A QUANTITATIVE ESTIMATION OF CHLOROPLAST THYLAKOID-BOUND COUPLING FACTOR 1 BY ROCKET IMMUNOELECTROPHORESIS

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1. Introduction

Chloroplast coupling factor 1 (CF_1) is the extrinsic membrane protein fraction of the chloroplast energy-transducing complex [1,2]. It contains the active site(s) for both ATP synthesis and ATP hydrolysis, as well as other binding sites for adenine nucleotides [3,4]. Many experiments performed with washed thylakoid membranes designed to either alter the activity of CF_1 or measure the binding of ligands to the protein are often expressed on a chlorophyll basis because quantitative estimations of the amount of CF_1 are difficult and time consuming. However, in [5] it is clearly shown that the amount of thylakoid-bound CF_1 is directly related to the amount of surface area of the thylakoid membrane exposed to the stroma phase. The fraction of exposed lamellae is dramatically influenced by the environment in which the plants are grown, and hence, the amount of CF_1 can vary substantially from different chloroplast preparations. It would, therefore, be desirable to have a fairly rapid, quantitative method to estimate the amount of thylakoid-bound CF_1.

Two methods that have been used to estimate the amount of thylakoid-bound CF_1 are electron microscopy [6] and the amount of trypsin-induced ATPase activity [7]. Here we describe a rocket immunoelectrophoresis system for the quantitative analysis of CF_1 and show how the system can be used to estimate the amount of thylakoid-bound CF_1.

2. Materials and methods

CF_1 was prepared from spinach leaves as in [8]. Final purification to homogeneity was accomplished by elution from a Sephadex A-50 column (1 × 30 cm) using a linear ammonium sulfate salt gradient (80–300 mM) as in [9]. The protein ran as a single band on analytical polyacrylamide gels [10] and had a heat-induced Ca^{2+}-dependent ATPase activity between 20–25 μmol ATP hydrolyzed/mg protein . min using 10 μM ATP as substrate. The protein was stored at 4°C as a suspension in 50% ammonium sulfate containing 4 mM ATP, 1 mM EDTA, and 20 mM Tricine/NaOH (pH 8.0). Prior to use, aliquots of the suspension were centrifuged, and the protein was redissolved in 20 mM Tricine/NaOH (pH 8.0) buffer. The ammonium sulfate was removed by chromatography on Sephadex G-50 as in [11]. Protein concentrations were measured as in [15] using bovine serum albumin as the standard.

Anti-CF_1 antiserum was prepared from rabbits immunized with CF_1 as in [5]. The activity of the serum was determined by measuring the ability of the serum to inhibit phenazine methosulfate catalyzed photosystem I cyclic phosphorylation [12]. Inhibition of cyclic phosphorylation of 50% was achieved by the addition of 0.12 mg antiserum/ml.

Spinach chloroplasts were routinely prepared as in [13] and suspended in 50 mM Tris–HCl (pH 8.0). Chlorophyll (chl) was adjusted to 1.5 mg/ml [14] and an equal volume of chloroform was added to a 1.0 ml suspension of thylakoids. The phases were separated by centrifugation (5 min at 2000 × *g*), and the clear aqueous phase withdrawn. An aliquot (usually 0.1 ml) was chromatographed on Sephadex G-50 [11] to remove residual chloroform. Protein concentration was determined [15] before and after chromatography to correct for sample volume changes. This fraction was used for immunoelectrophoresis.

Chloroplasts, depleted of CF_1, were prepared

essentially as in [8]. Thylakoid membranes were washed 3 times in 10 mM $NaPP_i$ (pH 7.8) and diluted to 20 μg/ml in buffer containing 2.0 mM Tris–Tricine (pH 7.8) and 0.3 M sucrose. Control chloroplasts contained, in addition, 0.1 M $CaCl_2$. The thylakoid membranes were stirred for 20 min at room temperature and then collected by centrifugation and resuspended in 20 mM Tricine/NaOH (pH 8.0).

Rocket immunoelectrophoresis was performed essentially as in [16]. A solution containing 0.8% agarose and 50 mM Tris–HCl (pH 8.0) was cooled from 100–43°C and made 40 μg/ml in serum protein containing anti-CF_1. The gel was poured into a rectangular mold (18.0 × 10.2 × 0.15 cm) and allowed to set for 1 h at 4°C. Circular wells 4 mM in diameter were punched into the gel and 10 μl samples were applied to each well. The slab was electrophoresed at 60 V for 15–18 h at 20°C. The slab was then dried and stained with Coomassie blue (R-250) to visualize the rockets.

3. Results and discussion

A standard curve for the calibration of CF_1, shown in fig.1, was determined by applying 10 μl of varying concentrations of CF_1 isolated from spinach to each well of the immunoelectrophoresis gel. This protein was ~95% pure as estimated by absorbance scans of Coomassie-stained polyacrylamide gels. Since CF_1 containing a significant amount of RuBP carboxylase had been used to elicit the antibody, some precipitation with RuBP carboxylase was observed. To eliminate this precipitation with thylakoid membrane extracts which might decrease the accuracy of the results, RuBP carboxylase [17] was added to the antibody, and the precipitate was removed before addition of antibody to the gel. Thus, the area enclosed by any given rocket should be proportional to the amount of CF_1 applied to the origin [16]. A close approximation to this relationship was observed by plotting the rocket peak height versus the amount of antigen as shown in fig.1b. Peak height was linearly proportional to the amount of CF_1 from 0 ~ 2.9 pmol CF_1. Peak height was measured to the nearest 0.1 mm enabling us to measure to a sensitivity of ±0.08 pmol CF_1. Statistical analysis of 16 standard curves gave a standard deviation of 2.0% of mean value for the assay.

Table 1 summarizes the results of 5 expt which determined:

(i) The CF_1/chl (w/w) ratio for chloroplasts isolated from spinach;

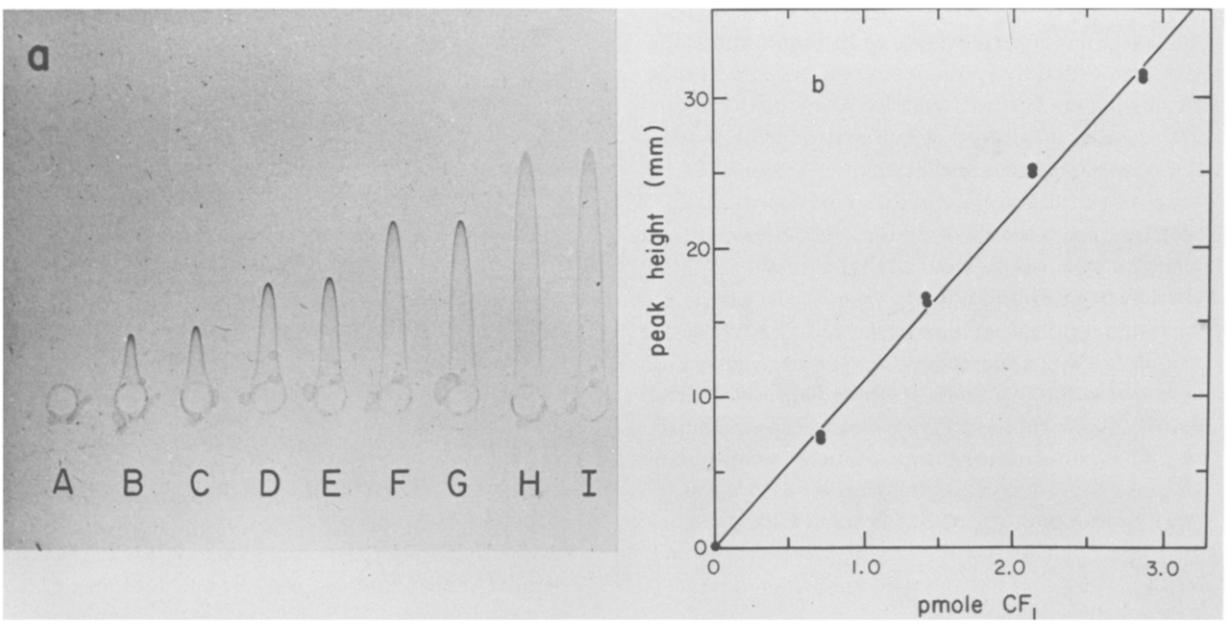

Fig.1. Rocket immunoelectrophoresis of soluble CF_1. (a) The following amounts of CF_1 (in pmol) were added in 10 μl aliquots to the wells of the gel; (A) 0; (B,C) 0.71; (D,E) 1.42; (F,G) 2.13; (H,I) 2.87. (b) Plot of the data in (a) as peak height versus amount of CF_1 added.

Table 1
Quantitative analysis of the ratio of CF_1 to chlorophyll (w/w) in isolated spinach thylakoid membranes

Expt. no.	Spinach age (days)	% Recovery of added soluble CF_1[a]	mg CF_1 / mg chl
1	31	82	0.32
		75	0.40
2	32	100	0.34
		91	0.35
3	37	86	0.42
		90	0.36
4	39	100	0.30
		93	0.34
5	–	92	0.28
		100	0.27
Mean		91 ± 5.2	0.34 ± 0.028
Corrected mean[b]			0.37

[a] To calculate the % recovery, 1 mg soluble CF_1 was added to the thylakoid suspension prior to the addition of chloroform. The peak height plus CF_1 was subtracted from the peak height of an identical sample without CF_1 and compared to the peak height of a standard equivalent amount of soluble CF_1

[b] The average ratio of mg CF_1/mg chl was divided by the average recovery of added soluble CF_1

(ii) The % recovery of soluble CF_1 added to suspensions of thylakoid membranes prior to their disruption with chloroform.

The mean CF_1/chl (w/w) ratio obtained by these measurements was 0.34 ± 0.028 mg CF_1/mg chl. The standard error associated with this value was 8.4%. However, the average % error between duplicate immunoelectrophoresis gels on any given day was 5.6%. The difference in error is due, presumably, to daily variations of the CF_1 content of the thylakoids. This variation did not appear to correspond to the age of the spinach. A known amount (1 mg) of soluble CF_1 was added to a portion of the thylakoid membranes prior to their disruption with chloroform to determine the fraction of CF_1 lost as a result of the extraction procedure. After subtracting the amount of CF_1 which had been associated with the thylakoids, the average recovery of the soluble CF_1 was 91 ± 5% (table 1). Assuming, then, that ~9% of the CF_1 associated with the thylakoids was also lost by chloroform extraction we estimate the ratio of CF_1/chl (w/w) to be 0.37.

Although the immunoelectrophoresis assay is a very sensitive technique for precisely determining small amounts of CF_1, the accuracy of the value of the CF_1/chl ratio obtained is still limited by the efficiency of the extraction procedure. For this reason the values of CF_1/chl (w/w) obtained by chloroform extraction were compared to a method which eluted CF_1 from the thylakoids in buffers of low ionic strength [7]. This method of elution of CF_1 from the thylakoids is non-destructive to the membranes and, thus, the thylakoids can be washed several times to thoroughly extract the protein. The data of fig.2 are a measurement of the amount of CF_1 released from chloroplasts following successive washes in a 10 mM Tris–Tricine buffer (low ionic strength) following 3 initial washes in $NaPP_i$ (high ionic strength): ~0.11 mg CF_1/mg chl was released on the first wash; with subsequent washes the ionic strength of the solution decreased further, releasing a total of 0.45 mg CF_1/mg chl.

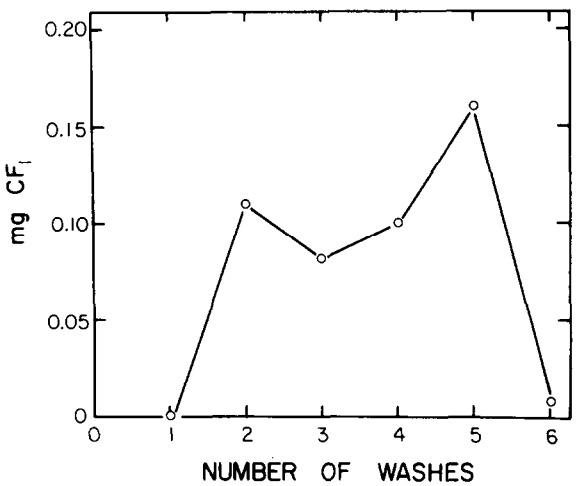

Fig.2. The elution of CF_1 from thylakoid membranes following successive washes in buffer of low ionic strength. Chloroplasts were prepared as in section 2, then washed 3 times in 20 mM $NaPP_i$ (pH 7.8) at 10 μg chl/ml. The last solution of $NaPP_i$ used to wash the chloroplasts was concentrated to a small volume and subjected to rocket immunoelectrophoresis for quantitation of CF_1 (wash no. 1). In washes 2–6 the chloroplasts were resuspended in 20 mM Tris–Tricine buffer (pH 7.8) at 10 μg chl/ml, then, after removal of the chloroplasts, these solutions were concentrated and the amount of CF_1 determined. The yield of CF_1 indicated on the *Y* axis was calculated as if 1 mg chloroplasts were used in each wash.

4. Conclusions

Microgram quantities of CF_1 can be determined easily, rapidly and quantitatively by rocket immunoelectrophoresis. The amount of thylakoid-bound CF_1 was measured using this procedure. The ratio of CF_1/chl (w/w) varied from 0.37–0.45 depending upon the method of extraction from thylakoids. Using CF_1 M_r = 326 000, we obtain a molar ratio of 1 CF_1/806–980 chl. These values favor the results in [7] where 1 CF_1/830–890 chl was measured (by determining the loss of Ca^{2+}-dependent ATPase activity from the membranes as a result of elution in low salt buffer) versus the molar ratio of 1 CF_1/100 chl as determined by electron microscopy [6]. The technique we employ has advantages over previous methods to measure the abundance of thylakoid bound CF_1 in that:

(i) The possibility that a portion of the CF_1 has lost activity during extraction is eliminated;
(ii) The immunoelectrophoresis allows for positive identification of CF_1 (not possible by electron microscopy).

Acknowledgements

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