Protein Sequence and Structure of N-terminal Amino Acids of Subunit Delta of Spinach Photosynthetic ATP-Synthase CF_1

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Amphipathy, Antibodies, Coupling Factor, Sequence, Secondary Structure

Chloroplast ATP-synthase (CF_1) subunit delta (δ) has been isolated from spinach thylakoids in the presence of SDS. By automated Edman degradation and online analysis of PTH derivatives the 35 N-terminal amino acid residues were sequenced. The mature protein starts with: NH_2–Val–Asp–Ser–Thr–Ala–Ser–Arg–Tyr–Ala–. This protein sequence allows alignment of spinach δ with the sequences of *Z. mays* 25 kDa polypeptide, the δ subunit of *Rps. blastica, Rsp. rubrum* and *E. coli* F_1, and of bovine OSCP, but not with mitochondrial δ.

Secondary structure calculations and helical wheel plots reveal a conserved secondary structure. The analyzed N-terminal sequences probably build a short amphipathic alpha helix with two adjacent turns. The such aligned polar residues around Tyr_8 of subunit δ are suitable to channel protons.

Introduction

During photosynthetic energy conservation in autotrophic higher plant chloroplasts light driven electron transport enables a thylakoid membrane bound protein complex to synthesize ATP. This ATP-synthase consists of the membrane integral CF_0 part and the peripheral CF_1 moiety, specifically attached to CF_0 [1].

CF_0 conducts H^+ [2] down the electrochemical gradient and CF_1 contains the active site for ATP hydrolysis and synthesis [3]. The energy conservation within the ATP-synthase complex probably occurs *via* an additional step. The question arises therefore where within the protein the proton motive force is transformed to a conformational change or movement of subunits [4, 5]; to find out in which form the energy is transmitted from CF_0 to CF_1, the structural details of the binding region between CF_1 and CF_0 have to be elucidated.

CF_1 subunit δ is in situ in contact both with CF_1 and CF_0 and is a possible intermitting link. This subunit is nuclear encoded and its sequence not known so far. We report here on protein sequencing of the N-terminal amino acids of CF_1 δ from spinach chloroplasts. Already from this N-terminal sequence the homology of CF_1 δ with *E. coli* F_1 subunit δ and bovine OSCP can be deduced, but not with mitochondrial F_1 δ which seems to be homologous to CF_1 ε [6].

Secondary structure plots and calculations reveal a conserved amphipathic alpha helix in the N-terminal residues of subunits δ of the six respective species sequenced so far.

From the sequence a hypothetical model of the secondary structure of the N-terminus of the δ subunit is deduced which shows an arrangement of polar residues suitable to conduct protons, *i.e.* to build an extension of the H^+ channel of CF_0.

Materials and Methods

The reagents for *sequence analysis* were purchased from Applied Biosystems; all other chemicals or solvents were of the highest purity available.

Abbreviations: SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin; CF_1, peripheral moiety of chloroplast ATP-synthase; CF_0, membrane integral moiety of chloroplast ATP-synthase; F_1, ATPase of oxidative phosphorylation; OSCP, oligomycin sensitivity conferring protein, a coupling factor in mitochondria; SSU, small subunit of chloroplast ribulose bisphosphate carboxylase.

The N-terminal sequences of CF_1 subunit delta from spinach and maize chloroplasts have been presented by us on a poster at the VIIth Intern. Congress on Photosynthesis, Providence 1986.

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Preparation of CF_1 was carried out by EDTA extraction of thylakoid membranes, isolated from market spinach, as described by Lien and Racker [7], except that DEAE Sepharose CL-6B (Pharmacia) was used instead of DEAE Sephadex A 50. *Protein determination* was done according to Lowry [8]. *SDS gel electrophoresis* was carried out according to Lugtenberg [9]. Acrylamide (Fluka) concentration was 13.5%. On analytical gels (1 mm thick, 7 cm separation length) the samples (10 μg) were run in 5 mm slots, 3 h, 27 mA, room temp.; on preparative gels (2 mm thick, 20 cm separation length) the sample (10–20 mg) was run as a continuous band, 30 cm wide, 24 h, 35 mA, 4 °C.

Staining of separated polypeptides was done with Coomassie brillant blue (0.17% Serva G 250 in 45% methanol, 10% acetic acid). Sometimes additional staining with silver dichromate according to Merril *et al.* [10] was carried out after destaining with 5% methanol, 7.5% acetic acid. Staining with Serva W was done with 0.2% Serva blue W in water, and destaining for 3 h in dest. water.

Electroelution of stained bands was carried out according to Hunkapiller *et al.* [11].

Immunization of rabbits was done as described [12]. About 200–400 μg CF_1 of electroeluted polypeptides were used for each injection; primary immunization by multisidal applications of 2.5 ml paste of antigen with compl. Freund's adjuvant (Difco); booster injection intraveneously at four weeks intervals with antigen in physiological buffer. The rabbits usually have been bled the 10th day after the second booster injection.

Western immuno blots were carried out essentially as described by Towbin *et al.* [13], but using horseradish peroxidase (HRP) conjugated 2nd antibody, as described by Hawkes *et al.* [14].

The amino acid sequence of the electroeluted polypeptides was determined by automated gas-phase Edman degradation (Applied Biosystems Sequenator) and by identification of the PTH derivatives [15].

Secondary structure predictions were calculated according to Wittmann-Liebold *et al.* [16]. The computer program was reformulated by K. H. Müller and J. Block, Max Planck Institut für Ernährungsphysiologie, Dortmund, from a Fortran like program running on a DEC/VAX computer to a Fortran 77 program running on a Perkin Elmer OF/32.

Results

Isolation of the delta polypeptide

Polypeptide δ was first shown to be a subunit of CF_1, purified from EDTA extracts according to the method by Lien and Racker [7] with an apparent molecular weight on SDS polyacrylamide gels of 17.5 kDa [17]. After staining with Amido black or Coomassie brilliant blue it takes up about 3–5% of the stain [18]. For specific detection and quantitative determination of δ only immunochemical techniques, *i.e.* specific antibodies, can be used. This technique also proved highly valuable for detection and identification of contaminations in preparations of CF_1 δ; in our laboratory we have produced antisera against all CF_1 and CF_0 subunits, except IV, and many other chloroplast proteins.

The procedures described for the isolation of δ using pyridine/urea, stepwise extraction and anion exchange chromatography on DEAE cellulose [19], or starting with a chloroform extract [20] do not lead to homogeneous preparations of spinach δ [21]. Only after combination of the pyridine/urea method with gel filtration on Sephacryl S 300 minute amounts of δ could be produced which showed on SDS polyacrylamide gel electrophoresis a single band.

These preparations were active; rebinding could be shown to membranes depleted of CF_1 by EDTA and in reconstitution of photophosphorylation by four-subunit CF_1 the dependency from the addition of this δ polypeptide was titrated [22]; immunization with this preparation of active δ yielded serum Bo 167 and 168 [21] which, however, show a multiple "background" reaction in Western immuno blot analysis. Thus the isolated subunit appeared not pure enough for sequencing by automated gas-phase Edman degradation and analysis of the PTH derivatives [15] and also for immunization [12]. For these purposes we isolated CF_1 δ with better purity, but denatured. Subunit δ was separated from the other subunits by electrophoresis of CF_1 on preparative polyacrylamide gels [9] in the presence of 0.1% SDS and 2.5 M urea; the δ containing region was excised and the polypeptide eluted [11]. The CF_1 had been prepared from spinach chloroplasts according to [7] except that the sucrose gradient centrifugation was omitted; contaminations by ribulose-bisphosphate carboxylase and ferredoxin-NADP reductase were observed, but can be tolerated for this isolation method of CF_1 δ.

The electroeluted polypeptide appeared pure on analytical SDS gels and was sequenced according to ref. [15]. Although more than 90% of the protein was blocked at the N-terminus (initial yield 22 out of 300 pmoles injected), the N-terminal and three more residues could be identified:

NH_2–Val–Asp–x–x–Ala–x–x–Tyr–x–x–.

Primary sequence of the N-terminal amino acids of the delta polypeptide

On the basis of the above results and the detection of proteolysis described in an accompanying paper the CF_1 preparation was carried out at 4 °C against the recommendation of Lien and Racker [7], urea was omitted in separating SDS slab gels, the resolving length was increased to 20 cm and the gel pattern was stained with Coomassie brilliant blue (Serva G) before the excision of the δ band.

With this preparation in the second run in the gas-phase sequenator 25 amino acid residues could be identified, in a third run with an independent preparation and online analysis of the PTH derivatives 35 residues (Fig. 1). Fig. 1 shows the yield of the respective residue in each cycle. The initial yield of 374 pmol was calculated using Val and Ala (open circles). From the plot the reliability of the identification of each residue can be judged. No synchronous changes in amount of other amino acids were detectable in the chromatograms of the PTH derivatives. There was complete agreement of residue 1–24 between the two runs.

The sequence is novel. The 35 residues contain 19 polar, including 9 charged, and 16 nonpolar amino acids. No clustering of hydrophobic residues is obvious, and the polar residues are distributed in a certain pattern (compare next chapter). The reported N-terminal sequence is not homologous to any partial sequence of the β subunit of CF_1. This could have happened since in standard preparations of CF_1 very easily a proteolytic breakdown of CF_1 β occurs and one product migrates at 21 kDa and is coeluted together with δ (R. J. Berzborn, J. Otto, W. Finke, unpublished). This contamination was below 10% and did not disturb the protein sequencing.

The reported sequence of spinach CF_1 δ can be aligned with sequences of a 25 kDa polypeptide of *Zea mays* [23], the sequences of *Rhodopseudomonas blastica, Rhodospirillum rubrum* and *Escherichia coli* δ subunit and of bovine OSCP [24–26] (Table I), but not with mitochondrial F_1 δ, which seems to be homologous to CF_1 ε [6].

Three residues are identical in all six sequences: Tyr_8, Ala_9 and Ala_{11}. The overall homology is low, but at several positions only conservative and compensating exchanges – not boxed in Table I – seem to indicate a very similar function. If Asp_{17} is switched with Val_{18}, it corresponds to Glu_{18} in the other sequences. The completely conserved residue Tyr_8 is homologous to Tyr_{11} of the δ polypeptide of *E. coli* F_1, the only Tyr in this subunit.

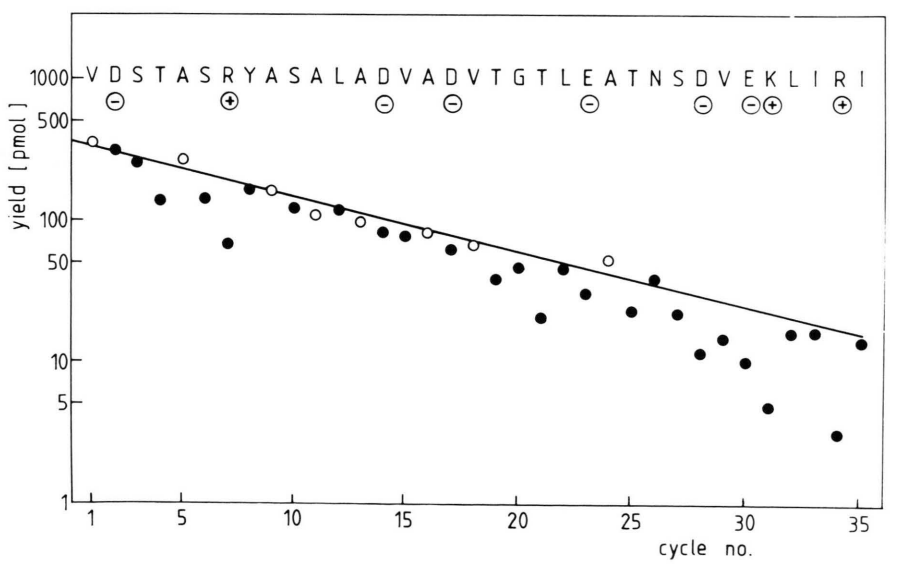

Fig. 1. N-terminal sequence of spinach CF_1 subunit δ. Identity and yield of each amino acid residue is indicated. Residues used for calculation of initial yield are shown as open circles.

Table I. N-terminal amino acid sequence of CF_1 polypeptide δ from *Spinacea oleracea*, and alignment with respective polypeptides from other species (*Spinacea oleracea* δ and *Zea mays* 25 kDa polypeptide from our laboratory, *Rhodopseudomonas blastica*, *Rhodospirillum rubrum* and *Escherichia coli* δ, and bovine OSCP from the literature).

Sp. ol.	δ	V D	S T . . A S R Y A S	A L A D V A D V T G	T L E A T N S D V E	K L I R I x x x x x
Z. mays	25 kDa	N A A . E	. . A A A E S Y A S	A L S E V A V E N G	L V E Q T V L D T L	R L T x x x x x x x
Rps. bl.	δ	M A E A A S I	S Q G I A E R Y A T	A L F E L S K E T G	A L K T L E T D I D	A L K D V L A G S P
Rsp. r.	δ	M S S H K A G	V T G V A E R Y A T	A L Y E L A E D R G	A L D Q V S A D L R	S L K A M L D E S G
Bos pr.	OSCP	F A K L V R P P V Q	I Y G I Q G R Y A T	A L Y S A A S K Q N	K L E Q V E K E L L	R V G Q I L . K E P
E. coli	δ	M S E	F I T V A R P Y A K	A A F D F A V E H Q	S V E R W Q . D M L	A F A A E V T K N E

Secondary structure analysis of the N-terminal sequence

A hydropathy analysis [27] of the first 35 N-terminal amino acids (window 9) showed very little variation in the average hydropathy index. This was found with the sequences of the 21 kDa δ subunit of spinach CF_1, the 25 kDa subunit of maize CF_1CF_0, the δ subunits of *E. coli*, *Rps. blastica* and *Rsp. rubrum* and of bovine OSCP (data not shown). The result from the Kyte/Doolittle program [27] is not in agreement with the conclusion by Ovchinnikov *et al.* [28] who suggested that OSCP may be anchored in the membrane by residues 7–26 which are mostly hydrophobic [28].

Folding prediction algorithms according to Scheraga [29], Chou, Fasman [30], Nagano [31], and Robson [32], combined to a consensus prediction program by Rawling, Ashman and Wittmann-Liebold [16] were calculated for the 35 N-terminal residues from spinach δ; the calculations were run on the Perkin Elmer OF/32 computer in the Max-Planck-Institut für Ernährungsphysiologie, Dortmund, by Dr. Jürgen Block (Fig. 2).

The consensus program predicts for spinach δ a turn at Ser_6, Arg_7, an alpha helix Leu_{12}–Ala_{16}, and another turn at Asn_{26}, Ser_{27}. Within the parameters used by this program the secondary structure of the other residues is unpredicted. The individual algorithms show more detail, however (Fig. 2).

The same calculations were run with the sequences of the homologous polypeptides (plots not shown). A turn was predicted by the consensus program in homologous regions for the peptide of *E. coli* (Arg_9, Pro_{10}), and OSCP (Gln_{15}, Gly_{16}, Arg_{17}, Tyr_{18}), but for *Rps. blastica* already in Gln_9, Gly_{10}, *i.e.* five residues before the conserved Arg_{14}, Tyr_{15}; for *Rsp. rubrum* and *Zea mays* no clear turn was indicated before the onset of the alpha helix by the consensus program, but by some of the individual programs.

All five algorithms show a more or less extended alpha helix starting at or including the conserved Tyr_8 in the δ polypeptides of all six species.

At Gly_{20} in spinach the Nagano and Robson program predicts a turn, at the homologous Gly_{22} in *Zea mays* (compare Table I) all four programs predict a turn, at *Rps. blastica* Gly_{27} three programs, at *Rsp. rubrum* Gly_{27} all four, at the Asn_{30} in OSCP only Chou Fasman and Nagano; in *E. coli* Gln_{23} Nagano yielded no prediction, whereas all the other calculations show the alpha helix continued. In summary, a

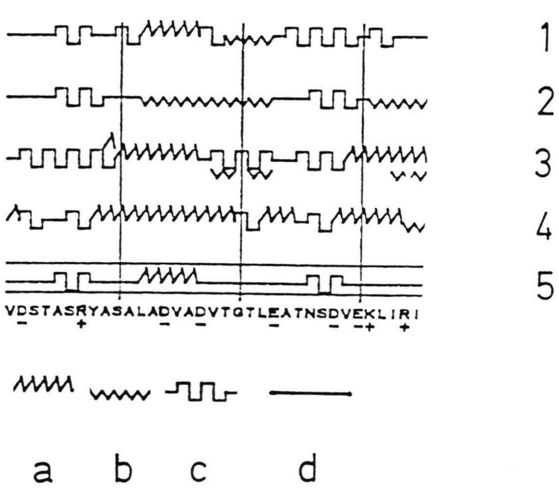

Fig. 2. Secondary structure predictions for the N-terminal sequence of spinach CF_1 polypeptide δ. Symbols denote: (a) alpha helix, (b) beta sheet, (c) beta turn, (d) unpredicted random structure. Calculations according to algorithms of (1) Scheraga *et al.* [29], (2) Chou, Fasman [30], (3) Nagano [31], (4) Robson, Suzuki [32], (5) Wittmann-Liebold *et al.* [16].

secondary structure detail seems to be conserved: An alpha helix with two adjacent turns.

The partial stretches of the six investigated N-terminal sequences which probably build alpha helices were plotted as helical wheels [33], the conserved sequence Y–A–x–A superimposed (Fig. 3). A common feature becomes immediately apparent: This partial sequence exhibits a strong amphipathic character in all six cases. From preliminary experiments with arginase C and monoclonal antibodies we conclude that Arg_7 and Tyr_8 are not accessible in CF_1CF_0 or in isolated CF_1 (W. Finke, R. J. Berzborn, unpublished). The N-terminus of the CF_1 δ seems to be located in the protein interior.

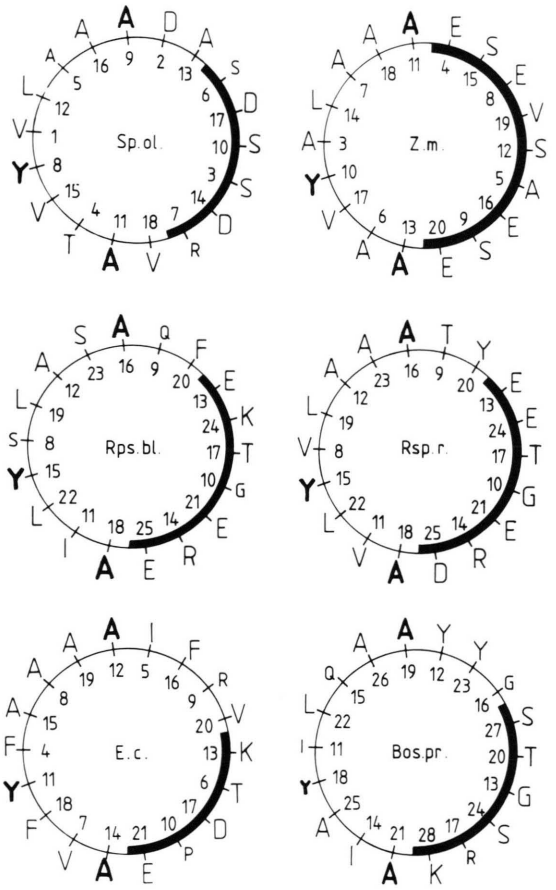

Fig. 3. Helical wheel plots of 18 N-terminal amino acids of spinach CF_1 subunit δ, and of homologous stretches from the five other species known. Abbreviations of species as in Table I. The residues are numbered according to their position in the actual sequence of the respective δ subunits. Conserved Tyr and Ala are shown in bold letters. When the consensus program by Wittmann-Liebold *et al.* [16] predicts alpha helix or does not come to a decision, large letters were used, when a higher probability for turn was calculated, smaller letters were used. Polar faces of the helices are shown as bold sector.

Discussion

To facilitate the understanding of the function of each subunit of the photosynthetic ATP-synthase CF_1CF_0 a detailed knowledge of the structure of the polypeptides is necessary. Finally one should have the positions of each amino acid residue within the tertiary structure.

Since functional amino acids and domains were conserved during evolution, a biochemical comparison of polypeptides helps to understand the function of particular amino acid residues, if identical or homologous residues or identical secondary structures are discovered.

The investigated polypeptide CF_1 δ is a subunit of the peripheral moiety of the photosynthetic ATP-synthase with its own identity. This was concluded from separation on SDS gels [17], immunological non-crossreactivity with the other subunits in Ouchterlony tests [18, 34], and immuno-electrophoretic analysis [35, 36], its amino acid composition [19, 37], and from the fact that reconstitution of photophosphorylation was dependent on its addition [19, 20]. In contrast to the α, β and ε subunit [38], δ and γ are encoded in the nucleus [39] and imported into the chloroplast [40, 41]. The new N-terminal amino acid sequence, reported by us (Table I), manifests that the 21 kDa polypeptide δ has its own identity.

To isolate homogeneous δ polypeptide for protein sequencing and production of monospecific antisera we have chosen separation on SDS slab gels and electroelution of the denatured protein. For large scale preparation of CF_1 subunits chromatography on diverse columns in the presence of SDS was necessary [37]. Isolation on hydroxylapatite in the presence of octylglucoside [42] did not lead to homogeneous preparations in our laboratory. In a second paper from McCarty's group [43] experiments using isolated active δ are missing. This isolation method of δ could also not be reproduced in other laboratories (S. Engelbrecht, pers. commun.).

For elution of δ from preparative SDS gels certain

contaminations in the starting material CF_1 can be tolerated, *e.g.* ribulose bisphosphate carboxylase large and small subunits and ferredoxin NADP reductase. But degradation of protein can be highly misleading. After we realized on SDS gels with good resolution in the respective region up to 4 bands running very close below δ by silver staining, we suspected a proteolytic digestion of this CF_1 subunit during isolation. The same suspicion has been mentioned by McCarty's group [44] and others [45]. In an accompanying paper it will be shown, however, by the use of monoclonal and polyclonal antibodies that the degradation of δ is negligible, if the isolation of CF_1 is done in the cold. Our analysis of immunological crossreactivity of most anti δ antisera, also from other laboratories [45], with the β subunit and of anti β with several smaller polypeptides, including one in the position of δ, and one just below δ, indicate a breakdown of the β subunit during CF_1 isolation. This contamination with breakdown products of β was less than 5–10% and did not disturb the protein sequencing of the N-terminal residues of CF_1 δ.

The reported protein sequence of δ was identical after isolation from SDS containing gels with or without urea (run one *vs.* run two and three). The protein sequence clearly shows where the precursor-processing occurs during import of the δ polypeptide [41]. The mature protein as isolated by us from EDTA extracted CF_1 on SDS gels starts with:

NH_2–Val–Asp–Ser–Thr–Ala–Ser–Arg–Tyr–Ala–Ser–Ala–Leu–.

Residues Tyr_8 to Ile_{35} are identical to the sequence of CF_1 delta deduced from cDNA (R. G. Herrmann, pers. comm.), but residues Val_1 to Arg_7 were still missing from the deduced sequence.

The alignment shown in Table I of spinach δ with the homologous polypeptides from 4 species show strong similarities to these polypeptides with established assignment and function. There is high homology to a 25 kDa polypeptide in *Zea mays* CF_1CF_0 reported by us [23]; no other sequence of CF_1 δ from plants is known.

Thus CF_1 δ is homologous to *E. coli* F_1 δ and OSCP, respectively, and not to mitochondrial F_1 δ, which is homologous to CF_1 ε [6]. The low degree of sequence homology explains why exchange of isolated δ from spinach and *E. coli* did not lead to reconstitution of activity (R. Tuttas, W. G. Hanstein, W. Finke, R. J. Berzborn, unpublished). CF_1 complexes containing δ from spinach and maize are interchangeable [46], however, although the δ subunits still are so different that there is no immunological crossreactivity [23].

The similarities in the N-terminal residues of the polypeptides from the six species are both seen in primary and secondary structure. Hydropathy plots and the distribution of polar residues show that the δ polypeptides do not contain hydrophobic sequences large enough to build alpha helical membrane spans; this is in agreement with the fact that δ polypeptides can be resolved from the membrane without the use of detergents. An exception is OSCP which is not removed by EDTA from the mitochondrial membrane.

We would like to draw attention to the conserved Tyr_8 (Table I) and the strong amphipathic character of a short alpha helix, predicted by several secondary structure algorithms (Fig. 3) for spinach δ. We would especially like to draw attention to the distribution of acidic residues and other amino acids able to form H-bridges and/or to associate or dissociate H^+ along the three windings of the alpha helix.

Turns before or at Tyr_8 and at Gly_{20}, *i.e.* before and after the discussed alpha helix, and homologous residues in the respective sequences of the other species lead us to propose a model for the tertiary structure of the N-terminal amino acids of δ from spinach CF_1 (Fig. 4). An alpha helix is drawn from Ala_9 to Val_{18}. On the basis of this structural feature we suggest a function for δ, hitherto not discussed; CF_1 δ may conduct protons.

The function of δ subunit within the CF_1CF_0 complex during photosynthetic ATP-synthesis is not clear. Two different passive roles have been suggested: δ was proposed one or the only binding protein between CF_1 and CF_0 [19, 20], in analogy to conclusions for *E. coli* F_1 δ [47]. Indeed δ is part of the resolved CF_1 complex; it is precipitated with anti CF_1 not containing anti δ antibodies and it does bind specifically to CF_0 [21]. Although δ is essential for reconstitution of activity [19], it was shown not to be necessary for binding CF_1 minus δ to EDTA treated thylakoids [22, 42]. Another passive role was proposed: Since membranes, from which some CF_1 is removed under special conditions without δ being found in the supernatant, are not uncoupled [48], the polypeptide δ was regarded a "stopper" against H^+ leakage through exposed CF_0; recently this function was changed to a "mobile stopper" [49].

The proposal that δ may play a thioredoxin like role, transmitting reducing equivalents to the γ subunit during activation of CF_1 [50], can be excluded due to the lack of cystein in δ [51].

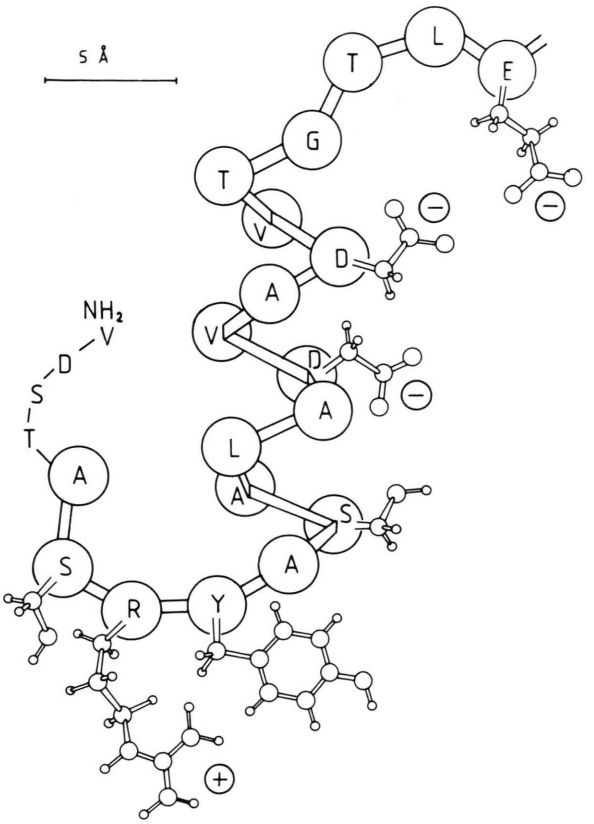

Fig. 4. Hypothetical model for the position of polar residues of the N-terminal sequence of spinach CF_1 subunit δ, deduced from secondary structure prediction algorithms, and from homology in primary and secondary structure with respective polypeptides from five other species.

If CF_1 polypeptide δ would actively participate in energy transduction, it could transduce the conformational change, if this "energy intermediate" is brought about in CF_0 already and transmitted to the active site in CF_1. The energy transformation from H^+ efflux to conformational change may take place in δ itself. Thirdly δ could be involved in H^+ translocation.

As mentioned, the N-terminus seems to be located in the protein interior. The model (Fig. 4) therefore allows the speculation that the polar residues of the N-terminus within the protein complex are conducting H^+ during photophosphorylation. A similar function was discussed [52] for polar residues of subunits a and c of *E. coli* ATPase F_0. Cox's model shows these polar residues located in the membrane.

Subunit CF_1 δ would extend the H^+ conduction and bridge part of the distance of about 90 Å between CF_0 and the active site of CF_1 [53]. Such an extension of the H^+ channel would mean that the conformational change is not brought about already in CF_0; this is in agreement with the conclusion by Weber *et al.* [54] who could not detect movements in CF_0 upon energization of the thylakoid membrane.

Acknowledgements

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Note added in proof

In a recent publication (A. L. Cozens and J. E. Walker, J. Mol. Biol. **194,** 359–383 (1987)) the organization and sequence of the genes for ATP-synthase subunits in the Cyanobacterium *Synechococcus* 6301 are described. The deduced amino acid sequence for subunit δ in the region discussed above (cp. Table I) reads: $-F-D-P-Y_{11}-A-E-A-L-M-A-I-A-R-E-Q-G-L-E-D-R-F-G-E-D-$. If plotted as helical wheel – the conserved Y–A–x–A– superimposed (cp. Fig. 3) –, this region shows the same amphipathic appearance, suggesting the same function of polar residues close to the homologous tyrosin (cp. Fig. 4).

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