both traces can be compared to the fluorescence pattern in Fig. 1e obtained when chloroplast membranes were solubilized in SDS before fluorescamine was added to the sample.

The major labeled polypeptides obtained from intact membranes include the cluster of bands 13—16, two of which (band 13 and 15) are subunits of the chlorophyll *a/b* light-harvesting protein complex [27,28]. Several major Coomassie staining bands including the α and β subunits of CF_1 band 5, the 57 000 molecular weight major subunit of ribulose 1,5-diphosphate carboxylase [29] and band 3, the 68 000 molecular weight polypeptide which derives from the chlorophyll · protein complex of Photosystem I [28], are not significantly labeled.

It can be seen from Figs. 1c and 1d that the labeling of bands 11 and 23 is greater in the dark than in the light. This difference in labeling pattern is seen in chloroplasts suspended either in the normal isolation medium (the conditions for Figs. 1c and 1d) or a low-salt medium such as 5 mM phosphate buffer (Figs. 2a and 2b). In contrast, when chloroplasts are suspended in 5 mM Tricine buffer, the pattern of labeling in the light is identical to that seen in the dark (Fig. 2c) and is similar to the labeling pattern in Fig. 2a, except that the labeling of band 11 has increased relative to that of band 23. A labeling pattern similar to Figs. 2a and 2b was obtained when chloroplasts were labeled after addition of either millimolar levels of divalent cations (Ca^{2+}, Mn^{2+}, or Mg^{2+}) or decimolar levels of K^+ or Na^+ to the 5 mM Tricine.

Dependence of labeling pattern on photosynthetic activity. Although the addition of electron acceptors such as ferricyanide may slightly increase the degree of light/dark labeling of bands 11 and 23, the phenomenon is easily observed with basal electron transport activity. The inhibition of electron transport with DCMU (3-(3,5-dichlorophenyl)-1,1-dimethylurea) diminishes the light/dark difference in labeling (Figs. 3a and 3b). The addition of such agents as FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), nigericin in the presence of KCl, gramicidin (Figs. 3c and 3d), and the divalent cation ionophore A23187, all of which uncouple photophosphorylation, inhibit the light-induced pH gradient and abolish the difference in the light/dark labeling levels of bands 11 and 23. In contrast, phloridzin, an uncoupler of photophosphorylation but not of light-induced pH gradients [30], had no noticeable effect on the labeling pattern.

Although we have often noted that all of the bands are labeled to a lesser degree (up to 30%) in the light than in the dark, only bands 11 and 23 show a complete loss of labeling in the light. It is this latter change which is sensitive

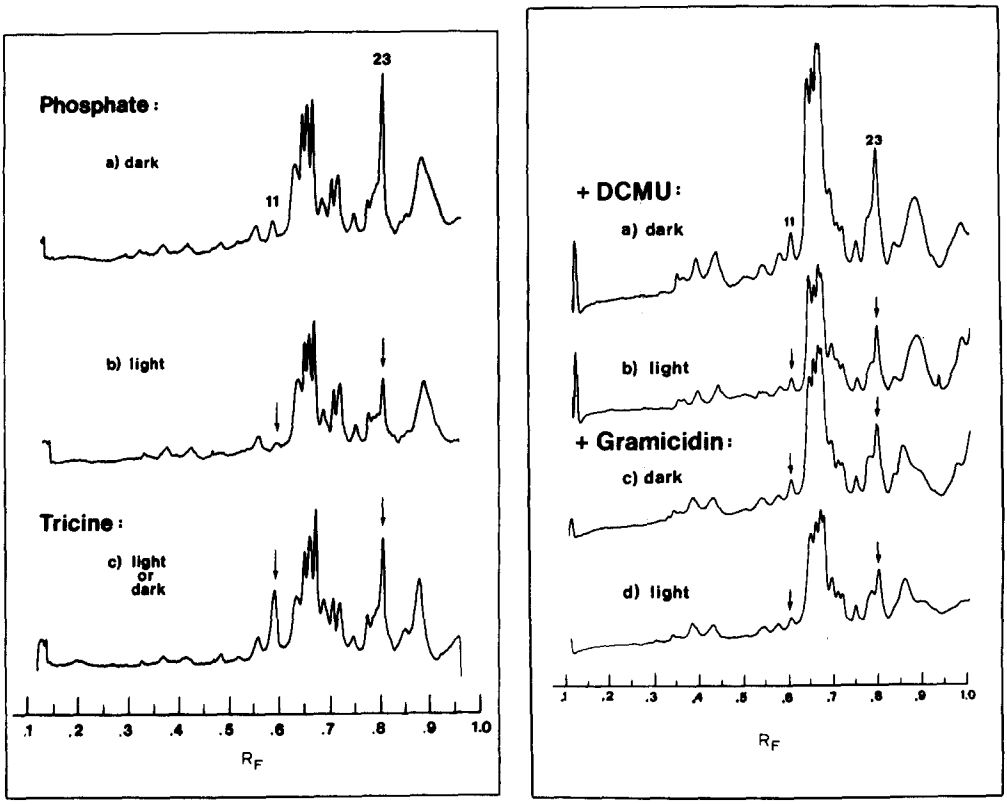

Fig. 2. Light/dark fluorescence labeling patterns of polypeptides obtained from chloroplasts suspended in 5 mM phosphate buffer, pH 8.0, labeled with fluorescamine in the dark (a) or in the light (b). (c) The pattern seen in either light or dark when chloroplasts are suspended in 5 mM Tricine, pH 8.0 instead of 5 mM phosphate buffer.

Fig. 3. Fluorescence labeling pattern of polypeptides obtained from chloroplasts suspended in 0.4 M sucrose/0.05 M Tricine (pH 8.0)/0.01 M KCl/2 mM $MgCl_2$ with: $5 \cdot 10^{-6}$ M DCMU added and labeled with fluorescamine in the dark (a) or in the light, (b); $5 \cdot 10^{-6}$ M gramicidin D added and labeled in the dark (c) or in the light (d).

to electron transport inhibitors and uncouplers.

Relation of light/dark labeling differences to the presence of CF_1. Removal of CF_1 by EDTA results in a loss of the light/dark difference in the extent of labeling of bands 11 and 23 (Figs. 4a and 4b). Since the loss of CF_1 also results in the loss of the pH gradient [4], the absence of the light/dark difference in labeling in CF_1-depleted chloroplasts is consistent with the effects of uncouplers described above. When the pH gradient is restored with DCCD, moreover, the light/dark difference in the labeling pattern returns (Figs. 4c and 4d). The magnitude of the labeling difference in CF_1-depleted, DCCD-treated chloroplasts was at least as large as the change observed in chloroplasts retaining CF_1 (Figs. 1c and 1d).

Artificial pH gradients. The decrease in labeling of bands 11 and 23 brought about in the light can be partially mimicked in the dark using an artificially generated pH gradient (cf. Figs. 5a and 5b). This observation was made when

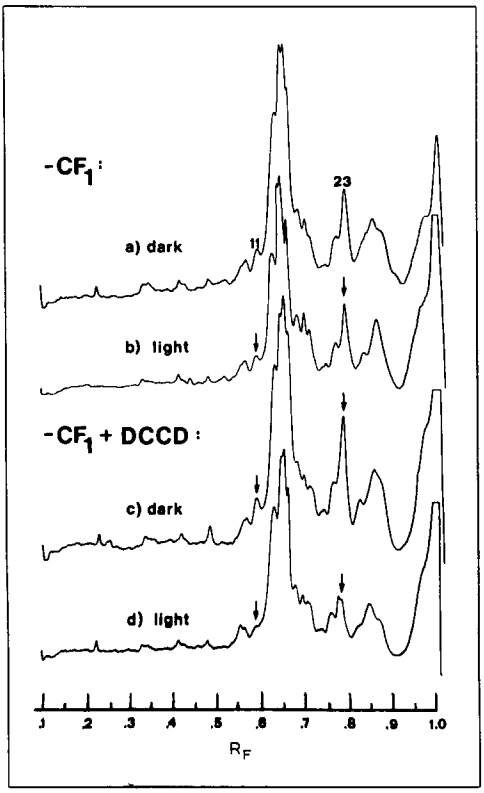

Fig. 4. Fluorescence labeling patterns obtained from chloroplasts depleted in CF_1 and labeled with fluorescamine in the dark (a) or in the light (b); CF_1-depleted chloroplasts incubated with $7.5 \cdot 10^{-5}$ M DCCD and labeled in the dark (c) or in the light (d). CF_1-depleted chloroplasts were prepared according to the procedure of McCarty and Racker [56].

the labeling reagent was added immediately after the addition of the base phase (Fig. 5b). However, when the labeling reagent was added to acid-base treated chloroplasts between 5 and 30 s after the addition of the base phase (Fig. 5a) the labeling of bands 11 and 23 had returned to a higer level characteristic of chloroplasts labeled in the dark.

Relation of labeling pattern to CF_o. Noting that the light/dark labeling difference of bands 11 and 23 apparently depends upon the generation of a pH gradient and not the presence of CF_1, we turned our attention to the relation of labeling patterns to membrane detergent fractions containing components which apparently include CF_o [6,31,32]. Chloroplast membranes which had been labeled either in the light or in the dark were extracted with 2% cholate according to the method of Winget et al. [32]. In order to examine the pertinent ammonium sulfate fractions analyzed by Winget et al. (33—39% [32]), and Younis and Winget (35—50% [6]), we looked at two fractions: 33—39% and 39—49% saturation in ammonium sulfate.

The 33—39% fraction is similar to that shown by Winget et al. [33]; it contains a considerable amount of CF_1, whose α, β, γ, δ, and ϵ subunits are identified in Fig. 6. In addition, three major bands, 10, 11, and 16, similar to those

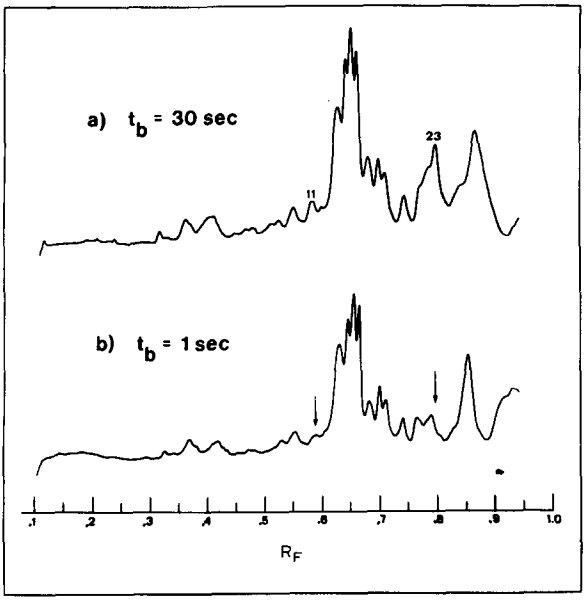

Fig. 5. Labeling pattern of polypeptides obtained from chloroplast membranes subjected to an artificial pH-jump and labeled (a) 30 s after addition of base and (b) 1 s after the addition of base. Chloroplasts (100 μg/ml chlorophyll) were initially suspended in 0.4 M sucrose/0.01 KCl/2 mM $MgCl_2$/3 mM succinic acid, pH. 5. An aliquot of 0.1 M KOH was added with vortexing to bring the final pH up to 9.5. Fluorescamine was added at a time t_b after the addition of base.

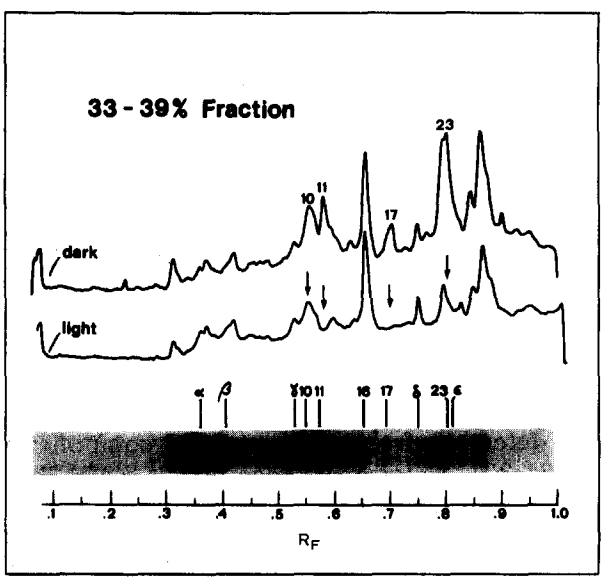

Fig. 6. The Coomassie staining pattern and fluorescence profiles of chloroplast membrane polypeptides extracted with 2% sodium cholate and precipitated between 33 and 39% saturation in ammonium sulfate. Chloroplasts were labeled either in the dark (upper curve) or the in the light (lower curve) before extraction with cholate. The Coomassie staining pattern for both samples was identical. Numbers above the Coomassie picture identify relevant polypeptides discussed in the text. The α, β, γ, δ, and ε subunits of CF_1 have also been identified.

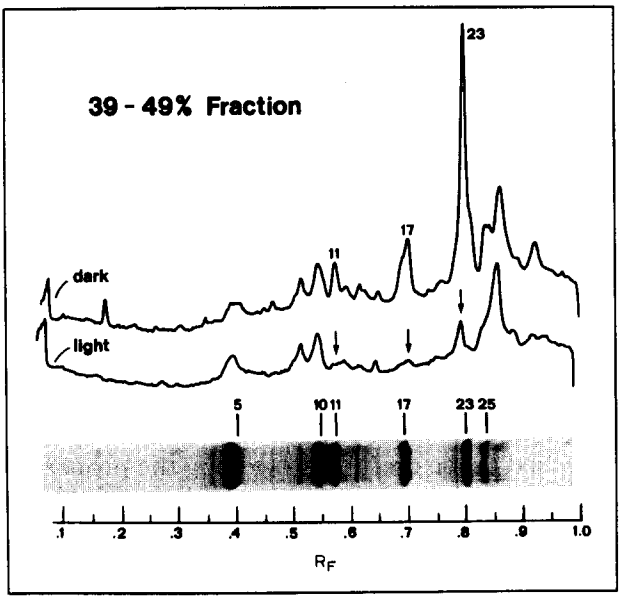

Fig. 7. The Coomassie pattern and fluorescence profile of chloroplast membrane polypeptides extracted with 2% sodium cholate and precipitated between 39 and 49% saturation in ammonium sulfate. Chloroplasts were labeled in the dark (upper curve) or in the light (lower curve) prior to extraction.

shown by Winget et al. [32] are evident. These three bands have apparent molecular weights of 35 000, 32 000 and 25 000, respectively. However, our estimated molecular weight of 35 000 for band 10 differs from the 42 000 value for the corresponding major polypeptide reported by Younis and Winget [6]. In contrast to their results, at least 10 minor bands, including band 23, are also observed, most probably reflecting the inherently greater resolution of a gradient slab gel system over a uniform tube gel system.

In contrast to the two bands (11 and 23) showing light/dark labeling differences in the whole membrane preparation (e.g., Fig. 1), three light/dark labeling differences in the fluorescence labeling pattern can be observed among the polypeptides recovered in the 33—39% fraction. Two of these correspond to the familiar bands 11 and 23, while a third, band 17, having an apparent molecular weight of 23 000, presumably corresponds to band 8 of Younis and Winget [6]. The light/dark labeling difference of this polypeptide is difficult to discern in whole membrane preparations (see Figs. 1c and 1d, for example).

Six major Coomassie staining bands appear in the 39—49% ammonium sulfate fraction (Fig. 7). Two of these, bands 5 and 25, having apparent molecular weights of 54 000 and 13 000, are the major and minor subunits of ribulose 1,5-diphosphate carboxylase [29]. The other four bands, 10, 11, 17, and 23 are all apparently hydrophobic components of the chloroplast membrane and may in part be identical to CF_o. Of these, 11, 17, and 23 show light/dark labeling differences.

Discussion

Fluorescamine has been used to label the proteins of intact cell membranes [34,35], and although some results have been interpreted as the labeling of external proteins [34,35], recent evidence [36] indicates that, because of its hydrophobic nature, fluorescamine partitions into the interior of lipid bilayers. We have found, for example, that polypeptide subunits of chloroplast membrane proteins such as CF_1 and ribulose 1,5-diphosphate carboxylase which are known to be externally exposed [37—40] are poorly labeled. On the other hand, we find extensive labeling of both of the polypeptide subunits of the light-harvesting chlorophyll protein, a complex which is believed to be buried within the membrane bilayer [41—44]. Thus, fluorescamine does not appear to be a reliable reagent for detecting proteins that are exposed on the surface of chloroplast membranes.

Fluorescamine has, however, proved to be useful for studying the properties of chloroplast proteins for which the number of groups available to react with the reagent vary depending on whether the membranes are in the dark or the light. When spinach chloroplast membranes are incubated in the light in the presence of fluorescamine, SDS polyacrylamide gel electrophoresis reveals that three membrane polypeptides, numbers 11, 17, and 23, having molecular weights of 32 000, 23 000 and 15 000, respectively show little or no fluorescence compared to that seen when incubation occurs in the dark.

The increased labeling in the dark can be mimicked in the light if CF_1 is removed prior to the addition of fluorescamine, and a similar result is obtained in the light when photosynthetic electron transport is inhibited with DCMU. Incubation in the light with uncouplers of photosynthetic phosphorylation which abolish the light-induced pH change also results in an increase in the fluorescamine labeling of these polypeptides. Furthermore, the addition of DCCD to CF_1-depleted chloroplast membranes restores the light/dark difference in labeling of the polypeptides, and the difference can be obtained in the dark when an artificial pH gradient is established across the chloroplast membranes. It appears, therefore, that the light/dark difference in the reactivity of polypeptides 11, 17, and 23 with fluorescamine is correlated with the occurrence of proton movement across the chloroplast membrane, but that the polypeptides involved are not derived from CF_1.

Bands 11, 17, and 23 appear in the same ammonium sulfate fractions of 2% cholate extracts of chloroplast membranes that reconstitute light-dependent ATP synthesis [6,32]. The molecular weights of these polypeptides also bear a striking resemblance to those of three polypeptides (molecular weights of 32 000, 23 000 and 11 000 [45]) which are major constituents of a cholate fraction containing the proton-translocating ATPase of bovine heart mitochondria [45]. It has been infered that these three polypeptides are a part of the membrane-bound sector of the mitochondrial, DCCD sensitive ATPase [45]. The polypeptide composition of the chloroplast DCCD-sensitive ATPase is less well-defined. A single proteolipid of molecular weight 8000 active in proton transport has been isolated from chloroplasts and identified as the site of DCCD sensitivity [46]. However, the nature of the CF_1 binding site and the total polypeptide composition of CF_o are not necessarily identical with

that of the DCCD-sensitive proteolipid. In view of the nature of the energy-dependent labeling differences and the detergent isolation properties of bands 11, 17, and 23, these polypeptides may be associated with CF_o.

The differences in the degree of light/dark labeling of bands 11, 17, and 23 may arise from two sources: (1) a conversion in the light of reactive $-NH_2$ groups to non-reactive $-NH_3$ groups and (2) a change in the light of steric conditions such that the ability of fluorescamine to react with previously exposed groups is blocked. With regard to the first possibility it is known that light activation of the chloroplast membranes results in a pH increase on the external side of the membrane [44], and therefore it is unlikely that any non-reactive $-NH_3$ groups would be formed on that side. On the other hand, either a pH decrease leading to increased formation of $-NH_3$ groups located at the inner surface of the membrane or the protonation of amino groups associated with a hydrophobic region of a protein could result in decreased labeling. Although it is not yet possible to distinguish whether the reduced degree of labeling in the light of bands 11, 17, and 23 is due to a protonation or steric blocking effect, there is a high probability that some form of conformational change occurs. Even in the case of a protonation event, the accommodation of a new charge on, or especially within, a protein will most likely cause some rearrangement in conformation.

A great deal of evidence suggests that CF_1 undergoes conformational changes in response to membrane energization [7—16,47]. The possibility that CF_o may also undergo some sort of conformational alterations is therefore very intriguing. If it can be shown that CF_o undergoes conformational changes related to the formation of proton gradients, a mechanical coupling of CF_1 to CF_o through their known binding interaction [6,32] could translate conformational changes of CF_o into alterations in conformations and enzymatic properties of CF_1. Such conformational changes in CF_o are compatible with the proton dependent conformational hypothesis of Boyer [48—52] and Slater [53], in which conformational changes and transmembrane proton gradients are coupled to ATP synthesis. We therefore propose that the hypothesis includes the possibility that conformational changes in polypeptides associated with CF_o play an important role in energy transduction. Accordingly, we suggest that the conversion of proton gradients into ATP synthesis includes the following steps:

1. Light-activated transmembrane proton transport leading to the localization of the proton gradient at CF_o (i.e., protonation and deprotonation within CF_o).

2. A change in the conformation of CF_o, inducing the capability of proton movement through the membrane proton channel (as for example, across a series of membrane-spanning hydrogen bonds [54]).

3. A change in the conformation of CF_1 coupled to that of CF_o resulting in a change in the enzymatic and nucleotide binding properties of CF_1.

4. Restoration of CF_o and CF_1 conformations to original states after utilization of protons, either in the synthesis of ATP or other proton binding events.

The necessary components for this hypothesis include a proton pump driven by photosynthetic electron transport, CF_o as the primary transducer of ΔpH into mechanochemical energy, the coupled interaction between CF_o and CF_1,

and the dependence of ATP synthesis on the conformation of CF_1.

This proposal is very similar to the model proposed by Boyer [49,50] but differs in part by our suggestion that conformational coupling could occur in association with CF_o. We also emphasize the point that these conformational changes in CF_o may occur whether or not CF_1 is present. Whether the important detail that conformational changes in CF_o are responsible for the observed conformational changes in CF_1 remains to be proven by future experimentation.

We would also like to note in passing that the anomalous behavior of low concentrations of Tricine toward fluorescamine labeling of band 11 (Fig. 2c) may be related to the observation by Gross [55] that similar concentrations of Tricine result in the uncoupling of photophosphorylation from electron transport.

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