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# TWO-DIMENSIONAL GEL ANALYSIS OF POLYPEPTIDE APPEARANCE IN FORMING THYLAKOID MEMBRANES

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SUMMARY: For the first time, two-dimensional gel electrophoresis is used to analyse the polypeptide composition of thylakoid membranes during chloroplast development. The number of polypeptide spots resolved on the two-dimensional gels is 3-4 times the number of bands previously resolved on one-dimensional gels. The pattern of polypeptide synthesis and insertion into the forming thylakoid membranes of dark-grown Euglena exposed to light conforms with previous biochemical measurements on the assembly of the electron transport chain located in these membranes.

Chloroplast thylakoid membranes of algae and plants house the reactions of photosynthetic electron transport which are basic to the production of food and oxygen by these organisms. Intensive studies have revealed the general pathways of these reactions as well as those of the associated photophosphorylation (1). In contrast, relatively little is known about the formation of the thylakoid membranes themselves. The polypeptide composition of forming thylakoics in developing chloroplasts has been analysed by one-dimensional SDS-polyacrylamide gel electrophoresis in certain higher plants (2,3) and algae (4,5). Such one dimensional gels resolve at best 40-50 polypeptide bands. A much higher degree of resolution of the polypeptides of fully developed thylakoid membranes is obtained with two-dimensional (2-D) slab gels especially when the technique of O'Farrell (6) is used (7-10). However, the 2-D gel approach has not been employed in studies of chloroplast development. This paper therefore represents the first such use of 2-D gels.

The alga Euglena gracilis is a particularly useful model system with which to study the formation of thylakoid membranes because, unlike higher plants, it contains "conditional" chloroplasts. In the dark Euglena can grow heterotro-

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phically, but its chloroplasts dedifferentiate to rudimentary proplastids which lack chlorophyll and thylakoids (11). When dark-grown Euglena are placed in the light, their proplastids develop into mature chloroplasts over a 72-96 h period (11). In the present communication, dark-grown Euglena exposed to the light are used to study thylakoid membrane formation. Specifically, for the first time, the appearance of polypeptides in thylakoid membranes forming during chloroplast development are analysed with 2-D gel electrophoretograms.

## MATERIALS AND METHODS

E. gracilis strain Z-UCLA (12) were grown in the dark for up to 3 months on a defined medium with 0.1 M ethanol as carbon source (10). Subculturing and other experimental manipulations were done under a green safe light (13) to prevent light induction of chloroplast development. For labeling experiments, dark-grown stationary-phase cells were concentrated by centrifugation, resuspended in defined sulfur-limited medium supplemented with 22.15 µg of $Na_2SO_4$ per ml (10) and 0.1 M ethanol. Cultures were grown in the dark to a density of $1.9 \times 10^6$ cells per ml, supplemented with 20 $\mu C_i$ [$^{35}S$]-$Na_2SO_4$ (New England Nuclear, carrier- free) per ml, and immediately placed in continuous white light (1600 lux) to induce chloroplast development.

After 12 and 72 h of exposure of the cells to isotope and light, chloroplasts were isolated as described (10) with KTM.2 buffer (25 mM KCl, 50 mM Tris-HCl, pH 7.6, and 0.2 mM $MgCl_2$) containing 0.25 M sucrose and 1 mM phenylmethyl sulfonyl fluoride as protease inhibitor. Thylakoid membranes were isolated from the chloroplasts as described (14). Thylakoid polypeptides were solubilized and resolved by 2-D polyacrylamide gel electrophoresis (isoelectric focusing for the first dimension, sodium dodecyl sulfate gel electrophoresis with a 7.5 to 15% linear gradient polyacrylamide gel for the second dimension), and the gels were fluorographed, all as described (9,10).

Chlorophyll was determined in 80% acetone (15) and protein according to Lowry et al. (16).

## RESULTS AND DISCUSSION

Following growth in the dark with ethanol as carbon source, cultures show little cell division when placed in the light for up to 72 h (Fig. 1). During this time, proplastids develop into mature chloroplasts (Fig. 1) as indicated by the increase in chlorophyll per cell (11). When chloroplast development is nearly completed, cell division resumes (Fig. 1).

[$^{35}S$]-labeled thylakoid membrane polypeptides were isolated by a well-characterized procedure (14) at 12 and 72 h in the light (early and late chloroplast development). Labeling of thylakoid polypeptides is complete within 12 h following exposure of dark-grown Euglena to the light and isotope (5). Equivalent amounts of labeled polypeptides from each isolation were analysed by

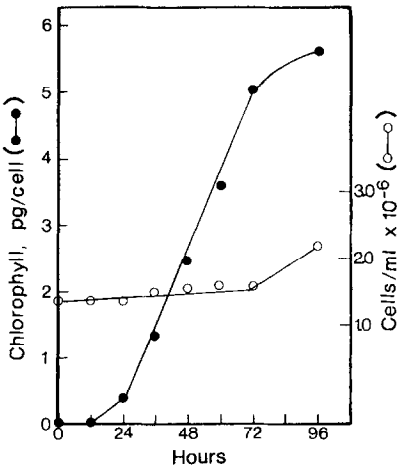


Fig. 1. Chloroplast development measured by the increase in chlorophyll per cell (11) in dark-grown Euglena exposed to light. Zero hour is the beginning of the light exposure.

2-D polyacrylamide gel electrophoresis (Fig. 2). Results demonstrate the high sensitivity of the 2-D gel method. In the 12 h sample, 125-130 thylakoid polypeptide spots are detected at apparent pIs ranging from 4.45 to 7.9 and apparent $M_r$s ranging from 8.4 to >100 kd (Fig. 2A,B). In the 72 h sample, 140-145 thylakoid polypeptide spots are detected at apparent $M_r$s also ranging from 8.4 to >100 kd but at apparent pIs ranging from 4.3 to 7.4 (Fig. 2C,D). The number of polypeptide spots resolved on the 2-D gels (Fig. 2) is 3-4 times the number of thylakoid polypeptides resolved on one-dimensional gels (5,10). The sensitivity of the 2-D gels is further demonstrated by the detection of groups of multiple polypeptide spots with the same $M_r$ but with differing pIs at both 12 and 72 h (Table 1). Also, among these groups (Table 1) are 20 spots detected at 12 h but not later and 29 at 72 h but not earlier.

The majority of polypeptide spots are found in common at early and late development; however, 39 are uniquely detected at 12 h and 45 at 72 h (Fig. 2). This suggests that the appearance of a large number of polypeptides in the thylakoid membrane is developmentally regulated. Indeed, early development (Fig. 2A, B) is especially characterized by a group of large basic polypeptides of >100 kd not detected later (Fig. 2C, D). In contrast, many acidic polypeptides of <30 kd not observed early are present at late development. Further, of the total number of polypeptide spots uniquely detected at each time point, 56%

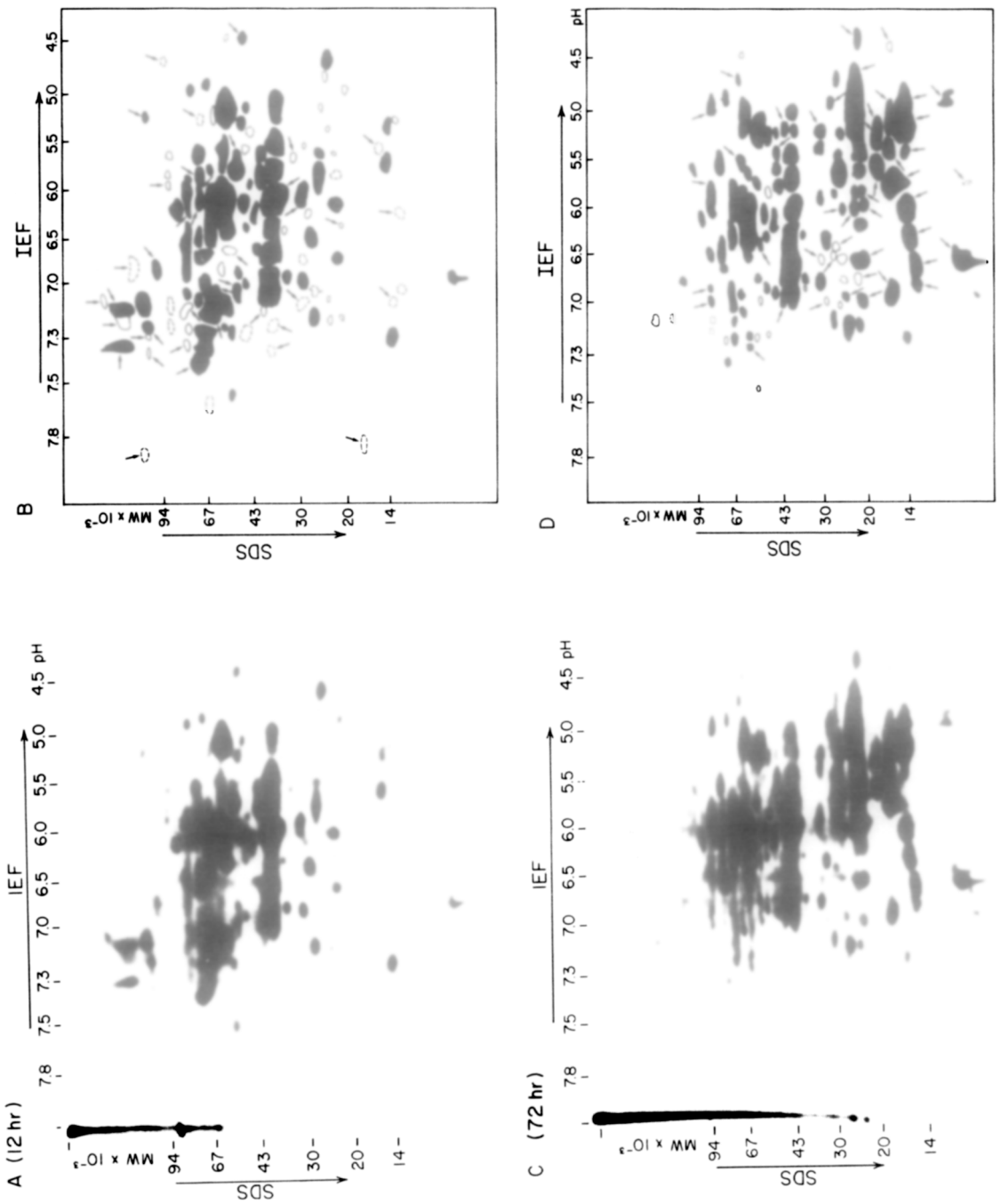
A (12 hr)
B
C (72 hr)
D
IEF
SDS
MW x 10⁻³
pH
7.8
7.5
7.3
7.0
6.5
6.0
5.5
5.0
4.5
94
67
43
30
20
14

TABLE 1

Multiple Thylakoid Polypeptides with the Same Apparent Molecular Weight but with Differing Apparent Isoelectric Points at 12 and 72 Hours of Chloroplast Development

| Apparent $M_r$ (kd) | Apparent pIs at 12 h | 72 h |
|---|---|---|
| 105 | 7.25 ,7.2 | - |
| 83 | 7.0,6.8,6.6,6.5,5.9 | 7.0,6.8,6.6,6.5,5.9,5.1 |
| 74 | 6.6,6.3,6.05 | 6.6 |
| 69 | 7.05,5.2 | 7.05 |
| 68 | 7.3,7.2,6.3 | 7.3,7.2,6.3,5.5 |
| 67 | 6.05,5.8 | 6.05 |
| 65 | 7.15,7.1,6.9 | 7.1,6.9 |
| 61 | 5.5,5.0,4.5 | 7.25,5.5,5.0,4.5 |
| 59 | 7.2,7.0,6.5,6.3,6.1 | 7.2,6.5,6.3,6.1 |
| 55 | 7.2,6.6,5.8 | 7.2,6.4,5.8 |
| 53 | 7.5,7.0,6.95 | 7.5,7.0 |
| 51 | 7.2,6.3,5.7 | 7.2,6.3 |
| 40 | 7.15,6.3,5.7,5.5 | 6.3,5.7,5.5 |
| 37.5 | 7.35,7.2 | - |
| 36 | 6.3,5.95 | 6.3 |
| 32 | 6.0,5.6,5.45 | 6.0,5.45,5.2 |
| 30 | 6.8,6.1 | 6.8 |
| 29 | 7.05,6.4 | 7.0,6.4 |
| 27 | 5.9 | 6.45,5.9 |
| 26 | 5.6,5.2,4.7 | 6.8,6.0,5.6,5.2,4.7 |
| 23.5 | 7.2 | 7.2,6.0 |
| 22 | 7.2,6.7,6.2,6.1,5.5,4.8 | 7.2,6.8.6.7,6.2,6.1, 5.9.5.5,5.1,4.8,4.3 |
| 20.5 | - | 7.05,6.7,6.0 |
| 17 | - | 7.0,5.8,5.45,5.0 |
| 14.5 | - | 6.3,6.0,5.1,4.9 |
| 14 | 7.3,7.1,6.1,5.2 | 7.3,6.1,5.2 |
| 13.5 | - | 6.9.6.7 |
| 13 | 6.9,6.15 | 6.9 |
| 9 | - | 6.5,5.8 |

[a]Polypeptides detected at 12 h or at 72 h, but not at both times, are underlined.

are larger than 60 kd and 49% show neutral or basic pIs at 12 h whereas 84% are smaller than 60 kd and 89% show acidic pIs at 72 h (Table 2). The data in Table 1 also show this developmental trend toward the synthesis and insertion of lower $M_r$ acidic polypeptides into the thylakoid membrane as a function of time.

Fig. 2. [$^{35}$S]-labeled thylakoid membrane polypeptides resolved by two-dimensional gel electrophoresis and fluorographed for 3 days, a period of time long enough to reveal even very lightly labeled polypeptide spots (10). A. Fluorograph of polypeptides (equivalent to 5 x $10^5$ cpm) isolated from cells labeled with isotope for 12 h in the light. B. Schematic interpretation of A. Arrows point to polypeptides not detected at 72 h. Unshaded areas enclosed by a continuous line or by a broken line appeared as light or very light spots, respectively, on the fluorograph. C. Fluorograph of polypeptides (equivalent to 5 x $10^5$ cpm) isolated from cells labeled for 72 h in the light. D. Schematic interpretation of C. Arrows point to polypeptides not detected at 12 h. Unshaded areas as in B. Schematic interpretations B and D were drawn from the original fluorographs.

TABLE 2

Thylakoid Polypeptide Spots Detected Only at 12 or Only at 72 Hours of Chloroplast Development

| Apparent $M_r$ range (kd) | Number detected only at 12 h[a] | 72 h[b] | Apparent pI range | Number detected only at 12 h[a] | 72 h[b] |
|---|---|---|---|---|---|
| 100 | 11 | 0 | 7.0 - 7.9 | 19 | 5 |
| 80-99 | 3 | 4 | 6.0 - 6.95 | 10 | 17 |
| 60-79 | 8 | 3 | 5.0 - 5.95 | 8 | 16 |
| 40-59 | 6 | 4 | 4.3 - 4.95 | 2 | 7 |
| 20-39 | 7 | 17 | | | |
| 9-19 | 4 | 17 | | | |

[a]Corresponds to polypeptide spots indicated by arrows in Fig. 2B.

[b]Corresponds to polypeptide spots indicated by arrows in Fig. 2D.

These latter data again support the view that the polypeptide composition of the thylakoid membrane is developmentally regulated.

Thylakoid polypeptide spots detected (Fig. 2) remain to be identified. However, the results in Figure 2 conform with biochemical measurements on the assembly of the electron transport chain in forming thylakoid membranes. In seedlings (17) and Euglena (18-19), photosystem I (PSI) activity appears before photosystem II (PSII) activity. The majority of polypeptides required for the development of PSI are synthesized during the first 10-12 h following exposure of dark-grown Euglena to the light (18). In contrast, the development of PSII activity requires continuous de novo synthesis of polypeptides (18) and is delayed until about the 20th h (19). The number of PSII reaction centers per cell then increases throughout the remaining period of chloroplast development. Polypeptides associated with PSI tend to be large and range in $M_r$ from 60 kd up to 95 kd with major species at 60-73 kd (7,20-21). Major PSII polypeptides including those in the associated chlorophyll-protein complexes tend to be smaller and range in $M_r$ from 20 to 48 kd (7,20-21). In the present results, thylakoid polypeptide spots of 60 kd and larger are well-represented by 12 h and many new polypeptide spots in the range of 20-59 kd appear at the later period of chloroplast development (Fig. 2C,D; Table 2). Therefore, the general pattern of polypeptides synthesized and inserted into the thylakoid membrane (Fig. 2) fits the biochemical pattern of development of this membrane, i.e., PSI polypep-

tides (60 kd and above) being synthesized and inserted in the first 12 h of light with PSII polypeptides (less than 60 kd) synthesized and inserted later.

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