Localization and Orientation of Subunit Delta of Spinach Chloroplast ATP-Synthase within the CF₀CF₁ Complex

2. Identification of C-Terminal Residues of Delta, Exposed on the Thylakoid Membrane

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Amphipathy, Antibodies, Coupling Factor, Proteolysis, Photophosphorylation

The amino acid residues of spinach CF₁ subunit delta are identified which are accessible and thus exposed within the quaternary structure of the ATP-synthase complex on the thylakoid membrane.

Abbreviations:
CF₀, peripheral moiety of chloroplast ATP-synthase; CF₁, membrane integral moiety of chloroplast ATP-synthase; F₁, ATPase of oxidative phosphorylation; F₀, membrane integral moiety of ATPase of oxidative phosphorylation; OSCP, oligomycin sensitivity conferring protein, a coupling factor in mitochondria; α, β, γ, δ, ε, subunits of CF₁; IV, I, II, III, subunits of CF₀; Chl, chlorophyll; DIFP, diisopropyl fluorophosphate; PMSF, phenyl methylsulfonylfluoride; 306-0, preimmune serum of rabbit 306; 306-1,2,..., successive bleedings of anti δ serum; ELISA, enzyme linked immuno sorbent assay; SSU, small subunit of ribulose biphosphate carboxylase.

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and the ATP synthesizing peripheral CF₁ are connected by specific recognition structures and binding forces. We analyze this contact region in molecular detail. It is not known how the energy of the H⁺ gradient is transduced to the active site in CF₁ and where within the ATP-synthase complex it is transformed to a conformational change [1–3].

The CF₁ subunit δ was shown to bind specifically to thylakoid embedded CF₀ after EDTA treatment [4] and to partially reconstitute photophosphorylation [5]; therefore within the quaternary structure of the ATP-synthase complex CF₀CF₁ subunit δ is in contact both to CF₁ and to CF₁ subunits, and may be a specific link in energy transduction [6].

In the previous publication [7], analyzing the accessibility of subunit δ to proteases and antibodies, we have shown that nearly all immunogenic surfaces on the tertiary structure of this subunit, all lysines and arginines and the residues Asp53, Glu₆₁, Glu₉₅,
and Glu\textsubscript{106} are shielded within the complex on the thylakoid membranes. But δ is not completely sandwiched between CF\textsubscript{0} and CF\textsubscript{1}; some antibodies of the monospecific polyclonal antiserum against spinach CF\textsubscript{1} δ (rabbit 306) inhibit the PMS mediated cyclic photophosphorylation and agglutinate suspensions of isolated thylakoids [7].

In this publication we separate two types of antibodies from the serum 306, one type which agglutinates and inhibits and is absorbed by thylakoid embedded δ, and another type which is not absorbed and only reacts in Western immuno blots and ELISA with CF\textsubscript{1} and isolated δ. Both types of antibodies are used to analyze the pieces of δ after proteolysis within the quaternary structure by trypsin, the \textit{Staphylococcus aureus} endoprotease V8, aminopeptidase M and carboxypeptidase Y.

The C-terminal residues Met\textsubscript{180}—Val\textsubscript{187} are identified this way to comprise a significant part of the epitope of serum 306, exposed \textit{in situ}. No other exposed epitope has been detected. \textit{Staphylococcus aureus} protease V8 has access to δ and digests it behind Glu\textsubscript{179} and Glu\textsubscript{173}, trypsin does not degrade δ behind Lys\textsubscript{169}.

Secondary structure calculations of δ reveal Val\textsubscript{167}—Asp\textsubscript{182} as an amphipathic α-helix. The hydrophilic face of this helix is exposed on the surface of the CF\textsubscript{0}CF\textsubscript{1} complex on the thylakoid membrane; the hydrophobic face, which is well conserved in homologous sequences, probably is oriented towards the interior of the ATP-synthase in spinach chloroplasts.

### Material and Methods

Preparation of CF\textsubscript{1} from market spinach [8], protein determination [9], and chlorophyll determination [10], SDS-polyacrylamide gel electrophoresis [11], staining with Coomassie brilliant blue (Serva G 250) or silver dichromate [12], immunization, agglutination and absorption [13], Western immuno blots [14] and decoration with peroxidase conjugates 2nd antibodies [15] and ELISA [16] were carried out according to standard procedures. Separation of a polypeptide mixture after V8 degradation was done by HPLC (Waters) on a reversed phase column (C8, Macherey & Nagel, 300 Å, 5 μm) by acetonitrile gradient in the presence of trifluoro acetic acid (0.1—0.08% gradient). The amino acid sequence of peptides was determined by automated Edman degradation (Applied Biosystems Sequenator) and online analysis of the phenyl thiodyantoin derivatives [17].

The antibodies from serum 306-5, which can be absorbed by CF\textsubscript{1} δ \textit{in situ}, were separated and recovered as follows: 0.5 ml of antiserum were incubated with 0.4 ml thylakoid suspension, 0.5 mg Chl in 10 mM NaCl, for 30 sec at 22 °C; the thylakoids were spun down, washed 3 times with 0.5 ml 10 mM NaCl, and resuspended in 0.5 ml 0.1 mM glycine-HCl buffer, pH 2.5; after incubation for 30 sec the thylakoids were spun down, 2 min 10,000 rpm, Eppendorf centrifuge 3200, and the supernatant, containing the desorbed antibodies, was titrated to pH 8.0 with 1 M Tris base.

### Results

\textit{Separation of antibodies only reacting with an epitope of CF\textsubscript{1} δ exposed within CF\textsubscript{0}CF\textsubscript{1}}

In the previous publication [7] we have described the production of the monospecific polyclonal antiserum 306 against spinach CF\textsubscript{1} subunit δ. Purification of electroeluted 21 kDa polypeptide δ on HPLC was essential to separate a minute amount of a specific breakdown product of CF\textsubscript{1} subunit β, comigrating at 21 kDa; this very immunogenic piece led with earlier antisera to an apparent immunological crossreactivity of the CF\textsubscript{1} subunits δ and β [18].

The \textit{anti} δ serum 306 contains antibodies that agglutinate suspensions of thylakoids and inhibit PMS mediated cyclic photophosphorylation [7]. The serum was absorbed with suspensions of isolated thylakoids, and the supernatants tested for remaining antibodies against CF\textsubscript{1} δ; they did not agglutinate any more, but in Western immuno blots the strength of the reaction at 21 kDa did not decrease (data not shown). Therefore most antibodies positive in Western blot had not reacted with epitopes of δ exposed \textit{in situ}.

The antibodies, that do react with exposed epitopes, were prepared after absorption to thylakoids by low pH treatment, as described in Materials and Methods.

\textit{Identification of amino acid residues of subunit δ accessible at the surface of the quaternary structure of CF\textsubscript{0}CF\textsubscript{1}}

In the preceeding paper we have published [7] that trypsin (bovine, Boehringer) had no effect on δ in
situ up to 50 μg protease/mg Chl, nor had treatment with aminopeptidase M (Boehringer); on Western immuno blots no decrease of the strength of the 21 kDa band occurred and no degradation products were decorated by anti δ. We repeated the experiments and extended the analysis: After both limited proteolysis with trypsin and aminopeptidase M treatment the thylakoids still were agglutinated by serum 306, and the agglutinating antibodies, separated as described, could still be absorbed. DIFP led to unspecific aggregation of thylakoid suspensions, therefore PMSF was used to terminate the limited proteolysis by all four proteases applied. Trypsin digested δ already after resolution of CF from CF, by EDTA, aminopeptidase M only after dissociation of δ from the other polypeptides of CF, (data not shown). Thus the N-terminal residues of δ seem not to be exposed, and no lysine or arginine seems to belong to the exposed epitope of δ. Soluble CF, δ is digested by trypsin at Lys161 and Lys169 (W. Finke, unpublished).

We also reported on four digestion sites of Staphylococcus aureus protease V8 in isolated δ, Asp53, Glu173, Glu179 and Glu196, which are not exposed in situ [7], as concluded from the amino acid sequence of 2 isolated peptides after V8 treatment of isolated δ. A third degradation product was separated by HPLC and sequenced:

V8 peptide 3 of δ: IAALQLE.

The peptide corresponds to residues Glu173/Ile174 up to Glu179 in the sequence of spinach CF, δ, deduced from cDNA [19].

This degradation next to Glu173 and Glu179 of δ also occurs when the unresolved ATP-synthase complex on the membrane is subjected to V8 treatment: If analyzed in Western blot with the two types of antibodies from serum 306, the residual antibodies after absorption of the serum with thylakoids (called 306-5, abs.) decorated a weak 20 kDa and a 19 kDa band, whereas the agglutinating antibodies, purified by their affinity to thylakoids (called 306-5, affi.), did not react any more (Fig. 1). Their corresponding accessible epitope has been degraded. After V8 treatment the thylakoids were also not agglutinated any more by anti δ serum 306. After protease V8 treatment of isolated CF, or isolated δ a multiple pattern of degradation products can be decorated on Western blots (data not shown).

To further proof the exposure of C-terminal residues of CF, subunit δ in the complex, the thylakoids were subjected to digestion by carboxypeptidase Y and analyzed in Western blot with antisemum 306 and with both types of separated antibodies (Fig. 2). The antibodies against the exposed epitope (306-5, affi.) did not react any more (Fig. 2h), whereas the non absorbable antibodies (306-5, abs.) indicate a decrease of the apparent molecular weight of the antigen δ (Fig. 2f). After carboxypeptidase Y treatment the thylakoids were not agglutinated any more by the complete anti δ serum 306, as after protease V8 treatment. If in comparison isolated δ or CF, were subjected to digestion by carboxypeptidase Y, a further decrease in molecular weight of δ can be seen in Western blot with complete serum 306-5 (Fig. 2c, d).

Secondary structure calculation of C-terminal sequence of CF, subunit δ

A hydropathy analysis [20] of the last 45 C-terminal amino acids of CF, δ (window 9) showed little...
Fig. 2. Analysis of proteolytic digestion of CF$_1$ subunit $\delta$ on the thylakoid membrane by carboxypeptidase Y (Boehringer). Western blot with specific antibodies, c, d: 30 $\mu$g isolated CF$_1$/slot; a, b, e–h: thylakoids 13 $\mu$g Chl/slot; a, c, e, g: untreated preparations; b, d, f, h: treated with carboxypeptidase Y (b: 130 $\mu$g protease/mg Chl, 2 h at 22°C; d: 25 $\mu$g protease/mg CF$_1$, 1 h at 22°C; f, h: 133 $\mu$g protease/mg Chl, 30 min at 22°C; all treatments stopped with DIFP, 10$^{-6}$ M final conc.). a, b, c, d: Blot with complete serum 306-5 against CF$_1$ $\delta$, 1:100; e, f: blot with serum 306-5, absorbed with thylakoids, 1:50; g, h: blot with antibodies 306-5, affinity purified on thylakoids, 1:5 (2nd antibody and incubation buffer as in Fig. 1).

Fig. 3. Secondary structure predictions for the C-terminal amino acid sequence of spinach CF$_1$ subunit $\delta$. Symbols denote: a, $\alpha$-helix; b, part of $\beta$-sheet; c, $\beta$-turn; d, unpredicted structure. Calculations according to the algorithms of 1, Scheraga et al. [21]; 2, Chou, Fasman [22]; 3, Nagano [23]; 4, Robson, Suzuki [24]; 5, Rawlings et al. [25].

Table I. Alignment of the C-terminal amino acid sequence of CF$_1$ subunit $\delta$ from spinach [19] with respective sequences (CF$_1$ $\delta$ of Anabaena 7120 [38], Synechococcus 6301 [39], $F_i$ $\delta$ of Rhodopseudomonas blastica [40], Rhodospirillum rubrum [40], Escherichia coli [41] and PS3 [42], Bos primigenius OSCP [43] and Saccharomyces cerevisiae ATPase 5 [44], letters a and d indicate hydrophobic residues in the heptad; triangles indicate digestion sites of the protease V8 from Staphylococcus aureus.

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amphipathic character of this C-terminal α-helix became apparent in all cases. The sites of proteolytic digestion by the St. aureus endoprotease V8 are situated on the hydrophilic face of this helix, Glu$_{173}$ and Glu$_{179}$ for spinach CF$_1$ δ.

**Discussion**

Subunit δ of the CF$_1$CF$_2$ complex is necessary for reconstitution of thylakoids, resolved with EDTA or NaBr [26–28]. We have suggested that δ may carry H$^+$ into CF$_1$ along its amphipathic N-terminal α-helix [6]; this N-terminus and other hydrophilic residues are inaccessible within CF$_0$CF$_1$ [7].

By separating antibodies from serum 306 against an exposed epitope we could specifically trace the degradation of this part of CF$_1$ δ by proteases, and thus identify the exposed residues:

- The epitope corresponding to the inhibiting and agglutinating antibodies from serum 306 [7], is situated on the C-terminal residues of δ. The protease V8 removes a significant part of it (Fig. 1) by cutting after Glu$_{179}$. This protease probably does not digest after Asp under the experimental conditions applied. The observation of a degradation product of δ at 20 kDa (Fig. 1c) excludes the possibility of a proteolysis after Glu$_{23}$, the first Glu in the N-terminal sequence.

- Until now the animals did not produce antibodies against this epitope; after absorption of the earlier antisera against δ with CF$_1$ subunit β, i.e. removal of antibodies with apparent cross-reactivity with CF$_1$ β [7, 18], no agglutination or inhibition was observed any more. Rabbit 306 has been immunized with a trimeric δ after HPLC purification [7].

- All agglutinating and inhibiting antibodies are absorbed from the serum by thylakoids in the dark. The non absorbed antibodies are directed against parts of δ, that are not destructed by carboxypeptidase Y; the strength of the band, decorated by these antibodies, did not decrease in Western blot (Fig. 2f).

- As far as can be concluded from Western immuno-blot with the antigen δ, run on SDS gels, or with the proteolysis products, the agglutinating and inhibiting antibodies are directed against the same epitope region on the exposed C-terminus, Met$_{180}$–Val$_{187}$, since all antibodies purified by their affinity to epitopes on the thylakoid membrane do not react any more with CF$_1$ δ after carboxypeptidase Y treatment of thylakoids (Fig. 2h).

- From this it follows that antibodies in serum 306 inhibit the PMS mediated cyclic photophosphorylation [7] by reacting with the exposed C-terminus of δ. We do not know yet, whether they act like uncouplers or energy transfer inhibitors. It is difficult to reconcile how a degradation product of δ which is suggested to have lost about 10 C-terminal residues, can still reconstitute photophosphorylation [5].

- From the analysis of the digestion products of δ in situ by trypsin and the protease V8, the accessibility of Glu$_{173}$ is shown and inaccessibility of Lys$_{169}$ is suggested. Thus subunit CF$_1$ δ seems to become shielded within the CF$_0$CF$_1$ complex between Glu$_{173}$ and Lys$_{169}$. The F$_1$ subunit δ from E. coli, which is digested by trypsin [29], contains two arginines on the C-terminal amphipathic α-helix, corresponding to Glu$_{170}$ and Glu$_{173}$ of spinach CF$_1$ δ (Table I).

If the 45 C-terminal residues of spinach CF$_1$ δ are aligned with the known sequences of homologous subunits from various organisms (Table I), without maximizing amino acid homologies and identities by introducing gaps, in the positions marked “a” and “d” only hydrophobic residues are found. Thus the hydrophobic face of the amphipathic C-terminal α-helix (Fig. 4) is conserved and rather narrow. It is oriented in situ towards the protein interior, since the opposite hydrophilic face is accessible to the protease V8 in spinach.

The corresponding hydrophobic binding structure could be part of subunit δ itself, part of a surface of another subunit of CF$_1$ or part of one of the CF$_0$ subunits. We favor the last possibility: Titrations of
the salt dependency [30—34] and the pH effects [32, 34] on resolution of CF$_1$ from CF$_0$ by EDTA and pyrophosphate suggest an hydrophobic interaction of CF$_1$, including $\delta$, with CF$_0$ after removal of electrostatic repulsion [33]. In contrast Cox et al. [35] suggested $\text{Mg}^{2+}$-bridges between certain residues of E. coli F$_1$ $\delta$ and F$_0$ $\beta$; these acidic residues are not conserved, however, in spinach CF$_1$ $\delta$ [19] and CF$_0$I [36]. It may be of significance that the highest number of amino acid identities, especially along the hydrophobic face of the amphipathic C-terminal helix is found between spinach CF$_1$ $\delta$ and PS3 F$_1$ $\delta$ (Table I); hybrid reconstitution between coupling factors of higher plants and PS3 is shown to be possible [37].

In a further publication of this series (W. Finke, R. J. Berzborn) we will characterize by monoclonal antibodies an epitope on the shielded surface of CF$_1$ $\delta$, probably involved in binding to a CF$_0$ subunit.

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