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Primary structure, deduced from cDNA, secondary structure analysis and conclusions concerning interaction surfaces of the δ subunit of the photosynthetic ATP-synthase (E.C. 3.6.1.34) from millet (*Sorghum bicolor*) and maize (*Zea mays*)

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λgt11 cDNA clones for the nuclear-encoded subunit δ of the chloroplast ATP-synthase from *Zea mays* and *Sorghum bicolor* were sequenced. The processing site for *S. bicolor* δ was established, and the sequence of the mature subunit δ from *Z. mays* was completed by N-terminal sequencing of the proteins isolated from chloroplasts. Only five amino acids are identical and not more than 16% conservatively exchanged in all sequences of δ subunits from higher plants and the corresponding proteins from alga, bacteria and mitochondria (OSCP) available. In binary comparison the comparatively high conservation of hydrophilic residues indicates the importance of the surface of δ. The degree in identities of surface residues correlates with the capacity in hybrid reconstitution of photophosphorylation. A hypothetical secondary structure model for a typical δ subunit can be deduced from prediction algorithms. Three putative amphipathic α helices and an antiparallel amphipathic β sheet seem to be conserved. These common secondary structure features should be significant for the function of the δ subunit of F_0F_1 ATPases.

Introduction

As in oxidative phosphorylation also for photosynthetic ATP production on the stroma side of thylakoid membranes the energy is provided by a proton motive force [1]. Protons, accumulated during illumination inside the thylakoid lumen by chloroplast electron transport components, pass back through the membrane integral CF_0 part of the enzyme, ATP-synthase (E.C. 3.6.1.34), to drive the reaction [2,3], that occurs about 90 Å away on the α and β subunits of the peripheral CF_1 domain (α_3, β_3, γ, δ and ε) [4]. Whereas the regulatory functions of γ and ε are established [5–7], the δ subunit, considered a mere binding protein between F_1 and F_0 [8], is not neccessary for rebinding of CF_1 to CF_0; its essential role [9,10] is not yet specified. Also in *Escherichia coli* the corresponding subunit δ is not required just for binding of F_1 to F_0, provided ε is present [11].

Isolated CF_1 δ binds to CF_0 [10,12] and in high concentrations is able to block the proton efflux through CF_0 after removal of CF_1 from thylakoid membranes by EDTA [13,14]. The addition of subunit δ is essential for reconstitution of ATP-synthase activity of EDTA- or NaBr-treated thylakoid membranes by δ-free CF_1 [15,9,16,13]. Since antibodies against δ inhibit photophosphorylation [17], probably by displacement of this polypeptide within the ATP-synthase complex, δ seems to be involved in the transduction of some energy of the proton motive force to the active sites. CF_1 δ is in close contact to the CF_0 subunit I [18]; either protons or a conformational movement thus could proceed via the δ subunit [19,20,3].

We want to elucidate conserved (essential) elements in the primary and secondary structure of this nuclear-encoded CF_1 subunit of higher plants, and also look for additional features in CF_1 δ possibly correlated to the light regulation of the chloroplast ATP-synthase [4] or the import of this polypeptide. Therefore we have cloned and sequenced cDNAs for the δ subunits from *Pisum sativum* [21], *Sorghum bicolor* and *Zea mays*, and compared them with the sequences of the δ sub-

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The sequence data are deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence databases under the accession numbers X66004 (*Sorghum bicolor*) and X66005 (*Zea mays*).

Abbreviations: OSCP, oligomycin sensitivity confering protein of mitochondrial ATPase; RACE-PCR, rapid amplification of cDNA ends polymerase chain reaction; bp, base pair(s).

unit from *Spinacea oleracea* [22] and of the corresponding, homologous proteins from mitochondria and photosynthetic and heterotrophic bacteria. Here we report our results concerning the δ subunits of *S. bicolor* and *Z. mays*.

The sequence of δ from *Z. mays* is of special interest: hybrid reconstitution of photophosphorylation with EDTA treated thylakoids and CF_1, including δ, from different species is possible. With the coupling factors of spinach and *E. coli* the hybrid reconstitution was only structural (for definition cp. Ref. 23); after plugging of H^+ leaks residual ATP-synthase can be energized again [24]. Hybrid reconstitution between spinach and maize led, in addition, to catalytic activity of the readded enzyme [25]. On the other hand there is no immunological crossreaction between CF_1 δ from spinach and maize, indicating that the essential interaction surface is rather small.

Materials and Methods

Materials

Restriction endonucleases and DNA-modifying enzymes were obtained from Gibco/BRL, Pharmacia/LKB and Boehringer-Mannheim, radiolabeled nucleotides from Amersham. Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus synthesizer using the phosphoramidite method (in collabora-

NAAE	**A**	**A**	**A**	**E**	**S**	**Y**	**A**	**S**	**A**	**L**	**S**	**E**	**V**	**A**	**V**	**E**	
1	A	A	A	E	S	Y	A	S	A	L	S	E	V	A	V	E	16
1	GCC	GCG	GCG	GAG	AGC	TAC	GCA	TCC	GCG	CTG	TCG	GAG	GTG	GCC	GTC	GAG	48
17	N	G	T	L	E	Q	T	V	S	D	L	E	K	L	E	K	32
49	AAC	GGC	ACC	CTC	GAG	CAG	ACG	GTC	TCC	GAC	CTC	GAG	AAG	CTG	GAG	AAG	96
33	I	F	S	D	E	A	V	A	E	F	F	D	N	P	T	V	48
97	ATC	TTC	TCC	GAC	GAG	GCC	GTC	GCC	GAG	TTC	TTC	GAC	AAC	CCC	ACC	GTC	144
49	P	R	E	E	K	A	E	L	I	G	E	I	A	K	S	S	64
145	CCG	CGC	GAG	GAG	AAG	GCC	GAG	CTC	ATC	GGC	GAG	ATC	GCC	AAG	TCG	TCC	192
65	E	L	Q	P	H	V	V	N	F	L	N	V	V	V	D	N	80
193	GAG	CTG	CAG	CCG	CAC	GTC	GTC	AAC	TTC	CTC	AAC	GTC	GTC	GTC	GAC	AAC	240
81	F	R	A	S	I	V	P	Q	I	V	V	E	F	E	N	V	96
241	TTC	CGC	GCG	TCC	ATC	GTG	CCG	CAG	ATC	GTC	GTC	GAG	TTC	GAG	AAC	GTC	288
97	Y	N	S	L	T	G	T	E	V	A	T	V	T	S	V	V	112
289	TAC	AAC	TCG	CTC	ACG	GGC	ACC	GAG	GTC	GCC	ACC	GTC	ACC	TCC	GTC	GTG	336
113	Q	L	E	S	Q	D	L	A	Q	I	A	Q	H	V	Q	K	128
337	CAG	CTC	GAG	TCG	CAG	GAC	CTC	GCG	CAG	ATC	GCG	CAG	CAC	GTC	CAG	AAG	384
129	M	T	G	A	K	N	V	R	L	K	T	Q	L	D	P	A	144
385	ATG	ACC	GGC	GCC	AAG	AAC	GTC	CGC	CTC	AAG	ACG	CAG	CTC	GAC	CCG	GCG	432
145	L	I	A	G	F	T	V	Q	Y	G	R	D	G	S	N	L	160
433	CTC	ATC	GCC	GGC	TTC	ACC	GTG	CAG	TAC	GGC	CGC	GAC	GGC	TCC	AAC	TTG	480
161	I	D	M	S	V	R	K	Q	I	E	E	I	A	S	E	F	176
481	ATC	GAC	ATG	AGC	GTC	CGG	AAG	CAG	ATC	GAG	GAG	ATC	GCG	TCC	GAG	TTC	528
177	E	L	P	A	V	P	L	E	V	*							185
529	GAG	TTG	CCC	GCC	GTC	CCG	CTC	GAA	GTC	TGA	GGC	CGG	CTT	GAG	GCG	TCC	576
577	ATC	CTC	GCC	CTC	GAA	GCT	ATA	GGT	CCT	TTT	TCT	GCT	GCC	TCT	CTC	CCT	624
625	GCT	CTG	CTA	TTC	ATC	TGT	ATC	ATT	CTC	TGG	TTG	TTG	AAT	GTT	TTG	AGT	672
673	GAA	TGG	TGA	ATG	CTG	CAT	ACC	TGG	ATT	TTA	GGA	TTC	ATA	TTT	GAA	TGA	720
721	ATG	GTG	AAT	ACA	CTG	CAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA		765

Fig. 1. Nucleotide sequence of the truncated cDNA and deduced amino acid sequence of the CF_1 δ subunit from *Zea mays*. The potential polyadenylation signal (bp 727 to 732) is underlined. The sequence of the complete protein was determined by comparing the cDNA sequence with N-terminal sequencing data of the *Zea mays* δ protein as isolated from chloroplasts [19]; they are given in bold letters above the cDNA sequence. Combination of the two independent sets of data reveals that the mature δ protein from *Zea mays* starts with **NAAEAAAESY**...

tion with the Department of Biochemistry, Faculty of Chemistry, Ruhr-Universität Bochum). All other chemicals were of p.a. grade.

Screening of cDNA libraries

Zea mays. A *Z. mays* λgt11 cDNA library (a gift of Prof. Dr. U. Flügge, Würzburg) was analyzed by ex-

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   1  GAG CGA GGC CGC ACT CCC CCC CGA GTC CAC TCC GAC CGC ACG CTC CGC   48

   1           M   A   A   L   R   L   A   S   F   T   L   R   P   A   14
  49  GAC GCG ATG GCC GCG CTC CGC CTC GCC TCC TTC ACC CTC CGA CCC GCC   96

  15  A   A   A   A   A   S   A   S   S   G   A   T   P   A   A   P   30
  97  GCC GCC GCC GCG GCG TCC GCC TCC TCC GGC GCG ACC CCC GCG GCG CCC  144

  31  R   S   A   S   F   A   R   A   A   R   G   L   P   S   L   R   46
 145  CGG TCC GCC TCC TTC GCC CGC GCG GCG CGG GGG CTA CCC TCC CTC CGC  192

  47  L   A   P   P   R   R   R   G   D   L   V   R   P   R   A   E   62
 193  CTG GCC CCG CCC CGC CGC CGC GGG GAC CTC GTC AGG CCC CGC GCC GAG  240

  63  A   A   A   D   S   Y   A   S   A   L   S   E   V   A   V   E   78
 241  GCA GCG GCG GAT AGC TAC GCG TCC GCG CTG TCG GAG GTG GCC GTC GAG  288

  79  N   G   T   L   E   Q   T   V   S   D   L   E   K   L   Q   K   94
 289  AAC GGC ACC CTC GAG CAG ACG GTC TCC GAC CTG GAG AAG CTG CAG AAG  336

  95  I   F   A   D   E   T   V   A   E   F   F   D   N   P   T   V  110
 337  ATC TTC GCC GAC GAG ACC GTC GCC GAG TTC TTC GAC AAT CCC ACC GTC  384

 111  P   R   E   E   K   T   A   L   I   D   E   I   A   K   S   Y  126
 385  CCG CGC GAG GAG AAG ACC GCG CTC ATC GAC GAG ATC GCC AAG TCG TAC  432

 127  E   L   Q   P   H   V   V   N   F   I   N   V   V   V   D   N  142
 433  GAG CTG CAG CCG CAC GTC GTC AAC TTC ATC AAC GTC GTC GTC GAC AAC  480

 143  F   R   A   T   I   L   P   E   I   V   V   E   F   E   N   I  158
 481  TTC CGC GCG ACG ATC TTG CCG GAG ATC GTC GTC GAG TTC GAG AAC ATC  528

 159  F   N   S   L   T   G   T   E   V   A   T   V   T   S   V   V  174
 529  TTC AAC TCG CTC ACG GGC ACC GAG GTC GCC ACC GTC ACC TCC GTC GTG  576

 175  Q   L   E   S   Q   D   L   A   Q   I   A   Q   H   V   Q   K  190
 577  CAG CTC GAG TCG CAG GAC CTC GCG CAG ATC GCG CAG CAC GTC CAG AAG  624

 191  M   T   G   A   K   N   V   R   L   K   T   Q   L   D   P   E  206
 625  ATG ACC GGC GCC AAG AAC GTC CGC CTC AAG ACA CAG CTC GAC CCG GAG  672
                      Amp.                RT
 207  L   I   A   G   F   T   V   Q   Y   G   R   D   G   S   S   L  222
 673  CTC ATC GCC GGC TTC ACC GTG CAG TAC GGC CGC GAC GGC TCC AGC TTA  720

 223  I   D   M   S   V   R   K   Q   I   E   E   I   T   S   E   F  238
 721  ATC GAC ATG AGC GTC CGG AAG CAG ATC GAG GAA ATC ACG TCC GAG TTC  768

 239  E   L   P   D   V   P   L   E   V   *                          247
 769  GAG TTG CCC GAC GTC CCG CTC GAA GTC TGA CTG GCT TGA GGC GTC CAT  816

 817  CCT CGC CCT TGA AGC TTA GCT GCT ACT GTC TGA CGC AAT GGC ATG AAT  864
 865  CAA GCG GGA ATG TCG GTC CTT TCC CCT TCA TAC CTG CAG ATC AAT GGT  912
 913  GGT GGC TCC AAA TCT CCA ATA CAT ATG AAC GGA CAT TCT TTT TTT GCG  960
 961  TTT CTG TTT TCC TTC TTT TTC TTT TTG GCG GGA ACC TGT GTA ATT CTT 1008
1009  TCT CCT TTT TTC TGT GGT TGA CAG ACA CTG TAA TAT GTT TAA AAC ACT 1056
1057  CAC TCA TCT ATA TAT AGC TGT GCA CCT CTT CTC TCA AAA AAA AAA AAA 1104
1105  A                                                             1105
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Fig. 2. Nucleotide sequence of the cDNA and deduced amino acid sequence of the complete import precursor of the CF_1 subunit δ from *Sorghum bicolor*. The N-terminal amino acids determined by automated Edman degradation of the mature protein as isolated from chloroplasts are marked by bold letters. The sequence parts that correspond to the oligo nucleotide primers used in the RACE-PCR (RT, Amp), the ribosome binding site (bp 51 to 59) and the potential polyadenylation signal (bp 1065 to 1069) are underlined.

pression screening [26], using an antiserum raised against the δ subunit from *Z. mays* [27].

Sorghum bicolor. A 765 bp *Eco*RI/*Eco*RI restriction fragment containing information for the δ subunit from *Z. mays* was radiolabeled with [^{32}P]dATP [28]. This probe was used to screen a λgt11 cDNA library from *S. bicolor* (a gift of Prof. Dr. P. Westhoff, Düsseldorf) [29].

PCR

It was tried to complete the 5′-ends of the *S. bicolor* and *Z. mays* cDNAs cloned by RACE-PCR [30]. The gene-specific primer oligonucleotides used correspond to bp 648–664 (reverse transcription) and bp 634–649 (amplification) for *S. bicolor*, and bp 317–332 (reverse transcription) and bp 285–302 (amplification) for *Z. mays*, respectively.

Subcloning and DNA sequencing

The DNA of the isolated λ clones was restricted by standard techniques [31]. The restriction fragments were isolated [32], cloned into M13 mp18/19 vectors [33] and sequenced by the didesoxynucleotide method [34] using Taq DNA Polymerase and 7-deaza dGTP at 72°C (Promega).

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Sor. bic. MAALRLASFTLRP...AAAAAASASSGATPAAPRSASFARAARGLPSLRL.....AP
Zea mays  ..........................................................
Pis. sat. MASLQHTTASLHSKHIPKTTNILTR......KPI.LNLSSSTFYSPKLKLKLKLPLTKTR
Spi. ole. MAALQNPVA.LQSRTTTAVAALSTSSTTSPPKPFSLSFSSSTATFNPLRLKILTASKLTA
Bos. pri. MA.................ALAVSGLSQQVR......CF......STSV...VRPFAKL..
Ipo. bat. MAMTGRARSMGFS.ILQKALSSAQRSNAHR......SILCPTLSNSEL...LRNYATASA
Sac. cer. M..........FNRVFTRSFASS.........................LRAAASKAA
Odo. sin. ..........................................................
Ana. 7120 ..........................................................
Scy. 6803 ..........................................................
Sco. 6301 ..........................................................
Sco. 6716 ..........................................................
Cya. par. ..........................................................
Rps. bla. ..........................................................
Rsp. rub. ..........................................................
PS3       ..........................................................
Bac. meg. ..........................................................
Bac. fir. ..........................................................
Vib. alg. ..........................................................
E. coli   ..........................................................

                    1        10        20        30        40
Sor. bic. PRRRGDLVRPRAEAAADSYASALSEVAVENGTLEQTVSDLEKLQ.KIFADETVAEFFDNP
Zea mays  .........NAAEAAAESYASALSEVAVENGTLEQTVSDLEKLE.KIFSDEAVAEFFDNP
Pis. sat. RSTGGALGARMSSLAAGSYAAALADLANSNNTLDAITADFDKIE.QLFSDPKVFDYFSSP
Spi. ole. KPRGGALGTRMVDSTASRYASALADVADVTGTLEATNSDVEKLI.RIFSEEPVYYFFANP
Bos. pri. .....VRPPVQIYGIQGRYATALYSAASKQNKLEQVEKELLR.VGQILKEPKMAASLLNP
Ipo. bat. SKEQKIKVPLTMYGVSGNYASALYLAAVKSNTLEKVESELYDLVEASKKSPTFSQFMRDP
Sac. cer. A.....PPPVRLFGVEGTYATALYQAAAKNSSIDAAFQSLQKVESTVKKNPKLGHLLLNP
Odo. sin. .....MSINPLASKIAAPYARALFDFSVDQNLMHQITADFQNLEVFLNKTPDLTEYLSNP
Ana. 7120 .....MTSKVANTEVAQPYAQALLSIAKSKSLTEEFGTDARTLLNLLTENQQLRNFIDNP
Scy. 6803 .....MKGSLYSSKIAEPYAQALIGLAQQQNLTEVFGDNLRSLLTLLQDSPDLSAVLSSP
Sco. 6301 ........MTSTSQLFDPYAEALMAIAREQGLEDRFGEDAALFRSTLAASADLRHLLENP
Sco. 6716 .....MMQTTIRGELVEPYAEALLSLAQSHNLADQFQQDSGLILDLLAASAELQEFLANP
Cya. par. .....MKQSAVVSKITQPYAEALLEMAQKYDIVETVNNDITLILNCLQNSTKLQQFLANP
Rps. bla. ....MAEAASISQGIAERYATALFELSKETGALKTLETDIDALKDVLAGSPDLGAMIASP
Rsp. rub. ....MSSHKAGVTGVAERYATALYELAEDRGALDQVSADLRSLKAMLDESGDLRRVIASP
PS3       .........MNQEVIAKRYASALFQIALEQGQLDRIEEDVRAVRQALAENGEFLSLLSYP
Bac. meg. .........MSQPAVAKRYALALFQLATEKQMIDEMQDQLQIVEEVFAKTPELMDVLTHP
Bac. fir. .........MSNQAVANRYRYALFQLAEEKSILSQVVQEMELVKEVVNTTPEFLQLLSHP
Vib. alg. ........MSDLTTIARPYAKAAFDFALEKDQLDQWG.QMLSFAAEVAKNEQMNELLTGS
E. coli   ........MSEFITVARPYAKAAFDFAVEHQSVERWQ.DMLAFAAEVTKNEQMAELLSGA
                          + +   #                       #     # #
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Fig. 3. Alignment of the deduced amino acid sequences of the CF δ subunits from *Sorghum bicolor* and *Zea mays* with the proteins corresponding in sequence from higher plants, bacteria and mitochondria using the program Clustal 4. The N-terminal residues additionally sequenced by us by Edman degradation are shown in bold. Numbering refers to the mature polypeptide δ of *Sorghum bicolor*. +, strictly conserved amino acids; #, conservative exchanges. Species abbreviations used: Sor. bic., *Sorghum bicolor*; Pis. sat., *Pisum sativum*; Spi. ole., *Spinacea oleracea*; Bos. pri., *Bos primigenius* (OSCP); Ipo. bat., *Ipomoea batatas* (F_1 δ); Sac. cer., *Saccharomyces cerevisiae* (ATPase 5); Odo. sin., *Odontella sinensis*; Ana. 7120, *Anabaena* PCC 7120; Scy. 6803, *Synechocystis* 6803; Sco. 6301, *Synechococcus* 6301; Sco. 6716, *Synechococcus* 6716; Cya. par., *Cyanophora paradoxa*; Rps. bla., *Rhodopseudomonas blastica*; Rsp. rub., *Rhodospirillum rubrum*; PS3, thermophilic bacterium PS3; Bac. meg., *Bacillus megaterium*; Bac. fir., *Bacillus firmus* OF4; Vib. alg., *Vibrio alginolyticus*; E. coli, *Escherichia coli*.

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          50         60         70         80         90         100
Sor. bic. TVPREEKTALIDEI.AKSYELQPHVVNFINVVVDNFRATILPEIVVEFENIF.NSLTGTE
Zea mays  TVPREEKAELIGEI.AKSSELQPHVVNFLNVVVDNFRASIVPQIVVEFENVY.NSLTGTE
Pis. sat. IVEDSTKRQLIGEF.ATTSGFQPHTHNFLNVLIDSKRIDMIIDIIKEFEFVY.NTLTDTE
Spi. ole. VISIDNKRSVLDEI.ITTSGLQPHTANFINILIDSERINLVKEILNEFEDVF.NKITGTE
Bos. pri. YVKRSVKVKSLSDMTAKEK.FSPLTSNLINLLAENGRLTNTPAVISAFSTMM.SVHRGEV
Ipo. bat. SVPVDTRVNAIKEICAQAK.FGDTTQNFLLILAENGRLKHIDRIVKRFKELT.MAHRGEV
Sac. cer. ALSLKDRNSVIDAIVETHKNLDGYVVNLLKVLSENNRLGCFEKIASDFGVLN.DAHNGLL
Odo. sin. LISAKSKEEVLNKTLKS..QINKETFKFLIVLVNRSRINLLEPIIASYLNLVY.NAASVK
Ana. 7120 FIAAENKKALIKQILSE..ASP.YLRNFLLLLVDKRRIFFLPEILQQYLALLR.QLNQTV
Scy. 6803 VVKDEDKKSVLRSVLGD..GGNGYLLNFLMLLVDKRRIVFLEAICEQYLALLR.QFTNTV
Sco. 6301 TLFSSQKKAVLNQVFGS..SVHPLVLNFLNLLVDRNRIAFLDGIADRYQALLR.KLRNVV
Sco. 6716 LINPDAKKNVLRQLTVD..KVHGYFLNFLMLLVDRRRINLLAAICQQYRALLR.KLRNIV
Cya. par. LVKKSSKKNFFEKTLAK..EIHPYTFKFLLLVIDRGRISCLEIIAQKYQSLIL.KLTKTE
Rps. bla. VISRGDQAKAVAAIAGKM.GLSPLMTNTLALMSEKRRLFALPQVLSALAGLIA.EEKGEV
Rsp. rub. VIGRDDQRKALTALAEKA.GFHEIVRNFLGVVAAKHRSFAVPGMIGAFLERLA.ARRGEV
PS3       KLSLDQKKALIREAFAG...VSTPVQNTLLLLLERHRFGLVPELAGTVSRPRSTTARGIA
Bac. meg. KITIERKKQFVSEAFAE...LSPTVQHTVLLLLERHRIQIVSEMVKEY.RFLANEVRGVA
Bac. fir. KVTTEKKRAFIENSFKD..SLSETSLHTLLLLVENKRIESLVDMIDSF.KEMSYEAQDMA
Vib. alg. VSADKMAEIFV..AVCGE.QVDTHGQNLLKVMAENGRLAALPDVCTEFYTL.KKEHEKEI
E. coli   LAPETLAESFI..AVCGE.QLDENGQNLIRVMAENGRLNALPDVLEQFIHL.RAVSEATA
                                  # # ## ## +      #

           110        120        130          140        150        160
Sor. bic. VATVTSVVQLESQDLAQIAQHVQK...MTGAKNVRLKTQLDPELIAGFTVQYGRDGSSLI
Zea mays  VATVTSVVQLESQDLAQIAQHVQK...MTGAKNVRLKTQLDPALIAGFTVQYGRDGSNLI
Pis. sat. LVVVTSVVKLESHHLAQIAKQVQK...LTGAKKVRTKTLLDPSLVAGFTVRYGNTGSKFI
Spi. ole. VAVVTSVVKLENDHLAQIAKGVQK...ITGAKNVRIKTVIDPSLVAGFTIRYGNEGSKLV
Bos. pri. PCTVTTASALNEATLTELKTVLKS..FLKKGQVLKLEVKIDPSIMGGMIVRIG...EKYV
Ipo. bat. KATVTTVIPLPADEEKELKATLQE..MVGQGKSVQIEQKIDPTILGGLVVEFG...QKVF
Sac. cer. KGTVTSAEPLDPKSFKRIEKALSASKLVGQGKSLKLENVVKPEIKGGLIVELG...DKTV
Odo. sin. MIEVSTAYAFTNLQKNTLIKKLKE...LTNAREIRLVITVDSSLIGGFLIKTN...SKVL
Ana. 7120 LAEVTSAVALTEDQQQAVTEKVLA...LTKARQVELATKVDSDLIGGVIIKVG...SQVI
Scy. 6803 LAEVTSALKLTDAQKDQVKERVKQ...LTGAQAVELETKVDGDILGGIVIKVG...SQVF
Sco. 6301 RADVSSAVPLTEAQVQVITEKVKQ...LTGAAGVEIESQVDADLLGGVIIKVG...SQVL
Sco. 6716 LAEVISAVELTEQQRHAVVEKVKT...MTGAADVELAIAIDPELLGGVVIKVG...SQIF
Cya. par. LAEVVTAVPLSSEQEAALNNIIKE...LTNANEVKLVFKIDQNLIGGFIINIG...SKVV
Rps. bla. TAEVTAATKLSAAQAKKLAETLKA...KVG.KTVKLNTTVDESLIGGLIVKLG...STMI
Rsp. rub. TARIVSATALTSAQKSALTTALNK...ATG.NTVTIDASVDPALLGGMVVRVG...SRMV
PS3       KAVAYSGAASTDEELRALSDVFAQ...KVGKQTLEIENIIDPELIGGVNVRIG...NRIY
Bac. meg. DATVYSVKPLSADEKRAISQSFAS...KVGKHTLNISNIVDKTVIGGVKLRIG...NRIY
Bac. fir. EAVVYSAKPLTSEEQAQIAVIFAK...KVNKAKLLVINVVNKDLLGGLKIRIG...DRIY
Vib. alg. DVEVISATELSDEQLANIGSKLEK...RLE.RKVKLNCSVDETLLGGVIIRAG...DLVI
E. coli   EVDVISAAALSEQQLAKISAAMEK...RLS.RKVKLNCKIDKSVMAGVIIRAG...DMVI
               #            #              #     ## ## #+  #

                          170        180
          Sor. bic.       DMSVRKQIEEITSEFELPDVPLEV (this paper)
          Zea mays        DMSVRKQIEEIASEFELPAVPLEV (this paper)
          Pis. sat.       DMSVKRKLEEIAAQIDLGDIQLAV [21]
          Spi. ole.       DMSVKKQLEEIAAQLEMDDVTLAV [22]
          Bos. pri.       DMSAKTKIQKLSRAMRQIL..... [61]
          Ipo. bat.       DMSIRTRARQMERFLREPLNF... [62]
          Sac. cer.       DLSISTKIQKLNKVLEDSI..... [63]
          Odo. sin.       DFTIKNQLQKLAKHLDSVLEI... [44]
          Ana. 7120       DSSIRGQLRRLSLRLSNS...... [64]
          Scy. 6803       DSSLRGQLRRVGLSLGTAL..... [65]
          Sco. 6301       DASLRGQLKRISISLAA....... [66]
          Sco. 6716       DASLRGQLRRLSVSLAQPV..... [67]
          Cya. par.       DASLLGQLLRIGNYLGLETV.... [68]
          Rps. bla.       DTSVKSKLASLQNAMKEVG..... [69]
          Rsp. rub.       DSSLSTKLKRLQLAMKGVG..... [70]
          PS3             DGSVSGQLERIRRQLIG....... [71]
          Bac. meg.       DGSISSKLETIHRGLLAHRS.... [72]
          Bac. fir.       DGRVKSQLDRLERQLIAGTR.... [73]
          Vib. alg.       DDSARGRLNRLSDALQS....... [74]
          E. coli         DGSVRGRLERLADVLQS....... [75]
                          +     #   #   #
```

Fig. 3 (continued).

Protein purification and N-terminal sequencing

S. bicolor CF_1 was isolated [35]. 30 mg of protein from the DEAE column was separated on 12.5% (w/v) SDS polyacrylamide gels [36] and stained by Coomassie Blue. The protein band at 23.5 kDa, positive in Western blots with an antiserum against the δ subunit of maize CF_1, was cut out, purified on a second 18% (w/v) SDS PAGE gel and transferred to a PVDF membrane (Millipore). Automated Edman degradation was done on an Applied Biosystems 477 A Gasphase sequenator [37] (in collaboration with Dr. H.E. Meyer, Faculty of Medicine, Ruhr-Universität Bochum).

Results and Discussion

Sequencing of CF_1 subunit δ from Zea mays

Screening of a λgt11 cDNA library of *Z. mays* was done by help of antibodies against a subunit of chloroplast CF_1 from *Z. mays*, with an apparent molecular mass of 25 kDa [27], which has been concluded to be the δ subunit by N-terminal protein sequencing [19]. Six independent, but identical cDNA clones were isolated from 10^5 λ phages analyzed.

The clones contain 555 bp of the open reading frame coding for 185 amino acids of δ and 210 bp of the 3'-untranslated region including a poly(A) tail of 29 bp (Fig. 1). The first deduced 18 amino acids are identical with residue 5 to 22 of the isolated and sequenced 25 kDa polypeptide of *Z. mays* CF_1 [19]. RACE-PCR attempts using gene-specific oligonucleotides at various reaction conditions to clone the 5'-end of the full length cDNA, including an expected transit sequence, were unsuccessful (data not shown). This was probably caused by a high GC content of the mRNA, which is 62% in the RNA for the mature δ (Fig. 1) and 85% in the corresponding transit sequence of *S. bicolor* (Fig. 2).

Adding the known four missing N-terminal amino acids for the δ subunit from *Z. mays* [19], the sequence of the complete mature protein (189 amino acids, 20592 Da) could be established (Fig. 1).

Sequencing of the CF_1 subunit δ from Sorghum bicolor

Using the isolated cDNA for the δ subunit from *Z. mays*, the cDNA for the δ subunit from *S. bicolor* was isolated by DNA hybridization. A single clone, 1046 bp in length, could be isolated from 10^5 λ phages analyzed. The information for the mature protein and part of the sequence of a transit peptide was present. Using RACE-PCR [30] under modified conditions (reverse transcription at 52°C) with mRNA from 3 weeks old *S. bicolor* plants, a 650 bp fragment could be amplified containing the 5'-end.

The combined cDNA sequence consists of 1105 bp, including 14 bp of the poly(A) tail at the 3'-end. An open reading frame of 741 nucleotides codes for a 247 amino acids (26736 Da) import precursor of the chloroplast δ subunit from *S. bicolor*. In addition the clone contains 54 bp of 5'-untranslated sequence with a potential ribosome binding site at bp 51 to 59 and 210 bp of 3'-untranslated sequence with a potential polyadenylation site at bp 1065 to 1069 (Fig. 2).

To localize the processing site of the import precursor, the δ subunit of *S. bicolor* CF_1 was isolated from chloroplasts; automated Edman degradation showed that the mature protein starts with the sequence AEAAADSY..., i.e., a transit peptide of 60 amino acids has been cleaved off (Fig. 2). The mature δ subunit of *S. bicolor* CF_1 consists of 187 amino acids (20644 Da).

Common features of δ sequences from higher plant chloroplasts

Comparison [38] of the deduced amino acid sequences of the δ subunits of *S. bicolor* and *Z. mays* with the known sequences of *Pisum sativum* [21] and *Spinacea oleracea* [22] showed that 40% (75 residues) of the amino acid sequences of the mature proteins are identical; including conservative exchanges 85% of the sequences are homologous (Fig. 3). Since catalytic reconstitution of photophosphorylation with CF_0 (EDTA treated thylakoids) from *Z. mays* and CF_1 from *Sp. oleracea* is possible [25], some of the 103 residues, identical in the δ sequences of spinach and maize CF_1, are involved in the interaction with CF_0. It is reasonable to expect these among the 50 hydrophilic residues, identical between the δ sequences of spinach and maize.

The δ sequences of higher plant chloroplasts do not contain cysteins; therefore CF_1 subunit δ cannot participate directly in the thiol modulated light activation of the enzyme [4]. Cysteins involved in this regulation were found in an insertion in the primary sequence of the CF_1 subunit γ, unique to higher plants [39].

The differences in apparent molecular weights observed on SDS gels do not result from the deduced sequences (Table I) or clusters of hydrophobic residues (Fig. 3); posttranslational modifications may have occurred.

Subunit δ of the chloroplast ATP-synthase is shown to be nuclear-encoded and imported in *Spinacea oleracea* [40,41] and *Spirodela oligorhiza* [42]. An import transit sequence was deduced from cDNA for *Sp. oleracea* [22], *P. sativum* [21] and *S. bicolor* (Fig. 3); it also exists for *Nicotiana tabaccum* δ (J.C. Gray, personal communication). We therefore conclude that in all higher plants CF_1 δ is nuclearencoded; also in the green alga *Chlamydomonas reinhardtii* an atpD gene coding for CF_1 δ seems to be absent from the chloroplast DNA [43]. In the diatom alga *Odontella sinensis*, however, CF_1 subunit δ is chloroplast encoded and

TABLE I

Comparison of the apparent molecular masses of the δ subunits of chloroplast ATP synthase and the protein molecular masses as deduced from the respective cDNAs

Species	Apparent molecular mass [a] (kDa)	Molecular mass calculated from cDNA	Ref.
Sorghum bicolor	23.5	20.644	this paper
Zea mays	24.8	20.592	this paper
Pisum sativum	22.0	20.598	[21]
Spinacea oleracea	21.6	20.486	[22]

[a] Apparent molecular masses of the δ subunits were determined by running isolated CF_1 and/or isolated δ on 14% SDS polyacrylamide gels [36] with standard molecular mass markers from Bio-Rad.

accordingly the sequence of a transit peptide is missing [44].

Only ten respective amino acids are identical in the transit sequences of the δ subunits from higher plants; but they are similar in length (60–70 residues) and display the characteristics of stromal transit peptides [45], i.e., they are rich in serine, threonine, lysine and arginine and contain nearly no acidic residues. As target for the proteinase, processing the imported precursor in the chloroplast stroma, positive residues around −2 and −10 have been proposed [45]; they are present in the new δ transit sequences, however also in the homologous OSCP precursor sequences of mitochondrial ATPase from *Bos primigenius* and *Saccharomyces cerevisiae* (mature bovine OSCP starts with FAKL..).

Analysis of amino acids in ATP-synthase subunits δ conserved during evolution suggests specific interaction surfaces

Comparison of the amino acid sequences of CF_1 subunit δ of higher plants with the sequences of the corresponding proteins from mitochondria (OSCP, not the socalled δ subunit of F_1) and bacteria using the Clustal program [38] reveals that only five amino acids out of 180 on the average (2.8%, marked by a (+) in Fig. 3) are identical among all species, in the δ sequence of *S. bicolor* Tyr-8 and Ala-11 close to the N-terminus, Arg-84, and Gly-150 and Asp-164 close to the C-terminus. Even including conservative exchanges (23 out of 180 on the average, marked by a (#) in Fig. 3), not more than 16% of the amino acids are homologous. However, the few conserved residues are clustered together with the conservatively exchanged amino acids (see below).

If a binary comparison is done considering the gaps in the alignment, i.e., species- or group-specific insertions in the polypeptides (Fig. 3), about 25% more similarities are seen between δ from plants and prokaryots than in the comparison of the plain sequences (data not shown). Among the corresponding proteins the δ sequence of *Cyanophora paradoxa* is closer to the higher plants than δ of the diatom alga *Odontella sinensis* (Table II; cf. discussion in Ref. 44).

An inspection of the δ sequences by the program Prosite (HUSAR) revealed a potential target site for caseinkinase II, i.e., a potential phosphorylation site at T_{103}xxE, identical in all higher plants but otherwise only contained in the δ sequence of *C. paradoxa* (cp. Fig. 3). After hydrolysis of isolated δ from spinach we found phosphoserine instead (D. Lohse, R. Oworah-

TABLE II

Number of identical residues in the primary structure of mature subunit δ of Spinacea oleracea, compared as aligned in Fig. 3, with δ from S. bicolor, Z. mays, P. sativum, Odontella sinensis, Cyanophora paradoxa and E. coli

Comparison of subunits δ from	Total identities	Small AG	Hydrophilic DETSKR HNQCY	Hydrophobic LVIMFP W
Spinach and	103	15	51	37
S. bicolor	55% of 187	65% of 23	53% of 96	54% of 68
Spinach and	103	16	50	37
Z. mays	54% of 189	57% of 28	53% of 94	55% of 67
Spinach and	106	18	55	33
Pisum sativum	57% of 187	72% of 25	57% of 96	50% of 66
Spinach and	42	6	19	17
Odontella sin.	23% of 187	38% of 16	20% of 97	23% of 74
Spinach and	51	9	20	22
Cyanophora par.	27% of 186	45% of 20	22% of 93	30% of 73
Spinach and	41	9	18	14
E. coli	23% of 177	26% of 35	22% of 81	23% of 61

Numbers and percentages of identities in relation to all residues within the respective group of amino acids of δ in the respective species are shown. Spinach δ contains 24 small, 95 hydrophilic and 68 hydrophobic residues.

Nkruma, W. Finke, H.E. Meyer and R.J. Berzborn, unpublished data).

A specific feature of higher plant CF_1 subunit δ sequences is an insertion of three residues close to G_{156}; this extension close to the C-terminus may be correlated to the import of this nuclear-encoded subunit into the chloroplast, since the chloroplast encoded *O. sinensis* CF_1 δ sequence does not have these three residues. The homologous OSCP sequences show a similar extension of 1–3 residues close to Lys-130 of the *S. bicolor* δ sequence (Fig. 3).

After dividing the amino acids in three groups (small,

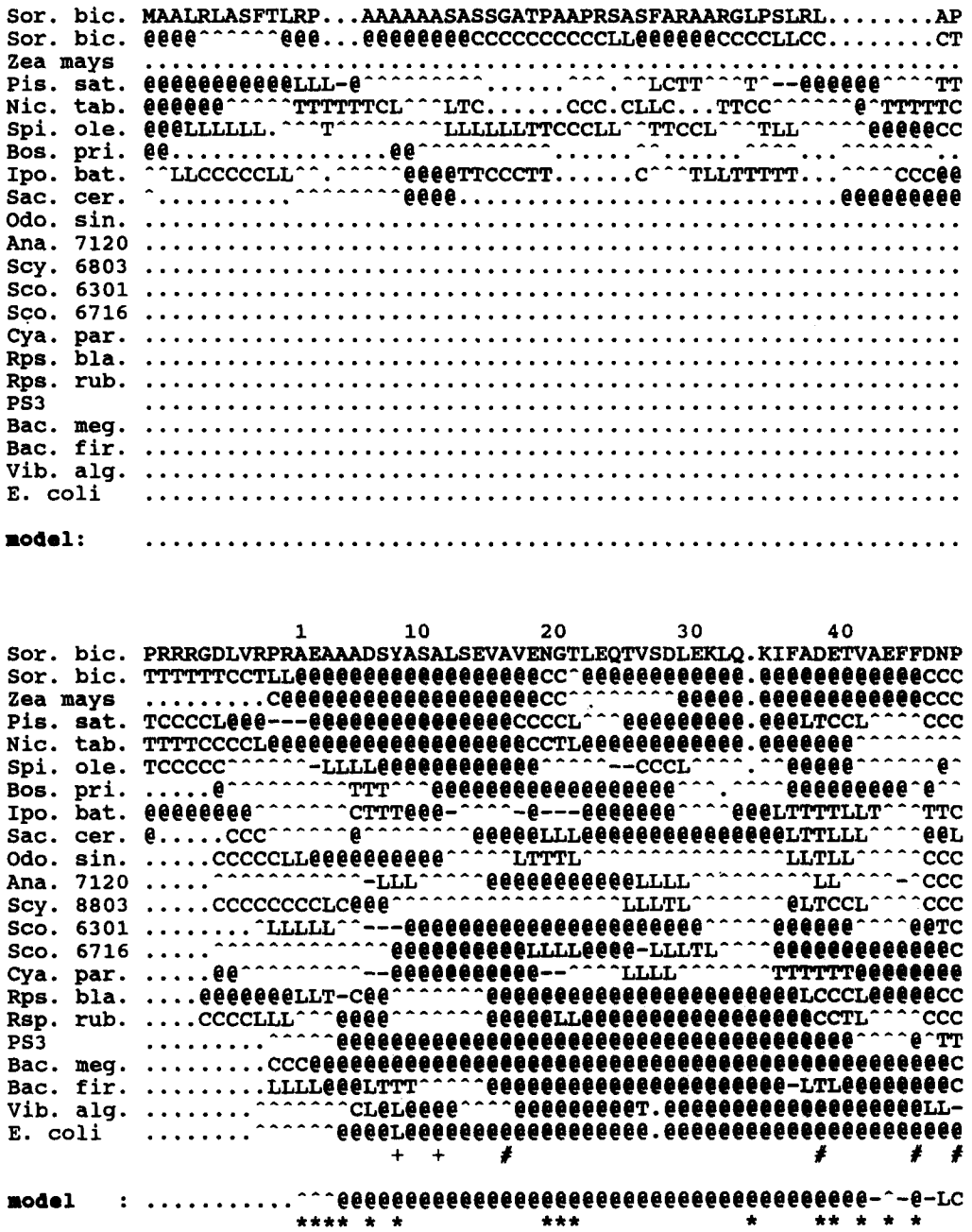

Fig. 4. Alignment of the predicted secondary structure consensus of CF_1 δ of each individual species and deduced overall consensus. Secondary structures for each δ sequence were calculated according to the algorithms of Chou and Fasman [52], Chou et al. [54] and Garnier et al. [53], repectively, as implemented in the program PROSIS (Pharmacia LKB). Consensus for each species were deduced, the consensus were aligned according to the amino acid alignment (Fig. 3) and an overall consensus (model) was constructed as described in the text; numbering refers to the mature polypeptide δ of *Sorghum bicolor*. The same species abbreviations are used as in Fig. 3. ,@, α helix; ^, β sheet; C, coil; T, turn; L, loop = turn or coil (cp. text); −, unpredictable by the applied method; . , gap in amino acid alignment; +, strictly conserved amino acids; #, conservative exchanges; *, less supported prediction in model (cp. text). Note: the secondary structure prediction of CF_1 δ for the species *Nicotiana tabacum* was calculated using the amino acid sequence provided by John C. Gray (now published in Plant Mol. Biol. 20 (1992) 549–554.).

hydrophilic and hydrophobic) the sequence of the mature CF_1 δ from *Sp. oleracea* was compared in detail with the new δ sequences from *S. bicolor* and *Z. mays* and with δ from *P. sativum, O. sinensis, C. paradoxa* and *E. coli* (Table II). As compared to *E. coli* δ all photosynthetic organisms have a larger δ with fewer small residues, that are well conserved among higher plants. The hydrophilicity has increased distinctly in higher plant δ. Hybrid reconstitution can be related to the detailed comparison of the δ sequence of spinach with *Z. mays* vs. *E. coli*: with 41 identities in *E. coli* only structural reconstitution was observed [24], with 103 identical residues in δ of *Z. mays* additional catalytic reconstitution was possible [25]. The number of identical hydrophilic residues is significantly higher than of identical hydrophobic ones; between spinach and *P. sativum* δ [21] even the percentage of identical hydrophilic residues is higher (Table II). Usually, in soluble proteins the hydrophilic residues are less well conserved than the hydrophobic ones in the protein core [46]. The surprisingly high degree of conservation of hydrophilic residues indicates the importance of the

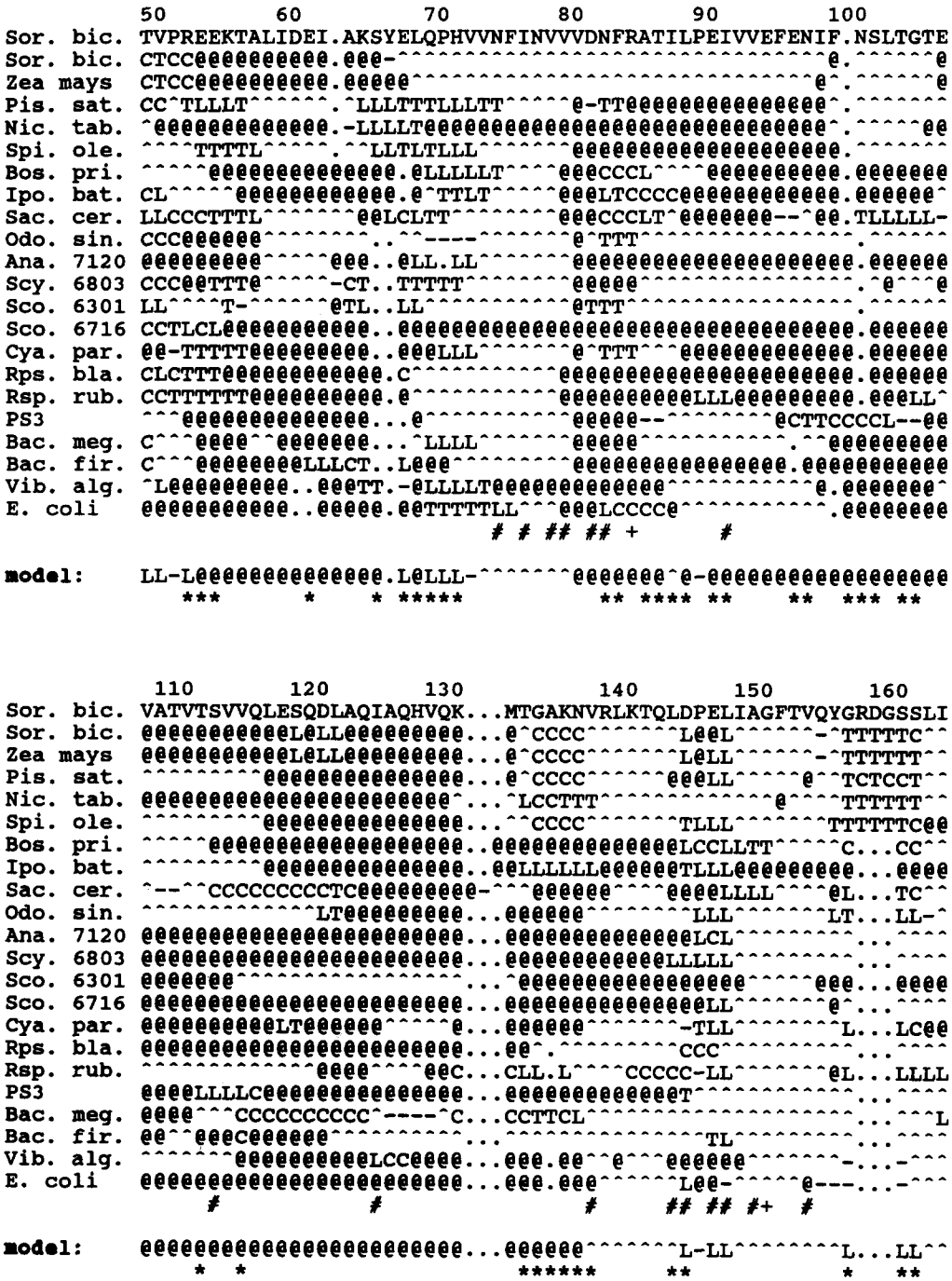

Fig. 4 (continued). (Last part overleaf.)

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                170       180
Sor. bic. DMSVRKQIEEITSEFELPDVPLEV
Sor. bic. @@@@@@@@@@@@@@@@@CC^@@@@
Zea mays  ^^@@@@@@@@@@@@@@@CC^@@@@
Pis. sat. @@@@@@@@@@@@@@@@@^^@@@@@
Nic. tab. @@@@@@@@@@@@@@@@@@@@@@@@
Spi. ole. @@@@@@@@@@@@@@@@@@@@@@@@
Bos. pri. ^^@@@@@@@@@@@@@@@@@.....
Ipo. bat. @@@@@@@@@@@@@@@@@@CCCC...
Sac. cer. ^^^^^^^^^^^^^^@@CCC.....
Odo. sin. ^^^^^^^^^^@@@@^^^^^@^...
Ana. 7120 ^^^L^^^^^^^^^^LCCC......
Scy. 6803 TLLL^^^^^^^^^^^^^^^^^.....
Sco. 6301 @@@@^^^^^^^^^^^^CC.......
Sco. 6716 ^^^^^^^^^^@@@@@@@@@.....
Cya. par. @@@@@@@@@^^^LL@@@@@@....
Rps. bla. ----@@@@@@@@@@@@@@C.....
Rsp. rub. ---L@@@@@@@@@@@@@@C.....
PS3       TTLLLTT^^^^^^@^^C.......
Bac. meg. TTTTCTTT@@@@LLL@@@CC....
Bac. fir. ^TT^^^@@@@@@@@@@@^^C....
Vib. alg. LLCCTTTCCC@@@@@@C.......
E. coli   LCCCTTC@@@@@@@@@@.......
          +     #   #   #

model:    @@@@@@@@@@@@@@@@@.......
          *******
```

```
@:   Alpha helix                    + : strictly conserved residue

^:   Beta strand                    # : conservative exchanges

C:   Coil                           * : less supported prediction

T:   Turn                             in model (cp. text)

L:   Loop = turn or coil

-:   unpredictable by the applied method

.:   gap in amino acid alignment
```

Fig. 4 (continued).

surface of δ. The function of δ and the capacity to reconstitute photophosphorylation activity seem to depend not only on its size and shape, that determine the position of the only five conserved residues in the tertiary structure, but on its specific and close interactions with other subunits within the ATP-synthase complex. Five of the 18 hydrophilic residues, identical in spinach and *E. coli* δ, are exchanged in *Z. mays* (Fig. 3 and Table II) and do therefore probably not participate in the specific interactions. Thus, the hydrophilic residues of δ, that are responsible for the additional catalytic reconstitution between spinach and *Z. mays*, should be among those 37 hydrophilic residues, which are additionally identical (Fig. 3 and Table II).

Analysis of secondary structure features conserved during evolution

Experimental data concerning the structure of the δ subunit of photosynthetic ATP-synthases are available: the location of this subunit within the ATP-synthase complex in situ was deduced from immunochemical experiments and limited proteolysis [17,47]; it is mostly inaccessible. Subunit δ was concluded to be part of the central mass depicted by electron microscopy techniques [48]. From sedimentation velocity and small angel X-ray diffraction [49] and from rotational diffusion [50] and circular dichroism measurements [51] of the protein in solution an elongated shape (2.8×10 nm) [49,50] and a mostly α helical structure [51] was deduced.

After deduction of a reasonable secondary structure or dissection of a tertiary protein structure into secondary structure domains it may become possible to calculate the protein folding pathway (P. Argos, personal communication). We therefore tried to develop a hypothetical secondary structure model: secondary structures for each of the 21 sequenced species were calculated using three different prediction algorithms [52–54], implemented in the program PROSIS, Pharmacia LKB (data not shown). Next, consensus were deduced: a certain conformation was assumed, if at least two of the three algorithms used agreed; if one

algorithm predicted 'coil' where another predicted 'turn' at a respective position, the term 'loop' was used. The single consensus were aligned (Fig. 4) according to the amino acid alignment (Fig. 3).

In the overall consensus (model) a certain structure is proposed, if at least 12 out of the 21 individual consensus agree. This is the case for 113 residues. If at a respective position less than 12 out of 21 consensus agree, the most prevailing prediction is taken; these less supported predictions are marked by an asterisk. If the single consensus does not allow for a distinction between 'coil' and 'turn', the term 'loop' is used in the model. For seven positions the secondary structure remains unpredicted (Fig. 4).

The conserved and conservatively exchanged amino acids of δ are located in regions for which the same secondary structures are predicted for most of the species, helical secondary structures for the N-termini, the middle parts and the C-termini and three short β strands, one in the middle and two near the C-termini, connected by a turn. Helical wheel analysis [55] of the three conserved putative helical parts shows that they would have the potential to form amphipathic α helices [19,47] in all δ sequences available (Fig. 5). The two C-terminal β strands could form an antiparallel amphipathic β sheet [56] connected by a turn (Fig. 6). The conservation of these amphipathies supports the putative overall secondary structure prediction in spite of deviations in the individual consensus predictions, which we think reflect the weakness of the prediction algorithms rather than differences in the real secondary structures of δ. The conserved Tyr, Arg and Asp are found at the N-terminal start of a putative amphipathic α helix in each case.

The insertion of three residues unique for higher plant δ sequences would be an extension of the loop connecting the antiparallel amphipathic β sheet and the amphipathic C-terminal α helix conserved in all species (Figs. 3 and 4).

Thus, the CF_1 δ subunits and the corresponding proteins from mitochondria (OSCP) and prokaryotes are a family of proteins with very similar secondary structure in spite of divergent amino acid sequences, and the typical δ subunit of F_0F_1 ATPases is predicted to be an α helical protein with only a few β strand structures; the predicted high proportion of α helical secondary structure even seems too low when compared to the values calculated from circular dichroism

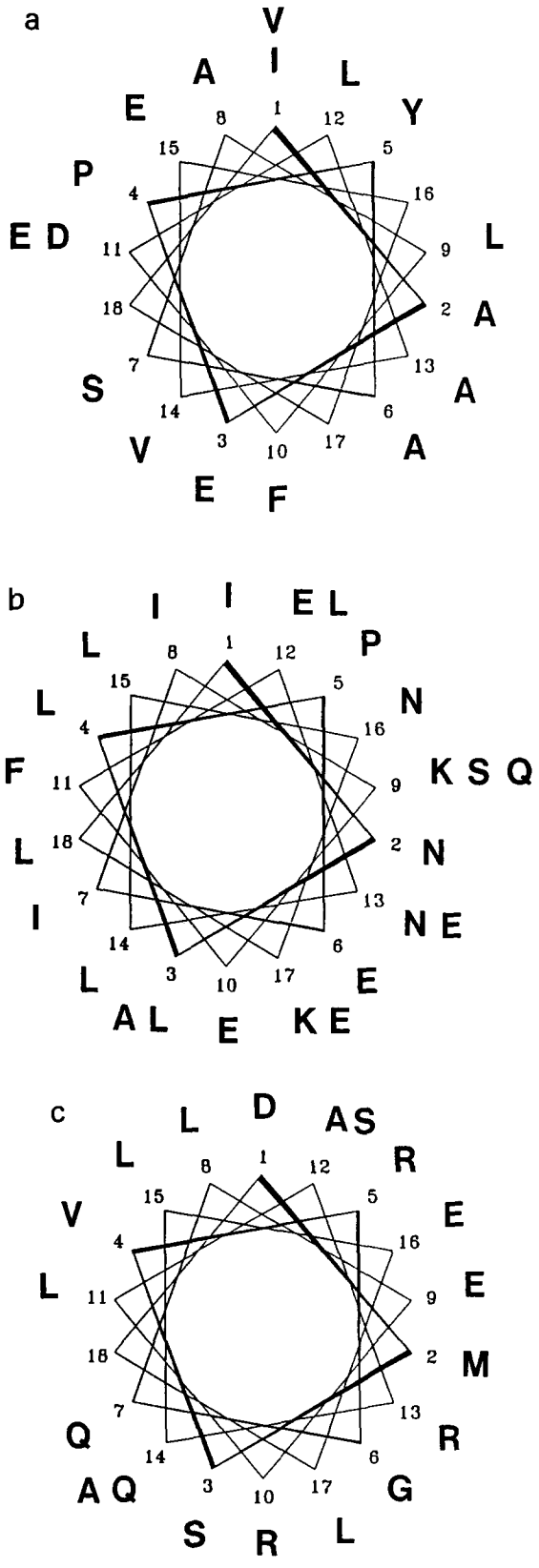

Fig. 5. Consensus helical wheels according to Schiffer and Edmundson [55] displaying the amphipathic character of the predicted N-terminal [19], middle and C-terminal [47] helices of a typical δ subunit. The amino acids were aligned as in Fig. 3 and the residue most common at each respective position was taken for the consensus wheels. (a) N-terminal putative amphipathic α helix, residues 5–19 of secondary structure consensus. (b) Middle putative amphipathic α helix, residues 87–105 of secondary structure consensus. (c) C-terminal putative amphipathic α helix, residues 164–180 of secondary structure consensus.

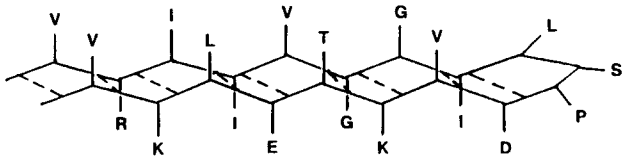

Fig. 6. Consensus β sheet and connecting turn for a typical δ subunit, displaying the amphipathic character of the putative C-terminal β sheet (residues 140–158 of alignment, Figs. 3 and 4). The amino acids were aligned as in Fig. 3 and the residue most common at the respective position was taken for the consensus sheet.

TABLE III

Comparison of the predicted secondary structure of subunit CF_1 δ (consensus in Fig. 4) with circular dichroism data [51]

Secondary structure	% Predicted consensus	% Calculated from circular dichroism	
		δ (spinach)	OSCP (bovine)
α helix	71.1	83.6±6.1	85.8±4.2
β sheet	16.7	8.6±7.5	0.3±0.7
Turn/coil (loop)	8.3	8.0±4.1	13.6±4.9
Unpredictable	3.9	–	–

data for spinach CF_1 δ and bovine OSCP [51] (Table III).

Although this CF_1 subunit contains several conserved hydrophilic residues, it is concealed within the quaternary structure of the enzyme, except for the C-terminus beeing exposed in situ [17,47]. A role in direct coupling [2,3], i.e., in H^+ translocation along the amphipathic structures [19] to the active sites, cannot be rouled out, but δ also may fulfill the structural requirements to act as a rather stiff transducer of a conformational 'wave' between the proton conducting and the ATP synthesizing moiety of the ATP-synthase: it can be crosslinked to CF_0 I [18], it also interacts with the α and β subunits of F_1 [57,58], and it is part of the central mass [48,59], where conformational movements have been depicted [60]. The specific functions of the conserved Tyr, Ala, Arg, Gly and Asp remain to be elucidated.

We have produced monoclonal antibodies against an epitope on the binding surface of *Sp. oleracea* CF_1 δ towards CF_0 (W.Finke, M.Wulf and R.J.Berzborn, unpublished); they crossreact with δ from *P. sativum*, but not with δ from *Z. mays*. Analysis of the sequences and secondary structures of this epitope in the different species is now possible and will lead to a partial characterization of the binding surface of δ.

Acknowledgements

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Note added in proof

The sequence of tabacco CF_1 δ has now been published (J.A. Napier, K.H. Larsson, F. Madueno and J.C. Gray (1992) Plant Mol. Biol. 20, 549–554). The recently published CF_1 δ sequence of the red alga *Antithamnion* sp. (Kostrzewa, M. and Zetsche, K. (1992) J. Mol. Biol. 227, 961–970) supports our speculation related to the *O. sinensis* δ sequence: it is also chloroplast-encoded and does not contain the insertion of 3 residues close to G_{156}.

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