Stable binding interactions among subunits of the chloroplast ATP synthase (CF_1-CF_0) as examined by solid support (nitrocellulose)-subunit reconstitution-immunoblotting

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The technique termed solid support-subunit reconstitution-immunoblotting has been applied to examine stable binding interactions among renatured subunits of the chloroplast ATP synthase. Reconstitution of the CF_1 subunits α, β and γ and subunit III of CF_0 with thylakoid membrane polypeptides separated electrophoretically and transferred to nitrocellulose filters followed by immunodetection of hybrid subunit complexes revealed interactions between α-ε, α-CF_0-I, α-CF_0-II, α-δ, β-γ, β-CF_0-II, β-ε, β-CF_0-III, γ-CF_0-II, γ-ε, γ-CF_0-III, CF_0-III-ε. Thus, the data confirm results on neighbour relationships of ATP synthase subunits obtained by cross-linking and suggest that the method employed is generally applicable to examine protein-protein binding interactions.

Chloroplast *ATP synthase* *Subunit renaturation* *Solid support-subunit reconstitution-immunoblotting* *Protein interaction*

1. INTRODUCTION

Though the structure and function of H^+-ATP synthases (F_1-F_0) have been studied extensively [1–3], the mechanism of H^+-driven ATP synthesis remains to be defined because of the lack of precise information about the structure, arrangement and interactions of subunits within and among the enzyme portion F_1 and H^+-translocating portion F_0. However, elucidation of the primary structure of subunits and polypeptide secondary structure predictions [4,5] together with biochemical and biophysical observations have brought about ideas on the possible organization of components in these multimeric proteins. It is now generally accepted that F_1 proteins contain $\alpha_3\beta_3\gamma\delta\epsilon$ subunits.

Abbreviations: CF_1 and CF_0, enzymatically active and membrane portions of the chloroplast ATP synthase; PAGE, polyacrylamide gel electrophoresis

The α and β polypeptides are most likely arranged alternately around the central γ subunit [6,7]. The precise location of the δ and ϵ subunits remains to be determined. The topology and neighbour relationships of the 3 types of subunits belonging to the membrane-integrated F_0 portion of the *E. coli* ATP synthase (subunits a–c) and CF_0 of the chloroplast enzyme complex (subunits I–III) have been predicted from the primary structure of the components [4,8] and cross-linking experiments [9–11]. Moreover, cross-linking experiments indicate that F_1-F_0 binding in *E. coli* is mainly due to β-F_0-b and β-F_0-a subunit interactions [9], whereas in the chloroplast ATP synthase subunits α-CF_0-II, β-CF_0-I, β-CF_0-II and γ-CF_0-II were found to interact directly.

To examine further stable binding interactions among chloroplast ATP synthase subunits using an independent method, the technique of solid support-subunit reconstitution-immunoblotting

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has been developed and employed in this study. The present version of this method is based on the presumption that SDS-polypeptide complexes noncovalently bound to nitrocellulose filters or in solution may be renatured at least partially upon removal of the detergent, thereafter again possessing binding sites for neighbouring components of the intact protein complex. Thus, reconstitution of a soluble polypeptide with proteins from the same source bound to the nitrocellulose filter induces the formation of hybrid subunit complexes which can be detected by labeling with antibodies directed against the reconstituted component and ^{125}I-protein A. This approach is thought to be generally applicable to investigate binding interactions among proteins and between proteins and other compounds such as lipids, nucleic acids and metabolites.

2. MATERIALS AND METHODS

Chloroplast thylakoid membranes from *Vicia faba* plants [12] and ATP synthase ([13] method 2) were prepared as described. ATP synthase preparations and thylakoid membranes were dissolved in 50 mM Na borate, pH 8.0, 4% SDS, 8 M urea and 5 mM dithiothreitol (DTT) and separated electrophoretically on 8–18% polyacrylamide gradient gel slabs containing 5 M urea as in [14]. The ATP synthase subunit zones on preparative gels were stained with 0.2% Coomassie blue G-250 in 60% ethanol, dissected and the proteins electroeluted in the presence of 0.1% SDS into no more than 5 ml Na borate, pH 8.0, containing 1 mM DTT. Subunits α, β and γ were precipitated by the addition of ice-cold ethanol and collected by centrifugation at 6000 × *g* for 10 min. The pellets were then washed with absolute ethanol containing 1 mM 2-mercaptoethanol until they appeared colorless and were dissolved to 1 mg protein/ml in 50 mM Tris-HCl, pH 8.0, 0.9% NaCl, 1 mM DTT and 0.05% Tween 20. Insoluble material was removed by centrifugation at 10000 × *g* for 5 min. Subunit CF_0-III (proteolipid) was isolated from ATP synthase immunoprecipitates [14] and dissolved in the same buffer.

For subunit reconstitution, thylakoid polypeptides were separated electrophoretically taking the whole breadth of a gel slab and afterwards electrotransferred onto a nitrocellulose filter (15 × 13 cm) as in [13]. The efficiency of protein transfer was checked by staining a small filter strip with 0.2% amido black in methanol/acetic acid/water (40:10:40, by vol.) and destaining with the same solution without dye. The nitrocellulose filter was washed twice in an excess of 60% ethanol prior to incubation for 3 h at 4°C in 50 mM Tris-HCl, pH 8.0, 0.9% NaCl, 1 mM DTT and 0.05% Tween 20. Tween 20 instead of bovine serum albumin was used to saturate free nitro groups of the cellulose filter. The nitrocellulose filter was cut into 4 mm wide strips which were incubated separately for 1 h at 4°C with 0.1–0.5 mg subunit α, β, γ or CF_0-III in 5 ml of the buffer mentioned above containing additionally 5 mM $MgCl_2$ and 5 mg bovine serum albumin. The strips were washed 3 times for 1 h with 10 ml of the same buffer without subunit protein following reconstitution. Immunoblotting with subunit-specific antisera and ^{125}I-protein A was done as in [13].

3. RESULTS AND DISCUSSION

An accompanying communication describes the analysis of chemically cross-linked, neighbouring subunits of the soluble chloroplast ATP synthase [11]. Since subunits of the enzyme complex may be inaccessible to cross-linkers either due to the absence of reactive amino acid residues on the solvent-exposed domains or because they are covered by other subunits, it is advisable to examine subunit interactions by additional independent methods. Subunit reconstitution has been applied successfully for this aim (review [15]). However, although subunit reconstitution experiments have demonstrated the minimum subunit composition of F_1 exhibiting ATPase activity and revealed subunits involved in F_1-F_0 binding [15], the number of stable binding interactions of a defined subunit within an oligomeric complex of reconstituted proteins cannot be determined by this approach. To circumvent this problem, we suggest the technique of solid support-subunit reconstitution-immunoblotting. The present version of this method is based on the presumption that removal of detergent molecules from polypeptides separated electrophoretically on SDS gels and transferred onto nitrocellulose filters or into buffer induces at least a partial renaturation of the secondary and tertiary structure of

components which then again possess binding sites for neighbouring subunits of the intact oligomeric protein. Thus, incubation of an intact-like subunit with proteins from the same source separated electrophoretically according to their apparent M_r values and bound to nitrocellulose filters is expected to induce the formation of at best as much hybrid subunit complexes as stable subunit-subunit interactions do exist in vivo. The subunit complexes formed can be detected by labeling with antibodies against the reconstituted protein and ^{125}I-protein A and binding proteins being identified simultaneously by their M_r values.

To obtain additional information on subunit binding interactions of the chloroplast ATP synthase, the present technique has been employed using the renatured subunits α, β and γ of CF_1 and subunit III of the CF_0 (proteolipid) which were reconstituted separately with chloroplast thylakoid polypeptides resolved on SDS-urea gels and electrotransferred onto nitrocellulose filters prior to detergent removal. To ensure optimal subunit reconstitution and to minimize nonspecific binding, reconstitution was carried out in the presence of $MgCl_2$ and bovine serum albumin. The subunit complexes formed on the nitrocellulose filters were labeled with subunit-specific antibodies and ^{125}I-protein A and visualized by autoradiography (fig.1). Despite nonspecific binding of antibodies against the α, β and γ subunits to the 26 kDa subunit of the light-harvesting Chl *a/b*-protein complex and polypeptides of 32 and 23 kDa as well as components of >59 kDa, this method allowed detection of binding interactions among subunits α-δ, α-CF_0-I, α-CF_0-II, α-ϵ, β-γ, β-CF_0-II, β-ϵ, β-CF_0-III, γ-CF_0-II, γ-ϵ, γ-CF_0-III, CF_0-III-β, CF_0-III-γ and CF_0-III-ϵ. Thus, these results confirm, at least in part, the data obtained by subunit cross-linking of the soluble chloroplast ATP synthase [11]. The findings further support the notion that the α and β components may interact directly with subunits I and II of the CF_0 portion but only subunit β was found to form a stable complex with CF_0-III. Evidence for a binding interaction between subunit α and/or β and F_0-c of the *E. coli* ATP synthase has also been obtained [16]. Of particular importance is the finding that subunits γ and ϵ possess binding sites for CF_0-III, suggesting that these components may form a common structural complex. This would

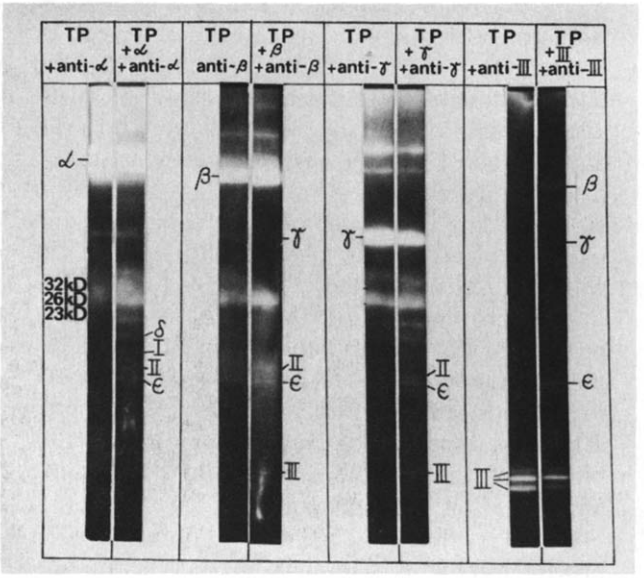

Fig.1. Reconstitution of the isolated subunits α, β, γ and III from the chloroplast ATP synthase with thylakoid membrane polypeptides bound to nitrocellulose filters. Thylakoid polypeptides (TP) were separated electrophoretically on SDS-urea gel, electrotransferred onto a nitrocellulose filter and washed as described in section 2. The filter was cut into equivalent strips which were incubated separately with or without one of the subunits mentioned above as described in section 2. Hybrid subunit complexes were labeled with antisera against subunits α (anti-α), β (anti-β), γ (anti-γ) and III (anti-III) and subunit-antibody aggregates detected with ^{125}I-protein A and autoradiography as in [13].

explain the observations that, in the intact ATP synthase, ϵ and CF_0-III can be cross-linked with imidoesters [11] and that dissociation of ϵ and CF_0-III from the membrane-bound enzyme complex is caused by proteolytic digestion of the γ subunit into two peptides [17].

Remarkably, under the conditions employed no binding interactions between CF_0-III and other CF_0 subunits could be detected. It is conceivable, however, that the formation of subunit complexes containing CF_0 components requires the presence of lipids prior to or during subunit reconstitution.

Although the present results demonstrate the feasibility of solid support-subunit reconstitution-immunoblotting as an approach to examine subunit binding interactions, the method has the disadvantages that: (i) optimal conditions for

subunit renaturation and reconstitution have to be determined; (ii) extensive washing of the nitrocellulose filters following reconstitution might disrupt hybrid subunit complexes; and (iii) dissociation of subunit aggregates may be caused by antibody binding. This might explain why a direct binding interaction between subunits β and CF_0-I was detected by cross-linking [11] but not with the present method.

To improve subunit reconstitution in future experiments, it is also advisable to renature the SDS-polypeptide complexes on gels prior to transfer onto the nitrocellulose filters in order to guarantee maximum renaturation as well as that protein binding to the support is caused by a minimum number of nitro groups only.

However, despite the remaining problems, the present technique is thought to be generally applicable for the investigation of stable binding interactions among subunits of multimeric proteins and, furthermore, of interactions of lipids, nucleic acids, metabolites, etc. with proteins.

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