**Cyanophora paradoxa:**
Nucleotide Sequence and Phylogeny of the Nucleus Encoded Muroplast Fructose-1,6-bisphosphate Aldolase

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**Cyanophora paradoxa**, Muroplast, Class-II fructose-1,6-bisphosphate aldolase

Immunoscreening of a *C. paradoxa* expression library against water soluble muroplast (“cyanelle”) proteins resulted in isolation of a clone encoding the nucleus-encoded muroplast class-II fructose-1,6-bisphosphate aldolase (class-II FBA). Its nucleotide sequence was determined. The 1432 bp insert, derived from a single-copy gene transcript, bears a reading frame of 1206 bp in length, representing 402 amino acids with 346 amino acids of mature protein. The leading amino acids match structural features necessary for precursor import across chloroplast envelope membranes. In phylogenetic tree topology, the investigated mature FBA clusters within type B FBAs with *Synechocystis* sp. as nearest neighbor. This is the first report of a Type B class-II FBA sequence of plastids.

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**Introduction**

Being part of fundamental pathways for conservation and utilization of energy and metabolites fructose-1,6-bisphosphate aldolase (FBA, EC 4.1.2.13) is present in most living organisms (Horcker et al., 1972; Plaumann et al., 1997). In glycolysis and gluconeogenesis it is the key tool at the hexose-triose-transition (Embden-Meyerhof pathway), where it is catalyzing the cleavage of fructose-1,6-bisphosphate (FBP) into triose-phosphates or vice versa, depending on metabolic situations. Within the Calvin-Benson cycle, aldolase plays a similar role preferentially leading the trioses towards hexose sink and CO$_2$-acceptor regeneration.

Two distinct species of FBAs, designated class-I and class-II, have been characterized (Jacobsha-

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**Abbreviations:** class-II FBA, class-II fructose-1,6-bisphosphate aldolase; cTPs, chloroplast transit peptides; FBP, fructose-1,6-bisphosphate; groEL, heat shock protein; IPTG, Isopropylthiogalactosid; petE, ferredoxin-NADP$^+$-oxidoreductase; psaE, subunit of PS-I complex; psaF, subunit of PS-I complex.

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shows, as other plastidic multifunctional class I aldolases, a comparable sedoheptulose activity (Flechner et al., 1999).

Phylogenetic analysis of class-II-FBAs led to separation of two types, designated type A and type B class-II FBA originating from gene duplication (Plaumann et al., 1997).

Discussing endosymbiosis theory in view of chloroplast evolution, it remains as yet an un-cleared and intriguing question whether C. paradoxa is an anagenetic model organism indicating the idea of a polyphyletic origin of primary plastids. Or a descendant of the missing link in the evolutionary chain to the protoplasm originating from the only endocytobiotic cyanobacterium having been able to undergo intertaxonic combination with its unique eukaryotic host.

The flagellate C. paradoxa normally bears two to four chromatophoric bodies. From the structural point of view, including the surrounding mucine sacculus (Schenk, 1970), these bodies seem to be still intracellular cyanobacteria (Pascher’s cyanelles, 1929). Considering the genomic level reveals a genome that is reduced (Herdman and Stanley, 1977) and structured like higher plant plastomes (Löffelhardt et al., 1980). Furthermore there are reports of protein import into this cell compartment (Bayer and Schenk, 1986; Bayer et al., 1990; Jakowitsch et al., 1996) together with the first direct proof of gene transfer from the originally endocytobiotic cyanobacteria’s genome into the nucleus of its host (Schenk et al., 1992).

That’s why we assigned to the glaucocystophytes “cyanelles” the status of a real cell organelle and altered their term into cyanoplasts (Schenk, 1977; 1990), also known as muroplasts (Schenk, 1994; Löffelhardt et al., 1999). Further analysis of single gene content of the C. paradoxa plastome (Löffelhardt et al., 1997a) revealed a set of genes which are no longer plastome-based in higher plants (e.g. psaE, psaF, petF, groEL and several ribosomal proteins). In view of a monophyletic evolution of plastids, C. paradoxa muroplasts may therefore be considered as a late stage of the postulated proto plastid (Löffelhardt et al., 1997b).

For C. paradoxa two isoforms of the nuclear encoded class II FBA, a cytosollic and a muroplastidic protein, have been identified by their different biochemical and biophysical properties (Gross et al., 1994; Flechner et al., 1999). In this paper we present the sequence for a nuclear coded type B class-II fructose-1,6-bisphosphate aldolase from C. paradoxa and provide indications that this protein is the muroplast’s enzyme. The phylogenetic implications based upon fba compartmentation and fba type are discussed.

**Materials and Methods**

**Strain and culture conditions and preparation of antibodies**

C. paradoxa (LB 555 UTEX, Pringsheim strain B 29.80 from SAG, Culture Collection of Algae, Göttingen, Germany) was grown and harvested according to Zook and Schenk (1986). To meet the specific needs of detecting unprocessed and mature proteins in various gel systems, polyspecific polyclonal antibodies were prepared in rabbits and purified over Protein A-Sepharose as described by Bayer (1991). Water soluble muroplast and membrane hosted proteins for immunizing were obtained according to Zook and Schenk (1986) and Bayer and Schenk (1989).

**Construction of cDNA library and screening of cDNA library**

Total RNA was isolated from a C. paradoxa culture 24 hours after dilution by the guanidinium thiocyanate method described in the “The Qiagenologist” application protocols (3rd. Ed., Qiagen, Hilden, Germany, 1990). The poly(A)+-RNA fraction was separated from total RNA by purification over Oligotex™-dT30 (Qiagen) and the cDNA library was constructed using the Uni-ZAP® synthesis kit (Stratagene, La Jolla, Ca., USA) following the suppliers instructions and amplified prior to screening.

Phages (10⁵ pfu) were incubated at 37 °C for 15 min in 100 μL E. coli XL1-Blue (OD₆₀₀=2.0) and cultured by the top-agar technique under 100 μg mL⁻¹ ampicillin selection and induction by 10 mM IPTG. When the plaques reached about 0.5 mm in diameter after for 3 to 4 h, expressed proteins from lysed cells were transferred to nitrocellulose membranes. Immunodetecation was performed according to the instruction manual of the ECL-western blotting detection system (Amersham Pharmacia, Freiburg, Deutschland) and posi-
tive plaques were rescreened until all plaques per plate were positive.

Recombinant phages were inserted into Bluescript SK(minus) plasmids by the "ZIP-Auto-

mated Excision Process" using the F1-ExAs-sist™ helper phage (Short et al., 1988) and sub-clones were generated according to Henikoff (1984) using the Nested Deletion Kit from (Amer-

sham Pharmacia, Freiburg, Deutschland).

Polymerase chain reaction (PCR)

The ABI Prism Dye Terminator Cycle Sequencing Kit with AmpliTaq® Polymerase (Perkin El-

mer, Foster City, CA., USA) was used to perform PCR using a Biozym Minicycler®.

DNA sequencing and sequence analysis

Clones were sequenced by the dideoxynucleo-

The following oligonucleotides were used as primers:

(a) 5'-ATCAAGTCGATCATGGAGGC-3'

and

(b) 5'-CTTCTGCATGTACTTCATCG-3'.

Using the HUSAR/GCG package (maintained by the Biocomputing Unit at DKFZ Heidelberg, Germany) and the BLAST Server (NCBI, Bethesda, MA., USA) the sequence was subject to further analysis. Alignment of amino acid se-
quences were based upon algorithms from the ClustalX 1.64/ClustalW 1.7.4 package (Thompson et al., 1994; Thompson et al., 1997), hydropathy plots of transit peptides – using the Kyte-Doolittle scale (1982) – and amino acid frequency calculations were performed with the DNASTar-package (Lasergene Inc., Madison, WI., USA). Phylo-
getic trees were constructed with the ClustalW 1.7.4 program using Dayhoff matrices for calculating NJ-distances. Bootstrap analysis was carried out with 1000 replicates.

Estimation of gene copy number

Gene copy numbers were estimated by Southern hybridization of noncutter-treated genomic DNA with an internal 808-bp-fragment (Fig. 3), la-

beled with α-[32P]-dCTP (ICN, Costa Mesa, Ca., USA) by the random prime labeling method (Feinberg and Vogelstein, 1983) using a kit from MBI Fermentas (Vilnius, Lithuania).
Results

Identification of transcripts in vivo and final cellular location of the mature FBA

Screening of the C. paradoxa cDNA library yielded a set of clones positive to a polyspecific antiserum against water soluble muroplast proteins. Subject to further investigation was a clone carrying a 1432 bp insert (see below), which was probed against C. paradoxa mRNA in Northern analysis (Fig. 1). This revealed a single band of 1520 bp, therefore the fragment captured seems to be about full length.

Southern blot analysis was performed to determine the number of FBA genes in the C. paradoxa genome. The blots were probed with the Xho I-fragment under high-stringency conditions, and yielded for Xho I and Sac I digestions one signal each, accounting for one single gene locus (Fig. 2). The double band observed on the Sal I lane originates from the Sal I side at position 1174 of the FBA gene (Fig. 3).

Sequence analysis

Sequence identification. Sequencing found the insert to be 1432 bp long (Fig. 3) with a reading frame for a polypeptide of 402 amino acids. Its deduced amino acid sequence in comparison to known FBA sequences led to the following findings:

The sequence positions 56 to 402 contain the complete sequence of a mature class-II FBA with amino acid sequence homology to the known class-II fructose-1,6-bisphosphate aldolases of up to 71% similarities with the Synechococcus PCC6308 protein (Table I).

A high degree of conservation (Fig. 4), particularly in domains crucial for enzyme activity (Fig. 3: metal binding sites His^{136}, His^{139}) as known from three-dimensional structure analysis of the E. coli enzyme (Blom et al., 1996, Cooper et al., 1996).

The same inserts that match full helical turns as the other FBAs residing in the same subclade (Fig. 4, region A to C) and other sequence features accounting for surface property patterns like hydrophobic or strongly charged positions in phase with helical turns (Fig. 4, region D and E: bold letters).

Although being incomplete, the leading stretch of amino acid pos. 1–55 can be assigned the function of a transit peptide. It shares important features (von Heijne et al., 1989) with other chloroplast transit peptides (cTPs) crossing the two envelope membranes, e.g. amino acid composition, extended ß-strand structures (not shown) and the hydropathy profile (Fig. 5).

Processing-site identification and molar mass. Comparison of the deduced protein with chloroplast aldolases from different species suggests an N-terminus for the mature monomer at Ala^{1} (Fig. 3). Processing would yield a mature protein with a predicted molecular mass (M_r) of 37.23 kDa. This is in good agreement with the molecular mass of 38 kDa previously determined for the cytosolic class-II FBA monomer of Euglena gracilis (Pelzer-Reith et al., 1994) and 37 kDa for the Ralsstonia eutropha enzyme (formerly Alcaligenes eutrophus: Schäferjohann et al., 1995). Further support is given by matching the (-3,-1) rule (von Heijne, 1983, 1985). In contrast to the prokaryotes where in most cases Ala is observed in these two positions, no strict preference for Ala is found in the eukaryotes (Nielsen et al., 1997). Therefore we suggest the cleavage site for FBA-cTP in C. paradoxa to be

...Glu-Val-Thr-Arg↓Met-Ala-Leu...

with the N-terminal methionine probably to be removed by a stromal methionyl-aminopeptidase after initial cleavage of the cTP, as with other plastidic nucleus-encoded proteins (Ikeuchi et al., 1989, Gavel and von Heijne, 1990).

Discussion

Phylogenetic considerations

Class-II FBA sequences have been reported from a variety of prokaryotic (e.g. Trach et al., 1988; Alefounder and Perham, 1989; Schäferjohann et al., 1995; Kaneko et al., 1995) and eukaryotic (e.g. Schwelberger et al., 1989; Muto and Hayashi, 1994; Plaumann et al., 1997) species. Amino acid sequence alignments (Fig. 3, Table I) of C. paradoxa FBA with other class-II aldolases show a clustering with the cyanobacterial group and in toto moderate similarities (59–71%) to type-B of cyanobacteria and α- and β-proteobacteria (exception: P. stutzeri, a γ-proteobacterium) and then to
Fig. 3. DNA- and amino acid sequence of *C. paradoxa* Type B class-II FBA. The arrow indicates the proposed site of precursor processing. The assumed first few residues of the mature protein are in bold face. The first eight nucleotides of the 5' end are the polylinker and the last six nucleotides of the 3' end are the *XhoI* cleavage site of pBluescript.
Table I. Relative amino acid similarities (%) of mature FBAs. Calculated with indels. Lower triangle shows the absolute number of identities by pairwise comparison. The sequence length is shown on the diagonal (See alignment Fig. 4).

| HAEIN | ECOLI | CAMJE | SYMPH | YEAST | EUGGR | CORLE | STRECO | BUBRU | HORTU | CYAPA | SYNEB | XANFL | RALCU | RHACA | NOEMO | NOSCO | PESTE | RHME | GIAIN | THEA | DEIRIA | TRIERA | BACSU | MYCEN | UREUR | ARATH |
|-------|-------|-------|--------|-------|-------|-------|--------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|

Fig. 4. Alignment of class-II-FBA proteins. Gaps are indicated by dashes, shading at positions with more than 65% similarities (•) and identities (*). Sequences were retrieved from GenBank, Swissprot and PIR databases. Accession to sequences through index numbers is for Arabidopsis thaliana (ARATH), AAF25990.1, Bacillus stearothermophilus (BACST), P94453, Bacillus subtilis (BACSU), P42420, Borrellia burgdorferi (BORBUR), O51401, Campylobacter jejuni (CAMJE), P53818, Corynebacterium glutamicum (CORGE), P19537, Cyanophora paradoxo (CYAPA), AJ227753, Deinococcus radiodurans (DEIRA), F75378, Edwardsiella ictaluri (EDWIC), O52402, Escherichia coli (ECOL), P11604, Euglena gracilis (EUGGGR), CA-A61912, Giardia intestinalis (GIAIN), AF079109.1, Haemophilus influenzae (HAEIN), P44429, Helicobacter pylori (HELPY), C71967, Hydrogenophilus thermodiluteus, BAA75221.1, Mycoplasma genitalium (MYCEN), P47269, Neisseria meningitidis (NEIME), AAF44203.1, Neurospora crassa (NEUCR), P53444, Nostoc commune (NOSCO), Q9XDP3, Pseudomonas stutzeri (PSEST), O87796, Ralstonia eutrophica (RALEU), Q59101, Rhodobacter sphaeroides (RHOKA), P29271, Saccharomyces cerevisiae (YEAST), P14540, Schizosaccharomyces pombe (SCHPO), P36580, Sinorhizobium meliloti (RHIME), P56888, Streptomyces coelicolor (STRCO), Q938R6, SynecococcusPCC6803 (SYNP6), Q55664, Thermoguineae (THEAG), G72397, Thermus aquaticus (THEAQ), AAF22441.1, Treponema pallidum (TREPA), O83668, Ureaplasma urealyticum (UREUR), AAF31010.1, Xanthobacter flavus (XANMA), Q56815.
Fig. 4. Legend see on page 996.
Fig. 5. Hydrophathy profiles for transit sequences (Kyte-Doolittle). Window size is 9 residues, positive (negative) peaks represent hydrophilic (hydrophobic) residues, arrows denote the putative cleaving site. Sequences were retrieved from GenBank, Swissprot and PIR databases. Accession to sequences through index numbers is for C. paradoxa FBA: AJ227753, C. paradoxa FNR: G1071829, Triticum aestivum GBSS: P27736, Brassica napus FBPase: Q07204, Mesembryanthemum crystallinum RbcS: Q08186, Arabidopsis thaliana rpl 12-C: P36212, Spinacea oleracea CP 12: Z72487, ditto Cyt b6f: S00454.
Fig. 6. Topology of gene tree for mature FBAs. Phylogenetic relations within the tested sequences (for data base accessions see legend to Fig. 4) by the neighbor joining method (Saitou and Nei, 1987), displayed with reference to substitution frequency per site (see scalebar). Probabilities for branching, as shown at the appropriate nodes were obtained through bootstrap analysis (Felsenstein, 1985) with 1000 replicates through the same distance estimation method. Eukaryotic species are marked by bold capital letters. The upper main branch includes the type B class-II FBA sequences, the lower house of the type A.
the other subgroups of the type-B sequences with similarity values of 24–38%. The next neighbor eukaryotes yet known are G. intestinales (formerly known as G. lambia) with 42% and A. thaliana with 28% similarity. The cellular compartmentation and expression status of the A. thaliana polypeptide is not known. The FBA isozyme of C. paradoxa is more related to that of Synechocystis than to the FBA of any other known bacterial organism or eukaryote. There was low similarity (15–20%, Table I) to the class-II type A aldolases from γ-proteo- and other bacteria, fungi and E. gracilis.

Similarity-Distance-Analysis of the available FBA class II sequence data suggests gene duplication and subsequent metabolic specification of the related products designated type A and type B aldolases (Gross et al., 1994; Plaumann et al., 1997). Type A FBAs are functional in glycolysis and gluconeogenesis pathways, whereas normally type B FBAs belong to the Calvin-Benson-Cycle, clearly indicated by their gene loci clustering with (Alefounder and Perham, 1989; Schwelberger et al., 1989; von der Osten et al., 1989; Mutoh and Hayashi, 1994; Fleischmann et al., 1995; Burucoa et al., 1995; Plaumann et al., 1997). This includes all investigated class-II FBA-genes found in the Calvin-Benson-Cycle operons of photoautotrophic proteobacteria and cyanobacteria (Meijer et al., 1990; Chen et al., 1991; Schäferjohann et al., 1995; Kang et al., 1995).

Sequence analysis and the high bootstrap support for the topology of the phylogenetic tree with the position of the C. paradoxa FBA in the type B subclade (Fig. 6) supports the view that the presented sequence is coding for the muroplast enzyme, which originated from transfer out of the endocyctobiotic cyanobacterium while turning into the real cell compartment stage. This is a further direct proof for gene transfer (Schenk et al., 1992).

We therefore assist the idea of Plaumann et al. (1997) that a third independent origin of plastid FBA must exist. Considering phylogeny we should bear in mind that class I and class II FBA isozymes must have originated differently according to reaction mechanism and sequence diversity (Plaumann et al., 1997).

Chlorophyta and embryophyta are found to have different class I FBA isozymes within the cytosol and their plastids with the original plastidic class II FBA gene thought to have been replaced by his host's gene. A similar situation was found recently for the rhodophyte Galdieria sulfuraria (Gross et al., 1999) which doesn't fit the observation of Ikawa et al., (1972) that other investigated red algae contain class I as well as class II aldolase activity.

E. gracilis contains a plastidic class I FBA and a cytosolic type A class II isozyme, which is explained by secondary symbiosis of a plastidic class-I-bearing chlorophyte (Plaumann et al., 1997). Class II FBA has been shown to exist in fungi (type A), including yeasts, and in few other unicellular eukaryotes like E. gracilis (type A), G. intestinialis, (type A), T. vaginalis, several marine planktonic algae and heterotrophic prokaryotes (type A or B). The C. paradoxa type B sequence is the first plastidic class II FBA known to date. On first sight this finding complies with the hypothesis of Gross et al., (1999) on chloroplast evolution with transfer of cyanobacterial class II FBA to the host nucleus as first endocyctobiotic step. In step 2 in green algae and higher plants (in G. sulfuraria and perhaps in the other red algae) this plastidic enzyme was replaced by a cytosolic class I FBA isozyme developing from the original cytosolic 'host' FBA by gene duplication. Would that be correct, then the cytosolic class II FBA in C. paradoxa might have been developed in a similar manner, e.g. following the described class II FBA gene transfer event (from cyanobacterial endocyctobioto to eukaryotic host).

If, in contrast, the cytosolic C. paradoxa class II FBA is original within this species, the monophyletic hypothesis for chloroplast evolution would be difficult to understand.

Referring to the fba gene as a tool to precise the position of C. paradoxa and the muroplast in the topology of the evolution and to back the challenge of the recent interpretation of the plastid evolution (Martin et al., 1998) to be monophyletic the cytoplasmic fba isoenzyme needs to be typed on the genetic level.

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