Localization and Orientation of Subunit Delta of Spinach Chloroplast ATP-Synthase within the CF_0 CF_1 Complex

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

Richard J. Berzborn and Werner Finke

Lehrstuhl für Biochemie der Pflanzen, Fakultät für Biologie, Ruhr-Universität Bochum, Postfach 102148, D-4630 Bochum 1, Bundesrepublik Deutschland

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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 CF_1 subunit delta are identified which are accessible and thus exposed within the quaternary structure of the ATP-synthase complex on the thylakoid membrane.

Two types of antibodies in the monospecific polyclonal antiserum 306 against CF_1 delta, described in the previous publication [Z. Naturforsch. **44c**, 153–160 (1989)], were separated by virtue of their different affinity to thylakoid membranes and used for specific analysis of the products of proteolytic digestion of delta *in situ*.

Polypeptide delta *in situ*, *i.e.* within the CF_0CF_1 complex on the membrane, is not susceptible to digestion by aminopeptidase M and trypsin, but is shortened by about 1 kDa by carboxypeptidase Y and digested at residues Glu_{173} and Glu_{179} by the *Staphylococcus aureus* protease V8. The epitope on delta reacting with the agglutinating antibodies from serum 306 is lost after these proteolytical treatments and therefore situated on residues Met_{180}–Val_{187}. Since trypsin destroys this epitope only after prolonged incubation and with at least 50 µg trypsin/mg Chl, residue Lys_{169} of delta probably is inaccessible *in situ*.

We conclude that the C-terminal amphipathic α-helix of spinach CF_1 subunit delta is exposed on the thylakoid membrane, with the hydrophilic face directed to the outside, and that CF_1 delta starts to be shielded within the quaternary structure of the CF_0CF_1 complex between Glu_{173} and Lys_{169}. The hydrophobic face of the c-terminal helix may be part of the binding surface towards CF_0.

Antibodies from serum 306 inhibit the PMS mediated cyclic photophosphorylation by reacting with C-terminal residues of δ.

Introduction

The ATP-synthase complex CF_0CF_1 transforms and conserves in higher plant chloroplasts part of the energy of light driven vectorial electron transport. The H^+ conducting membrane integral CF_0 moiety and the ATP synthesizing peripheral CF_1 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 CF_1 and where within the ATP-synthase complex it is transformed to a conformational change [1–3].

The CF_1 subunit δ was shown to bind specifically to thylakoid embedded CF_0 after EDTA treatment [4] and to partially reconstitute photophosphorylation [5]; therefore within the quaternary structure of the ATP-synthase complex CF_0CF_1 subunit δ is in contact both to CF_1 and to CF_0 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 Asp_{53}, Glu_{61}, Glu_{95},

Abbreviations: CF_1, peripheral moiety of chloroplast ATP-synthase; CF_0, membrane integral moiety of chloroplast ATP-synthase; F_1, ATPase of oxidative phosphorylation; F_0, 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 CF_1; IV, I, II, III, subunits of CF_0; 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 bisphosphate carboxylase.

Reprint requests to Prof. Dr. R. J. Berzborn.

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and Glu_{106} are shielded within the complex on the thylakoid membranes. But δ is not completely sandwiched between CF_0 and CF_1; some antibodies of the monospecific polyclonal antiserum against spinach CF_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_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 *Staphylococcus aureus* endoprotease V8, aminopeptidase M and carboxypeptidase Y.

The C-terminal residues $Met_{180}-Val_{187}$ are identified this way to comprise a significant part of the epitope of serum 306, exposed *in situ*. No other exposed epitope has been detected. *Staphylococcus aureus* protease V8 has access to δ and digests it behind Glu_{179} and Glu_{173}, trypsin does not degrade δ behind Lys_{169}.

Secondary structure calculations of δ reveal $Val_{167}-Asp_{182}$ as an amphipathic α-helix. The hydrophilic face of this helix is exposed on the surface of the CF_0CF_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_1 from market spinach [8], protein determination [9], and chlorophyll determination [10], SDS-polyacrylamide gel electrophoresis [11], staining with Coomassie brillant 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 thiohydantoin derivatives [17].

The antibodies from serum 306-5, which can be absorbed by CF_1 δ *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 M 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

Separation of antibodies only reacting with an epitope of CF_1 δ exposed within CF_0CF_1

In the previous publication [7] we have described the production of the monospecific polyclonal antiserum 306 against spinach CF_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_1 subunit β, comigrating at 21 kDa; this very immunogenic piece led with earlier antisera to an apparent immunological crossreactivity of the CF_1 subunits δ and β [18].

The *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_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 *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.

Identification of amino acid residues of subunit δ accessible at the surface of the quaternary structure of CF_0CF_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_1 from CF_0 by EDTA, aminopeptidase M only after dissociation of δ from the other polypeptides of CF_1 (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_1 δ is digested by trypsin at Lys_{161} and Lys_{169} (W. Finke, unpublished).

We also reported on four digestion sites of *Staphylococcus aureus* protease V8 in isolated δ, Asp_{53}, Glu_{61}, Glu_{94} and Glu_{106}, 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: I A A Q L E.

The peptide corresponds to residues Glu_{173}/Ile_{174} up to Glu_{179} in the sequence of spinach CF_1 δ, deduced from cDNA [19].

This degradation next to Glu_{173} and Glu_{179} 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_1 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_1 subunit δ in the complex, the thylakoids were subjected to digestion by carboxypeptidase Y and analyzed in Western blot with antiserum 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_1 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).

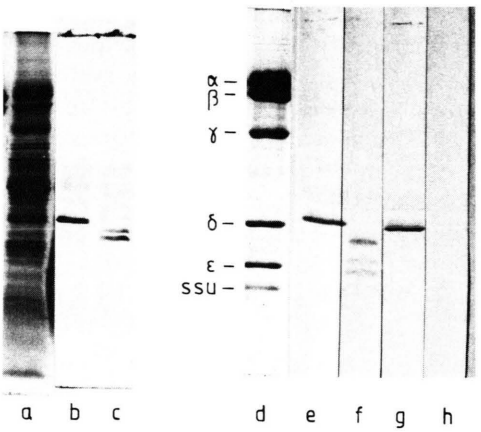

Fig. 1. Analysis of proteolytic digestion of CF_1 subunit δ on the thylakoid membrane by the *Staphylococcus aureus* protease V8 (Boehringer). a, d: SDS polyacrylamide gel electrophoresis, stained with Coomassie brilliant blue; b, c, e, f, g, h: western immuno blot with specific antibodies; d: 15 μg CF_1 as molecular weight standards; a, b, c, and e, f, g, h: thylakoids with 12 μg Chl/slot; a, b, e, g: untreated thylakoids in 10 mM NaCl; c, f, h: thylakoids treated with V8 (c: 17 μg V8 in NH_4-carbonate buffer, pH 7.8, 50 mM final conc./mg Chl, 5 min at 25 °C; f, h: 33 μg V8/mg Chl, 30 min, 25 °C, both stopped with DIFP, 10^{-6} M).
b, c: Blot with complete serum 306-5 against CF_1 δ, 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 (second antibodies (Medac) 1:3000; all incubations in 1% gelatine and TBS, 20 mM Tris buffer, pH 7.5, 500 mM NaCl).

Secondary structure calculation of C-terminal sequence of CF_1 subunit δ

A hydropathy analysis [20] of the last 45 C-terminal amino acids of CF_1 δ (window 9) showed little

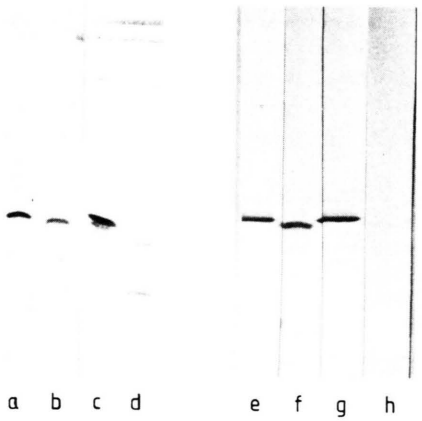

Fig. 2. Analysis of proteolytic digestion of CF_1 subunit δ on the thylakoid membrane by carboxypeptidase Y (Boehringer). Western blot with specific antibodies. c, d: 30 µg isolated CF_1/slot; a, b, e–h: thylakoids 13 µg Chl/slot; a, c, e, g: untreated preparations; b, d, f, h: treated with carboxypeptidase Y (b: 130 µg protease/mg Chl, 2 h at 22 °C; d: 25 µg protease/mg CF_1, 1 h at 22 °C; f, h: 133 µg 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 δ, 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).

variation in the average hydropathy index; in addition to CF_1 δ from spinach [19] we analyzed subunit δ from *E. coli, Anabaena 7120, Synechococcus 6301, Rhodopseudomonas blastica, Rhodospirillum rubrum* and *bovine* OSCP (J. Block, R. J. Berzborn, and W. Finke, unpubl.). A conserved increase in hydrophobicity is observed in this stretch, for spinach between residues 139 and 153; but there are not enough non-polar residues to build a span in subunit δ of any organism sequenced.

Next we calculated secondary structure algorithms (Fig. 3). The consensus program [25] predicts for residues 144–187 of spinach CF_1 δ: β-turn, β-sheet, β-turn, unpredicted random structure, α-helix, starting at Val_{167} and an unpredicted C-terminus. The stretch Asp_{144}–Asn_{157} (cp. Fig. 3) is probably part of an antiparallel β-sheet in the interior of CF_1 δ (J. Block, R. J. Berzborn, unpubl.).

The same calculations on homologous δ sequences (cp. Table I) suggest very similar patterns in secondary structure (data not shown). A conserved α-helix starts at the residue next to the conserved Ser_{166}. This C-terminal helix was plotted as an helical wheel for spinach δ (Fig. 4) and the other species. A strong

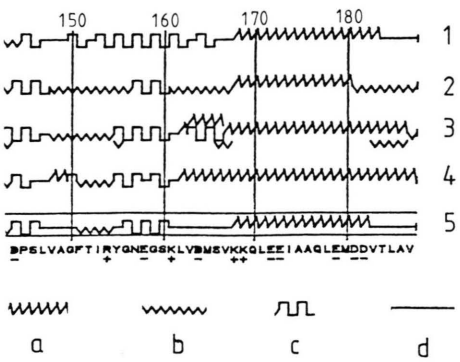

Fig. 3. Secondary structure predictions for the C-terminal amino acid sequence of spinach CF_1 subunit δ. Symbols denote: a, α-helix; 6, part of β-sheet; c, β-turn; d, unpredicted structure. Calculations according to the algorithms of 1, Scheraga *et al.* [21]; 2, Chou, Fasman [22]; 3, Nagano [23]; 4, Robson, Suzuki [24]; 5, Rawlings *et al.* [25].

Table I. Alignment of the C-terminal amino acid sequence of CF_1 subunit δ from spinach [19] with respective sequences (CF_1 δ of *Anabaena 7120* [38]. *Synechococcus 6301* [39], F_1 δ 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 heptade, triangels indicate digestion sites of the protease V8 from *Staphylococcus aureus*.

		d a d a	
Sp. ol.	D P S L V A G F T I R Y G N E G S K L V D	M S V K K Q L E E I A A Q L E M D D V T L A V	187
Ana. 7120	D S D L I G G V I I K V . . . G S Q V I D	S S I R G Q L R R L S L R L S N S	183
Syn. 6301	D A D L L G G V I I K V . . . G S Q V L D	A S L R G Q L K R I S I S L A A	180
Rps. bl.	D E S L I G G L I V K L . . . G S T M I D	T S V K S K L A S L Q N A M K E V G	186
Rsp. r.	D P A L L G G M V V R V . . . G S R M V D	S S L S T K L K R L Q L A M K G V G	186
E. coli	D K S V M A G V I I R A . . . G D M V I D	G S V R G R L E R L A D V L Q S	177
PS3	D P E L I G G V N V R I . . . G N R I Y D	G S V S G Q L E R I R R Q L I G	179
Bos pr.	D P S I M G G M I V R I . . . G E K Y V D	M S A K T K I Q K L S R A M R Q I L	190
Saccharo.	K P E I K G G L I V E L . . . G D K T V D	L S I S T K I Q K L N K V L E D S I	212

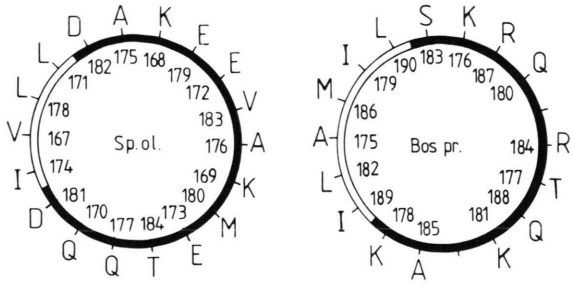

Fig. 4. Helical wheel plots of the C-terminal residues Val_{167}–Thr_{184} of spinach CF_1 subunit δ and of Ala_{175}–Leu_{190} of bovine OSCP. The residues are numbered according to their position in the actual sequence. These stretches of residues are predicted to fold into α-helix in both species by the algorithms applied. For the significance of the amphipathic distribution of residues around the α-helix see text.

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_0CF_1 complex is necessary for reconstitution of thylakoids, resolved with EDTA or NaBr [26–28]. We have suggested that δ may conduct H^+ into CF_1 along its amphipathic N-terminal α-helix [6]; this N-terminus and other hydrophilic residues are inaccessible within CF_0CF_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 crossreactivity 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 blots 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_0CF_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 Gln_{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 δ, with CF_0 after removal of electrostatic repulsion [33]. In contrast Cox *et al.* [35] suggested Mg^{2+}-bridges between certain residues of *E. coli* F_1 δ and F_0 b; these acidic residues are not conserved, however, in spinach CF_1 δ [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 δ and PS3 F_1 δ (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 δ, probably involved in binding to a CF_0 subunit.

Acknowledgements

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