THE ISOLATION OF SOME POLYPEPTIDES FROM THE THYLAKOID MEMBRANE, THEIR LOCALIZATION AND FUNCTION

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1. Introduction

The lamellar system of chloroplasts consists of approx. 60% of proteins. By means of terminal group determinations and sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis it was demonstrated that the thylakoid membrane contains more than 10 polypeptides of different molecular weights [1,2]. In the present paper we report on the isolation of polypeptides with the apparent mol. wts 66 000 (polypeptide 66 000), 62 000, 33 000 and 24 000 as well as on their localization in the thylakoid membrane and on their function.

2. Materials and methods

Stroma-freed chloroplasts from *Antirrhinum majus* [3] were dissolved in 0.01 M sodium phosphate buffer (pH 7.2), containing 1.1% SDS and 1% mercaptoethanol. 100 ml of this solution was used per 700 mg chloroplast dry weight. In order to remove the lipids, five times the volume of acetone was added; the precipitated protein detergent mixture was washed several times with acetone and was subsequently dissolved in 0.01 M sodium phosphate buffer (pH 7.2) which contained 1.4% SDS and 1% mercaptoethanol in order to yield a final protein concentration of 1%.

Fractionation of the polypeptide mixture was achieved by repeated gel filtration of columns of Sepharose 6B (Pharmacia) or Sephadex G-150 (Pharmacia) and adsorption chromatography on Hydroxylapatit-SC (Serva) [4]. For the isolation of the polypeptide 33 000 the SDS was first removed and the polypeptide was adsorbed in 6 M urea onto CM-cellulose (Serva). Elution was done with increasing concentrations of a urea containing ammonia formate buffer (pH 3.5). Removal of the SDS and renaturing of the proteins was carried out according to Weber and Kuter [5]. The success of the fractionation was verified by SDS polyacrylamide gel electrophoresis [6,2]. The optical density of the gels was measured after staining with Coomassie Brilliant Blue R 250 on a Zeiss PMQ II spectrophotometer fitted with a Disc Ansatz ZK 4.

The preparation of the antisera has been described earlier [7]. As the polypeptides usually aggregate in SDS-free solutions, the agarose for the Ouchterlony double diffusion test, for both the immune electrophoresis and the antigen solutions themselves, were supplemented with 0.1% SDS in 0.01 M sodium phosphate buffer (pH 7.2) [8,9].

Partial reactions of photosynthetic electron transport were carried out all as described earlier [7,10,11]. Photophosphorylation reactions were done according to the Avron procedure by using P^{32}-labelled phosphate [12].

3. Results and discussion

The polypeptides dissolved in SDS-containing buffer are separated by various chromatographic methods. After removal of the SDS under renaturing conditions their localization in the thylakoid membrane and the elucidation of their function in photosynthesis was achieved by means of specific antisera [13].

The purified fractions give only one band each when tested by SDS polyacrylamide gel electrophoresis (fig.1). The antisera to all 4 polypeptides agglutinate stroma-freed chloroplasts. Consequently,

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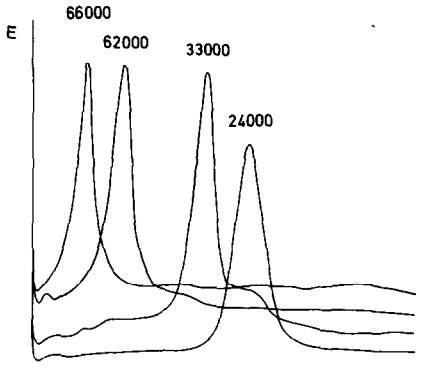

Fig.1. Photometric curves of stained polyacrylamide gels. The 33 000 molecular weight component shown in the graph is not pure. For immunization this preparation was rechromatographed.

the antigenic structure is not irreversibly destroyed by SDS. Furthermore, it follows that the antigenic determinants of the polypeptides are located on the outer surface of the thylakoid membrane. Besides the antiserum to the 66 000 polypeptide the antisera yield, when run against a solution of stroma-freed chloroplasts in the SDS Ouchterlony double diffusion test or in the SDS immune electrophoresis, only one single percipitation band. The antiserum to the 66 000 molecular weight component yields no precipitation line in these tests because the antigenic structure of the polypeptide is destroyed by the detergent. The bands of the polypeptides 62 000 and 24 000 unexpectedly appear to be identical. In contrast, the polypeptides 24 000 and 33 000 are serologically not identical. The immunological properties of the polypeptides 62 000 compared to 33 000 are not entirely clear yet. However, it appears already certain that they are not completely identical. If the reactions are carried out in the absence of the detergent, results are obtained the interpretation of which appears difficult due to the aggregation of the antigens.

The polypeptide 66 000 tends stronger to aggregation than any of the other polypeptides of the lamellar system. The antiserum to this compound inhibits only the photosystem I mediated photoreduction of methylviologen in chloroplasts (fig.2a). PMS (phenazine methosulphate) -mediated cyclic photophosphorylation and non-cyclic photophosphorylation with ferricyanide as the electron acceptor are not inhibited (table 1). As the concentration of the electron donor 2,6-dichlorophenol indophenol (DPIP) was chosen so high that electron transport is independent of plastocyanin [14] the inhibition site could be between the primary acceptor of photosystem I and ferredoxin. Antibodies to the polypeptide 33 000 inhibit the chloroplast catalyzed photoreduction of methylviologen. The inhibition is only observed if the concentration of the electron donor DPIP is sufficiently low (fig.2a). The sigmoid shape of the inhibition curve hints at some co-operative effect. Polypeptide 33 000 is certainly

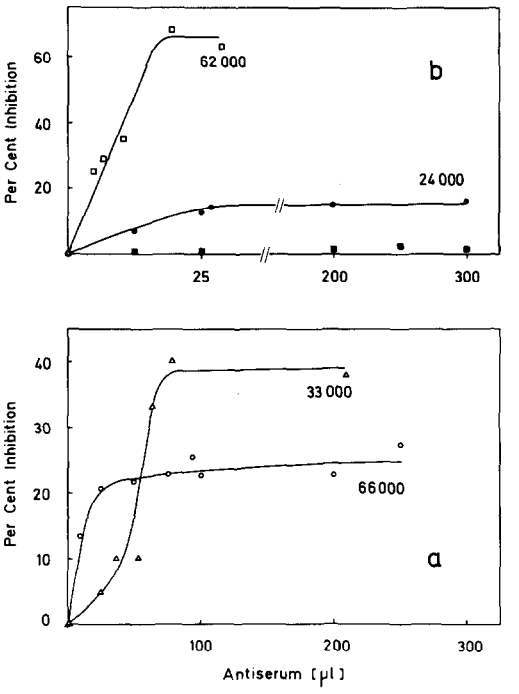

Fig.2. Inhibition of photosynthetic reactions of tobacco chloroplasts by antisera. In a total vol of 3 ml, chloroplasts corresponding to 9 µg total chlorophyll were used. All measurements were corrected for the effect of the control serum. a) Methylviologen Mehler reaction with DPIP/ascorbate as the electron donor in 20 000 ergs·sec^{-1}·cm^{-2} of red light and in the presence of 3-(3,4-dichlorophenyl)-1,1′-dimethylurea (DCMU). (○) Polypeptide 66 000. (△) Polypeptide 33 000. b) (□) Photoreduction of NADP$^+$ with water as the electron donor in the presence of antiserum to the 62 000 molecular weight component. Illumination with 100 000 Lx white light for 3 min. (■) Same reaction in the presence of $5\cdot10^{-2}$ M methylamine or 10^{-3} M NH_4Cl. (●) Photoreduction of methylviologen with water as the elctron donor in the presence of antiserum to the polypeptide 24 000. Conditions as in fig.2a.

Table 1
Effect of antisera to polypeptides isolated from the lamellar system of *Antirrhinum majus* on photophosphorylation reactions in chloroplasts from *Nicotiana tabacum*

Antiserum to polypeptides of molecular weight	[μMoles $AT^{32}P$ formed· (mg Chlorophyll) $^{-1}\cdot h^{-1}$] Non-cyclic $H_2O \rightarrow K_3Fe(CN)_6$	Non-cyclic $H_2O \rightarrow$ Methylviologen	Cyclic PMS-mediated
66 000	48.1	8.8	477
Control serum	43.1	11.3	515
% Inhibition	0	22	7
62 000	16	4.4	24.3[a]
Control serum	47	12.5	187
% Inhibition	66	65	87
33 000	47	14.8	156[a]
Control serum	47	12.6	222
% Inhibition	0	0	30
24 000	47.3	9.0	331
Control serum	49	8.8	308
% Inhibition	3	0	0
Control without serum addition	31	10.3	395 162[a]

The reaction was carried out at 15°C in 120 000 Lx white light. Chloroplasts for the reaction were prepared from *N. tabacum* var. John William's Broadleaf.
a) are results from a different series of chloroplast preparations. All values are the average of at least 8 individual determinations.

different from plastocyanin or cytochrome *f* because these have an apparent mol. wt of 9800 and 13 000, respectively. Moreover, the antiserum inhibits PMS-mediated cyclic photophosphorylation by 30% (table 1). Non-cyclic photophosphorylation with methylviologen as the electron acceptor is not inhibited. From this we conclude that the inhibition is located on the electron donating side to P_{700}. The effect of the antiserum to the polypeptide 24 000, however, could not be located. The electron transport from water to $NADP^+$ was inhibited between 10–16% (fig.2b). All reactions tested on the photosystem I or II side were inhibited to this extent. Either this preparation is a mixture of polypeptides with the same or only slightly different molecular weights or the observed inhibition is unspecific (fig.2b and table 1).

The antiserum to the polypeptide 62 000 inhibits the photoreduction of $NADP^+$ with water as the electron donor by 65% (fig.2b). If electron transport is uncoupled from photophosphorylation no inhibition is observed. In addition the antiserum inhibits PMS-mediated cyclic photophosphorylation by 87% and inhibits non-cyclic photophosphorylation with either ferricyanide or methylviologen in the Hill reaction by 65% (table 1). From this it appears obvious that our preparation is identical to the 62 000 [15] and 59 000 [16] molecular weight component of the coupling factor.

From our experiments it clearly follows that our antisera contain antibodies to native conformational states of thylakoid membrane proteins. This is demonstrated by the facts that the antisera agglutinate stroma-freed chloroplasts and inhibit photosynthetic electron transport. By the positive outcome of the serological precipitation tests in the presence of SDS it becomes evident that the antigenic structure of the polypeptides remains at least partially preserved in this detergent solution.

Despite the fact that all fractions appear uniform with respect to their molecular weight, it must be assumed, however, in order to explain some of the precipitation reactions, that the fractions contain in reality more than one polypeptide of the same or of slightly different molecular weights. The experiments clarifying this point are in progress.

Disregarding the detailed results presented in this paper we should like to note that we believe to have found a method by which all membrane proteins, even those with low solubility, can be isolated and subsequently localized in the thylakoid membrane where also their function can be determined by means of specific antibodies.

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