The chloroplast ATP synthetase consists of the subunits α, β, γ, δ, ϵ and proteolipid only

Evidence that the components termed subunit I and II are proteolytically altered light-harvesting proteins

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The subunit stoichiometry of the ATP synthetase (CF_1–CF_o) immunoprecipitated from Triton X-100 extracts of chloroplast thylakoid membranes was determined to be α_3, β_3, γ, δ, ϵ (CF_1) and $I_{0.3}$, $II_{0.6-0.9}$, $III_{4(6)}$ (CF_o). Antibodies against the polypeptides α, β, γ, δ, I, II and ϵ combined specifically with the isolated subunits as analysed by the protein blotting method. Applying this technique, antibodies against the CF_1 subunits were found to form complexes with the corresponding polypeptides of thylakoids, whereas those against I (M_r 20 000) and II (M_r 17 000) combined with M_r 26 000 and M_r 24 500 membrane polypeptides, respectively. The M_r 26 000 polypeptide was identified as the major subunits of the light-harvesting chlorophyll *a*/*b*–protein (LHCP) complex and the M_r 24 500 component seems to be functionally connected with this complex. From the results it is concluded that the chloroplast ATP synthetase consists of the subunit of the α, β, γ, δ, ϵ and III (proteolipid only and that proteolytically altered LHCP polypeptides bind artifically to the protein complex during isolation.

1. INTRODUCTION

Independent of the isolation method used, chloroplast ATP synthetase (CF_1–CF_o) preparations contained the CF_1-ATPase subunits α to ϵ and polypeptides designated I, II and III ascribed to the transmembranal, proton translocating entity CF_o [1–4]. Subunit III was identified as an M_r 7500 proteolipid [1,2,4] which may form the membrane channel through which protons flow during photophosphorylation [5,6]. The bacterial ATP synthetases (F_1–F_o) also seem to consist of 8 nonidentical polypeptides [7–10]; although the enzyme complex of a thermophilic bacterium contained only 7 kinds of subunits, 2 of them were found to be associated with the F_o including the proteolipid and a F_1-binding component [11]. Consistent with most of the results obtained for bacterial and mitochondrial ATP synthetases [5], the subunit stoichiometry of the chloroplast–protein complex of plants uniformly radiolabeled with $^{14}CO_2$ was estimated to be α_3, β_3, γ, δ, $I_{0.3}$, $II_{0.7-0.9}$, ϵ, and $III_{4.6-4.8}$ [12]. However, since the stoichiometric ratios of the subunits I and II were always found to be smaller than 1 compared with the γ subunit [12], it was a matter of discussion whether these components indeed represent subunits or tightly bound impurities, thus implying that the ATP synthetase consists of only 6 or 7 instead of 8 kinds of subunits.

In this work, applying the 'protein blotting' technique [13] in combination with the use of monospecific antibodies raised against all ATP synthetase subunits excluding the proteolipid, it is shown that the polypeptide termed subunit I (M_r 20000) is a proteolytically altered polypeptide of the major subunit (M_r 26000) of the light-harvesting chlorophyll *a*/*b*–protein (LHCP) complex. Subunit II of the ATP synthetase (M_r 17000) was identified as a degradation product of an M_r 24500 membrane polypeptide which seems also to belong to a light-harvesting entity of the thylakoid membrane although it was absent in the isolated LHCP complex. The CF_1 subunit chains are not altered during the isolation of ATP synthetases. The results are consistent with the view of only 6

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non-identical subunits to be associated with the chloroplast ATP synthetase including α, β, γ, δ, ϵ and proteolipid. From these findings the question arises whether the bacterial and mitochondrial ATP synthetase also consist of a smaller number of subunit types as previously established.

2. MATERIALS AND METHODS

Non-labeled and U-^{14}C-labeled chloroplast thylakoid membranes of *Vicia faba* (field bean) were isolated in the presence of 2% bovine serum albumin as in [12]. In order to inhibit protease activities of the chloroplast preparations, 1 mM di-iso-propyl fluorophosphate (DFP) and 1 mM phenylmethanesulphonyl fluoride (PMSF) were added to all buffers. The CF_1-ATPase of *Vicia faba* and antisera against the protein were prepared as in [14]. The LHCP complex was isolated according to [15] using gel permeation chromatography as the major step of purification. Two different methods of immunoprecipitation were applied to isolate the ATP synthetases. Immunoprecipitation of the protein complex from Triton X-100 thylakoid extracts (method 1) was performed as in [2]. In method 2, antiserum against the CF_1 was directly added to a thylakoid suspension containing 0.2 mg chlorophyll/ml in 50 mM Tricine–NaOH (pH 8.0) and incubated at 4°C for 3 h leading to the antibody cross-linking of ATP synthetases. The membranes were sedimented by centrifugation at 4000 × *g* and washed twice with the same buffer prior to solubilization with Triton X-100 as in [2]. The immunoprecipitates and thylakoid membranes were dissolved in 50 mM Na-borate (pH 8.9), 2% SDS, 8 M urea and 5 mM dithiothreitol (DTT) and electrophoretically separated on 8–18% polyacrylamide gradient slab gels containing 5 M urea [16]. The ATP synthetase subunit bands stained with Coomassie Brilliant Blue G-250 were dissected from the gels, homogenized and subcutaneously injected into rabbits. Injection of ~1 mg of each subunit in Freunds adjuvant was done 3 times every 20 days. The activity and specificity of the antisera precipitated at 50% saturation of ammonium sulphate were checked by the 'protein blotting' method as in [13]. The antibody-binding components were detected by the use of [^{125}I]protein A and autoradiography [13].

The efficiency of protein electrotransfers from polyacrylamide gels to nitrocellulose (0.45 μm,

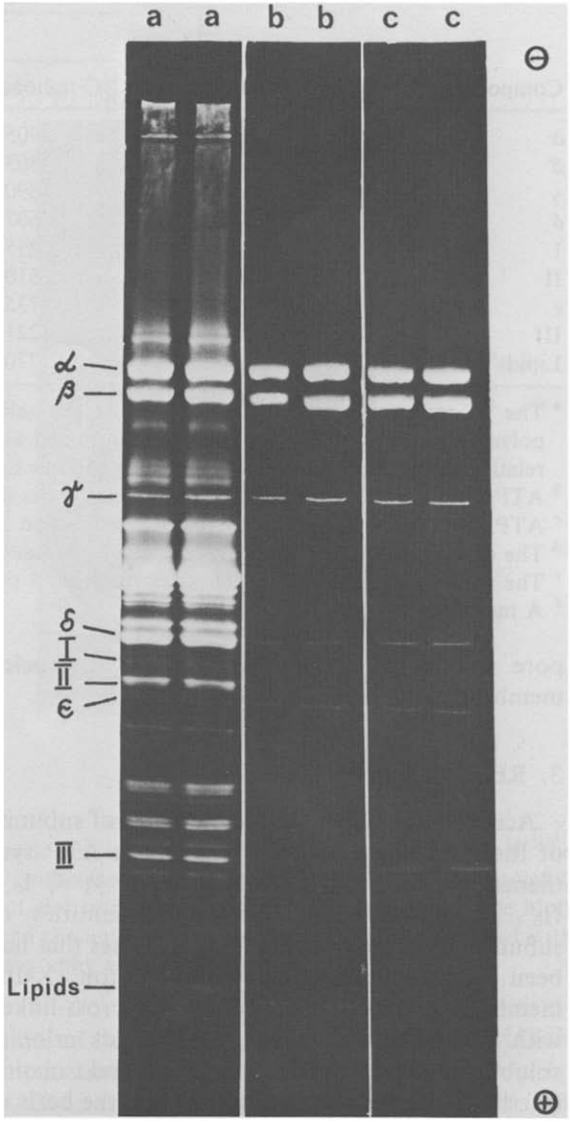

Fig.1. SDS–urea polyacrylamide gel electrophoresis of chloroplast thylakoid membranes and ATP synthetase immunoprecipitates. Two slightly different amounts of U-^{14}C-labeled thylakoid membranes of *Vicia faba* (a) and ATP synthetases cross-linked with antibodies at the membranes prior to isolation (method 2) (b), as well as immunoprecipitates from Triton X-100 thylakoid extracts (method 1) (c) were electrophoretically separated on 8–18% polyacrylamide gradient gels containing 5 M urea as described in section 2. The gels were scanned for radioactivity by autoradiography.

Table 1

Subunit stoichiometries of chloroplast ATP synthetases isolated by two different methods of immunoprecipitation

Component	$M_r \times 10^{-3}$	Observed stoichiometry of subunits[a]			
		Method 1[b]		Method 2[c]	
		^{14}C-radioactivity[d]	Stoichiometry[e]	^{14}C-radioactivity[d]	Stoichiometry[e]
α	59	7905	3.2	14610	3.2
β	55	7303	3.1	14065	3.3
γ	33	1390	1.0	2559	1.0
δ	21	607	0.7	1781	1.1
I	20	255	0.31	537	0.35
II	17	610	0.86	847	0.64
ϵ	16	735	1.1	1231	1.0
III	7.5	1221	3.9	3495	6.0
Lipids[f]	1.3	1770	32.7	–	–

[a] The ^{14}C-radioactivity (dpm) associated with the uniformly labeled ATP synthetase subunits separated by SDS–urea polyacrylamide gel electrophoresis was estimated as in [12]. The ratios of subunits were calculated by dividing the relative amount of radioactivity for each subunit by its M_r and normalizing all values to subunit γ

[b] ATP synthetases cross-linked with CF_1 antibodies at the membranes prior to isolation

[c] ATP synthetases immunoprecipitated from Triton X-100 thylakoid membrane extracts

[d] The distribution of radioactivity among the subunits of a representative experiment

[e] The values represent mean values derived from 3 different preparations

[f] A mean M_r of 1300 was assumed

pore size) strips was observed with ^{14}C-labeled membrane polypeptides and autoradiography.

3. RESULTS AND DISCUSSION

According to [12], the stoichiometry of subunits of the immunoprecipitated chloroplast ATP synthetase was estimated to be α_3, β_3, γ, δ, $I_{0.3}$, $II_{0.7-0.9}$, ϵ and III_5. Here, the quantities of subunits associated with ATP synthetases that had been immunoprecipitated from Triton X-100 membrane extracts (method 1) and cross-linked with antibodies at the intact thylakoids prior to solubilization with detergent solution and isolation (method 2) were compared (fig.1). On the basis of ^{14}C incorporation, the distribution of radioactivity among the subunits α–ϵ was very similar, whereas quantitative differences became detectable in the case of the components II and III (table 1). Polypeptide I appeared only at a ratio of 0.3 as compared to the γ subunit in immunoprecipitates obtained with both methods of isolation. Polypeptide II appeared in slightly greater amounts to be associated with the ATP synthetases prepared by method 2 and quantitatively more subunit III with immunoprecipitates isolated by method 1. The differences in the stoichiometric ratios of subunits seem to be connected with the presence of lipid or detergent molecules bound at the hydrophobic part of the enzyme. The ATP synthetase molecules obtained by method 2 contained about 30 lipid molecules (but not pigments) while the lipids were completely replaced by detergent molecules in immunoprecipitates isolated by method 1. The lipid–detergent exchange reaction probably induces the binding of two further proteolipid-like components not entirely associated with the membrane-bound enzyme, although it may not at present be excluded that two proteolipid subunits tend to dissociate if a significant amount of lipids is still bound at the CF_o. The results obtained on the stoichiometries of subunits led, however, to the question whether the components appearing in molar ratios smaller than 1, particularly I and II, are really subunits of the ATP synthetase or impurities tightly bound to the enzyme complex. In order to get information about this problem, the polypeptides of immunoprecipitated ATP synthetase preparations excluding subunit III were isolated by SDS–urea gel electrophoresis and used to prepare rabbit antisera against them. The subunits isolated have proved to be pure by re-

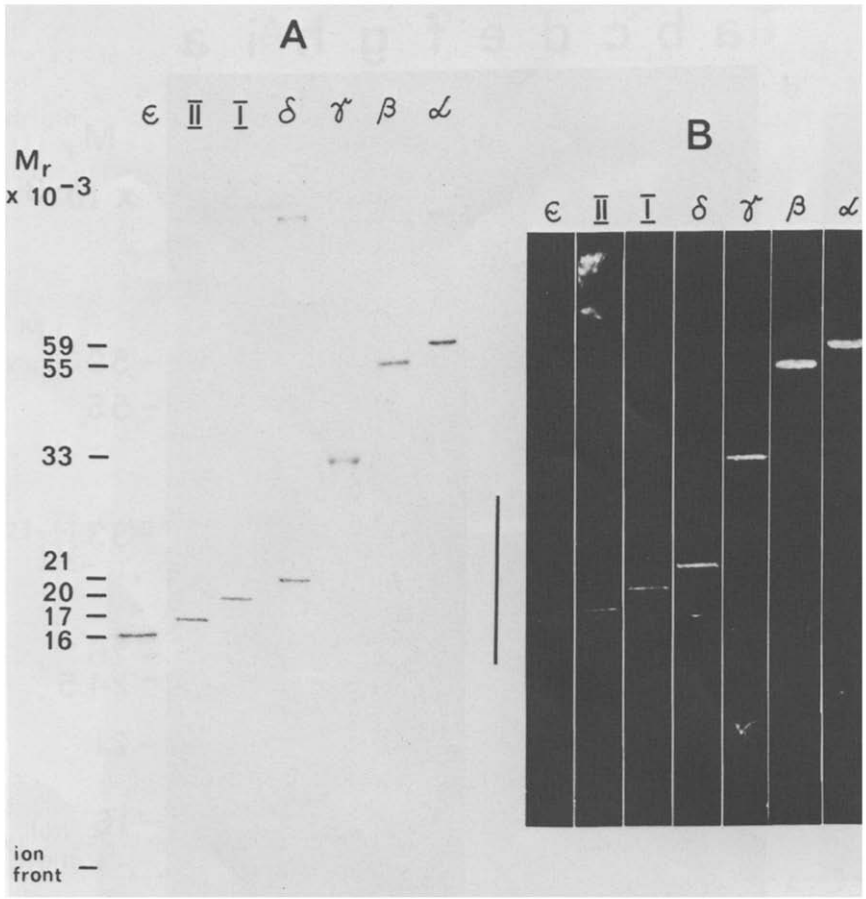

Fig.2. (A) Re-electrophoresis on SDS–urea polyacrylamide gels of the isolated ATP synthetase polypeptides α, β, γ, δ, I, II and ϵ. The gel was stained for protein with Coomassie brilliant blue G-250; (B) Demonstration of the combination of specific antibodies with the corresponding ATP synthetase polypeptides α, β, γ, δ, I, II, ϵ. The isolated polypeptides as indicated were separated electrophoretically and electrotransferred to nitrocellulose strips. The blots were then reacted with the corresponding, specific antiserum and the antibody–polypeptide complexes revealed with [^{125}I]protein A and autoradiography as in [13].

electrophoresis (fig.2A) and the antibodies prepared against them appeared to be specific as checked by 'protein blotting' [13] (fig.2B). Using the method of 'protein blotting' [13] of SDS–urea polyacrylamide gels applied to separate the *Vicia faba* chloroplast thylakoid polypeptides and antibodies raised against the subunits α–ϵ, only a single polypeptide band has been observed in each case by autoradiography of [^{125}I]protein A labeled antigen–antibody complexes (fig.3). The M_r of polypeptides were found to be the same as those of the isolated subunits indicating that no proteolytic degradation had occurred during the isolation of ATP synthetases and that no unprocessed polypeptides of the subunits are associated with thylakoid membranes.

Contrasting results were obtained for the polypeptides I and II. The antibodies raised against I (M_r 20000) and II (M_r 17000) combined also with only a single component of the gel electrophoretic thylakoid polypeptide spectrum but their M_r was estimated to be 26000 and 24500, respectively. The first one has been identified as the subunit a of the LHCP complex [2,15,17], and no further component became labeled. Because the result was still explainable by a subunit-specific proteolysis of the components I and II during thylakoid membrane extraction with Triton X-100

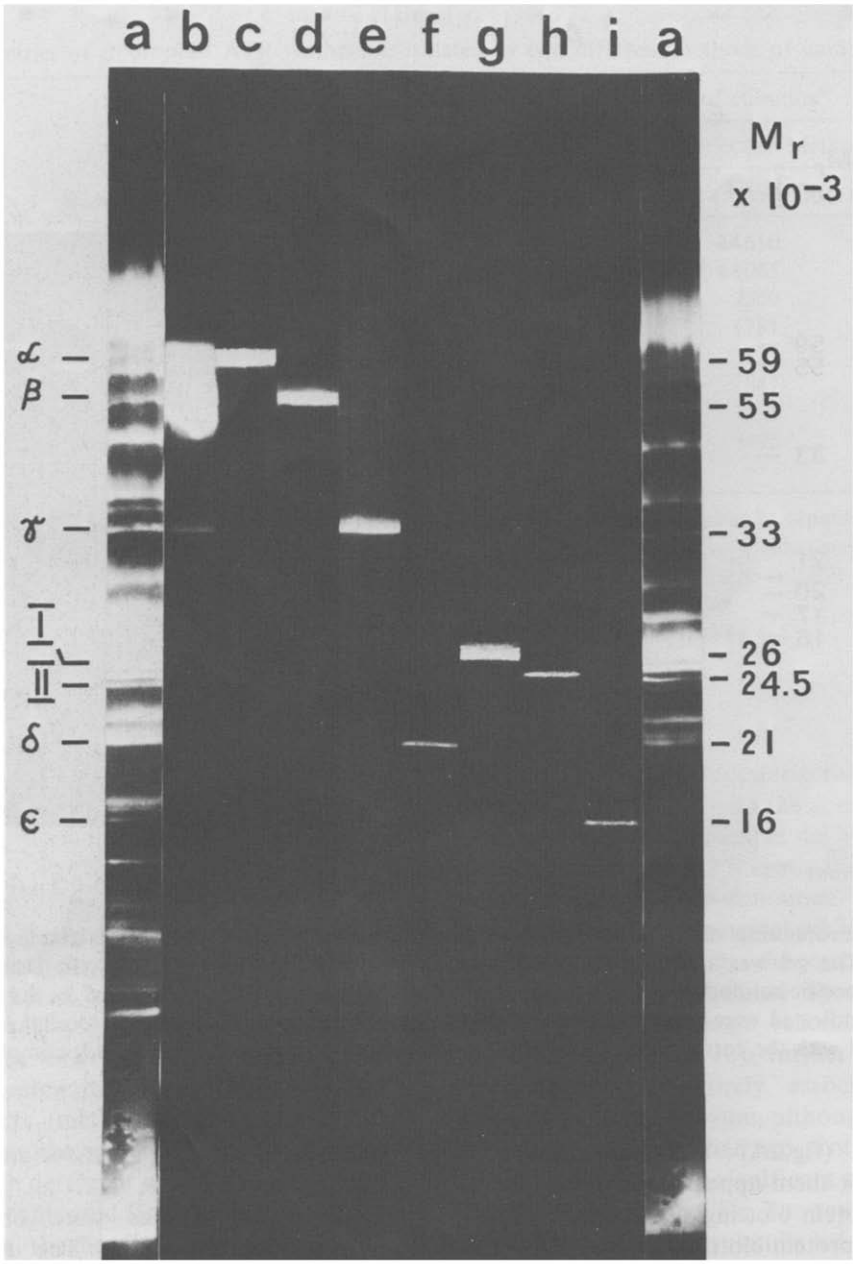

Fig.3. Identification of chloroplast thylakoid membrane polypeptides combining with antibodies directed against the CF_1 (b), and individual ATP synthetase subunits (c–i). ^{14}C-labeled (a), or non-labeled (b–i) thylakoid polypeptides were electrophoretically separated on 8–18% polyacrylamide gradient gels containing 0.1% SDS and 5 M urea and electrotransferred to nitrocellulose strips. The blots (b–i) were reacted in order with antiserum against the CF_1 and the polypeptides α, β, γ, δ, I, II and ϵ, respectively, and the antibody–polypeptide complexes were revealed with [^{125}I]protein A and autoradiography as in [13].

and/or immunoprecipitation of the ATP synthetase, the binding of antibodies to the polypeptides of the purified LHCP complex gel electrophoretically separated and electrotransferred to nitrocellulose has been investigated. Fig.4A shows that the antibodies against component I now com-

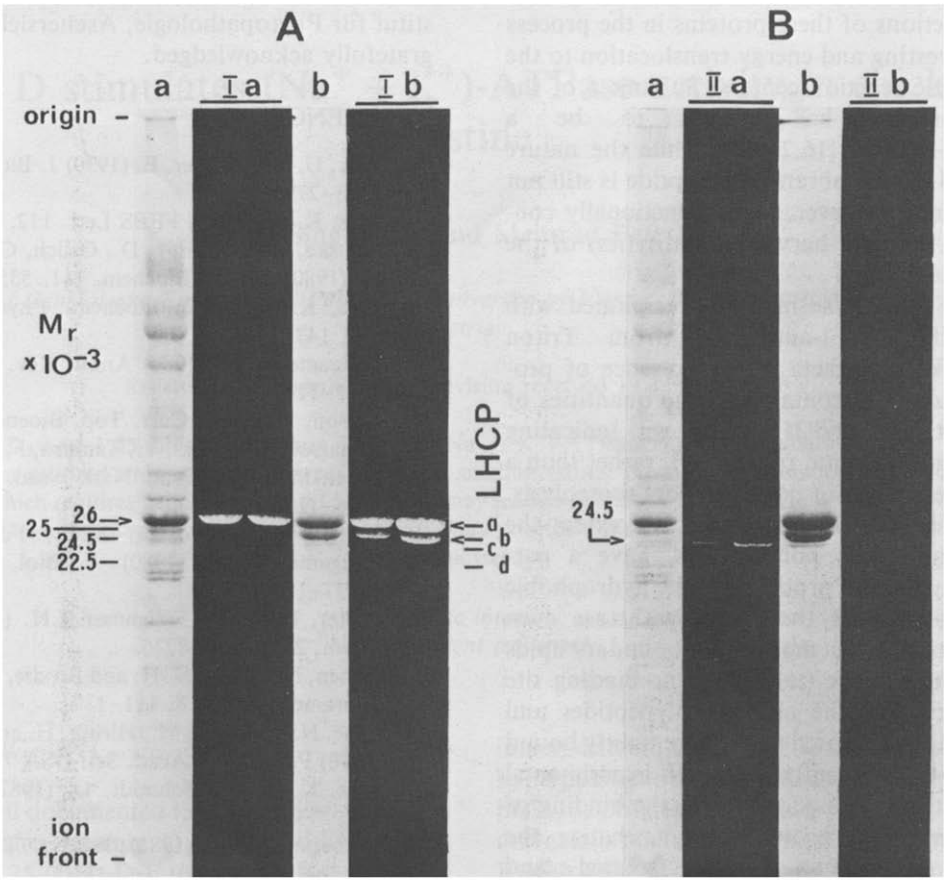

Fig.4. Identification of the mature ATP synthetase polypeptides I and II. Two different quantities of thylakoid polypeptides (Ia, IIa) and of the isolated LHCP complex (Ib, IIb) were separated electrophoretically on SDS–urea polyacrylamide gradient gels and electrotransferred to nitrocellulose strips I and II, respectively. The strips were reacted with antiserum against the ATP synthetase polypeptide I (A) and II (B) and the antibody–polypeptide complexes detected with [^{125}I]protein A and autoradiography as in [13]. The gels a and b show the polypeptide pattern of thylakoid membranes and the isolated LHCP complex, respectively, as revealed by staining with Coomassie blue.

bined with the two major subunit bands of the LHCP complex previously referred to as subunit a and b [2] or chlorophyll *a*/*b*–apoprotein 2a and 2b [18]. Since only subunit a was labeled by the same antibodies in the polypeptide profile of thylakoid membranes that had been isolated under conditions where proteolytic processes were inhibited, the second component is a proteolytically altered polypeptide of subunit a of the LHCP complex. The altered component showed the same M_r as the polypeptide previously termed LHCP subunit b [2,15,16]. One conclusion from this finding is that subunit b does not really exist in the thylakoid membrane but appears during the isolation of the LHCP complex in the absence of protease inhibitors. This is consistent with previous results showing an identical peptide map of the two major LHCP subunits after trypsin digestion [19,20,21].

The antibodies against component II of the ATP synthetase combined with an M_r 24500 polypeptide of the thylakoid membrane (fig.4B) but not with any polypeptide of the isolated LHCP complex. It should be noted that the antisera against both polypeptide I and II decreased the delayed fluorescence of thylakoid membranes in the absence of $MgCl_2$ by ~30% and increased the fluorescence by the same value (Goltsev, V.N., Yordanov, I. and Süss, K.-H., unpublished), in-

dicating functions of these proteins in the process of light-harvesting and energy translocation to the photosynthetic reaction centers. Subunit a of the LHCP complex has shown to be a chlorophyll–protein [16,21–23] while the nature of the M_r 24500 membrane polypeptide is still not clear. It seems, however, to be functionally connected with the light-harvesting entity(ies) of the thylakoid membrane.

The ATP synthetase immunoprecipitated with affinity-purified CF_1-antibodies from Triton X-100 thylakoid extracts in the presence of protease inhibitors still contained some quantities of the polypeptides I and II (not shown) indicating that a membrane-bound protease(s), rather than a serum protease, is responsible for proteolysis. Components I and II, and to a lesser extent the corresponding intact polypeptides, have a particular ability to bind probably to the hydrophobic part (proteolipid) of the ATP synthetase even under conditions when most of the boundary lipids remain at the enzyme (table 1). One binding site seems to exist for the altered polypeptides and component II is supposed to be more tightly bound to this site. Consequently, although experimental results are at present not available, the binding of the light-harvesting proteins might change the functional properties of the isolated and reconstituted ATP synthetase preparations.

In conclusion, our data suggest that the chloroplast ATP synthetase consists of only 6 authentic subunits occurring with stoichiometries of α_3, β_3, γ, δ, ϵ and proteolipid 4 [6]. Although the 'gel blotting' method and subunit-specific F_1-antisera have been used to show the authenticity of ATP synthetase subunits of chloroplasts, yeast and rat liver mitochondria as well as *E. coli* membranes [24,25], it is not yet clear whether the remaining components of bacterial and mitochondrial ATP synthetases are really nonaltered subunits and whether other membrane proteins become artificially associated during the isolation of ATP synthetases.

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