Ferredoxin-NADP$^+$ Oxidoreductase of *C. paradoxa* Nucleus Encoded, but Cyanobacterial Gene Transfer from Symbiont to Host, an Evolutionary Mechanism Originating New Species

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The nucleus encoded cyanoplast ("cyanellar") ferredoxin-NADP$^+$ -oxidoreductase (FNR) of *Cyanophora paradoxa*, characterized by an N-terminal amino acid sequence, is compared with homologous sequences of other photoautotrophic organisms. The high degree of similarity to the cyanobacterial sequences indicates a cyanobacterial origin. This could be a first direct demonstration of an intertaxonic combination: a gene transfer from an original endocytobiont (cyanobacterium) to the nucleus of its host, one of the most important demands of the Endosymbiosis Theory, an evolutionary mechanism leading to the origin of a new species.

Introduction

The origin of eukaryotic cells and some of their organelles is described by (a) the autogenic (or endogenic) Compartmentation Hypothesis (*e.g.* [1–3]) and (b) the exogenic Symbiosis Theory [4–6] based on ideas of Schimper [7] and Altmann [8]. Neither prokaryotic characteristics of cellular organelles, nor *e.g.* the cytochrome c phylogeny [9] must be interpreted as support for either hypothesis. Acceptance of either hypothesis has been more or less a matter of belief; both have been viable alternatives ([10–12]; but see [13]), assuming different evolutionary rates and longlasting horizontal gene transfer between the protist ancestors. Though recent arguments in favour of a symbiotic origin of the complex (phylogenetically secondary) chloroplasts are overwhelming [14–17], strong evidence for gene transfer from a phylogenetically primary endocytobiont to the host genome is lacking. Such an indication would be more stringent contrarily to implications received by studies on eukaryotes due to uncertain phylogenetic origin of their organelles [18–20]. And even this is one of the most decisive objections against the Endosymbiosis Theory (EST). The biflagellated, unicellular and photoautotrophic eukaryote *Cyanophora paradoxa* Korsch. is particularly well suited for studying a potential gene transfer. It represents the best investigated species within the class Glaucocystophyceae [21, 22], a group of unicellular photoautotrophic organisms with blue-green assimilators, so-called "cyanelles". *Per definitionem* [23] a "cyanelle" is an intracellular symbiotic (endocytobiotic) cyanobacterium, defined in analogy to the term "zooxanthelle" or "zoochlorelle" (the latter representing an endocytobiotic *Chlorella* cell). Investigations over the past years have shown that the "cyanelle" genome of *C. paradoxa* (more detail see [24, 25]) is greatly reduced in size [26, 27], *e.g.* 80 to 90% of the soluble "cyanellar" proteins are encoded on the nuclear genome [28, 29]. Therefore, the "cyanelles" of *C. paradoxa* can no longer be regarded as "symbiotic" cyanobacteria, the eukaryote no longer as "host"; the original cyanobacterial ancestor has changed into an actual cell organelle, now termed cyanoplast [30–32] (a cyanoplast is a symbiogenetic eukaryotic cell organelle, descending from a cyanelle (an endocytobiotic cyanobacterium) and genetically depending on nucleus DNA impart). In contrast to the plastids of other photoautotrophs (including the red algae) the following characteristics of the *C. paradoxa* cyanoplast indicates clearly its original cyanobacterial nature: a) the light microscopic morphological structure [33], b) the presence of a murein sacculus [30, 34–36] in the envelope, c) the electron micro-

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scopic structure of the inner envelope membrane [37]. Unlike the chloroplast genome, the cyanoplast genome encodes the small subunit of RUBISCO [38], ferredoxin [39], and various ribosomal proteins [40–42]. The nucleotide sequence of 5S RNA [10, 43, 44] and the amino acid sequences of RUBISCO and ferredoxin [45–47] are most similar to those of cyanobacteria. These characteristics make *C. paradoxa* an important analogous model organism [48] for studying mechanisms of plastid evolution with regard to the proposals of the EST or SET ("Serial Endosymbiosis Theory") [49–51].

Recently, we demonstrated that ferredoxin-NADP oxidoreductase (FNR), a central photosynthetic enzyme, is one of the nucleus encoded cyanoplast proteins and interpreted this result as a first direct indication of "gene transfer" from an endocytobiotic prokaryotic cell to a eukaryotic host nucleus [52], the type of transfer required by the Endosymbiosis Theory [50, 53]. In order to follow up this assumption we here compare the N-terminal FNR amino acid partial sequence of *C. paradoxa* [54] with corresponding FNRs of prokaryotes and eukaryotes.

Materials and Methods

Organism, FNR purification, amino acid sequencing

C. paradoxa B 29.80, Pringsheim strain (SAUG, Institute of Plant Physiology, University of Göttingen) was continuously grown as previously described [55]. Cell harvesting and homogenization, crude extract preparation, protein purification and amino acid sequencing was essentially done by procedures described in [45, 56]. Details on methods will be given elsewhere [54].

FNR sequence alignment and comparison

The alignment of the mature *C. paradoxa* FNR (N-terminal amino acid sequence) with two cyanobacterial and three dicotyledonic FNRs (see Table I for species and references) was done by eye using the mature FNR of *Spinacea oleracea* as reference, in particular the location of charged residues. Jacard's similarity coefficient S [57] was calculated by computer program as $S = (n-d) \cdot 100/n$ (= identity in Table I); n is the sum of all amino acid positions compared and d represents the sum of detected differences. Calculations were based on (a) all positions taking gaps into account ($n = 41$), (b) excluding positions with a gap in either one or both sequences ($N < 41$), and (c) on positions remaining after cutting out all sites with a gap in any one of the sequences ($n = 19$). Several alignments giving a high overall score in the binary comparison were also checked in pairwise comparisons for structural similarity using a distance weighing matrix based on the superposition of homologous proteins according to their atomic coordinates [58] as well as for the minimal number of DNA mutations required for observed amino acid substitutions [59]. Bivariate analysis for interspecific distances of FNR and ferredoxin sequences [45, 46] followed the method described in [60].

Results and Discussion

FNR, N-terminal amino acid sequence

In vitro translation of *C. paradoxa* polyA$^+$-mRNA and detection of the translation products after electrophoresis by immunoblotting with a monospecific polyclonal FNR antibody [52] demonstrates that the FNR is synthesized as a preprotein. From this we infer that the translation product is processed to the mature protein during or after import into the cyanoplast. After enzyme purification [54] up to six FNR specific polypeptides are visible in 2D PAGE electropherograms having approximately identical mole masses (SDS-PAGE), but small differences in charge (NEPHGE). Amino acid sequencing of FNR preparations from different fractions after anion exchange chromatography revealed two different N-termini, a longer N-terminal sequence AVDAK KKGDI PLNLF RPANP YIGK (Table I) with 24 amino acids and a shorter polypeptide lacking the first four amino acids. Since there is no biochemical indication for further amino acids on the N-terminus we assume that the longer partial sequence represents the N-terminus of the mature enzyme and used it in the alignment.

Pairwise comparisons of the total length of five other known FNR sequences have shown a high number of invariant sites within the group of higher plants (identity scores up to 90% [61]), and similar values were determined for the cyanobacteria. Since the number of identical amino acids between prokaryotes and eukaryotes are relatively high also (*e.g.* the sequences of *S. oleracea* and *Spirulina sp.* have 57% amino acids in common [61–63]),

Table I. Ferredoxin-NADP$^+$ oxidoreductase (FNR): N-terminal amino acid partial sequence of mature *C. paradoxa* FNR aligned with the N-terminal part of published sequences*.

Organism	Position 1	11	21	31	41	Identity a)	b)	c)
A:	x--AVD-----	---AK-----	KK-GD-IPLN	LFRPANPYIG	K..	100	100	100
B:	----------	---AK-----	---TD-IPVN	IYKPKNPYIG	K..	73	68	68
C:	--MTQ-----	---AK-----	AKHAD-VPVN	LYRPNAPFIG	K..	71	54	63
D:	XQIASDVEAPP	PAPAKVEKHS	KKMEEGITVN	KFKPKTPYVG	R..	32	54	47
E'):	X-VASDVEAPV	---AKVEKHS	KKMEEGVIVN	KYKPKNPYTG	R..	39	50	42
F:	XQVTT--EAP-	---AKVVKHS	KKQDENIVVN	KFKPKEPYVG	R..	30	48	47
Con:	-.....-...-	---AK	KK...I.VN..KPK.PY.G		:			

* Sequence notation and source: A: *Cyanophora paradoxa* (this paper); B: *Spirulina* sp. [73]; C: *Anabaena* PCC 7119 [74], and *A. variabilis* (underlined [75]); D: *Spinacia oleracea* [59, 74]; E: *Mesembryanthemum crystallinum* [61]; F: *Pisum sativum* [62]; Con: Consensus sequence depicting positions with identical amino acids in more than half of all sequences considered (threshold = 4; − denotes a gap); invariant positions in all sequences are underlined; variable positions are denoted by colons; double colons refer to variable sites with conserved negative or positive charge; X denotes preceding known transit sequence; x denotes an assumed unknown transit sequence. (a)−(c)) Similarity indices with respect to FNR of *C. paradoxa:* a) similarity index for positions 1−41 taking insertions/deletions into account; b) similarity index for positions 1−41 excluding sites with an insertion/deletion in either one or both of the two compared sequences; c) similarity index based on 19 sites remaining after omitting all positions with a gap in anyone of the sequences; ') localization of the cleavage site within the subsequence ..TIRAVASD.. after [61] three amino acids before the here given position −V (between T and I).

FNR seems to be a well conserved protein. The conservative nature might also be inferred from the positive cross reactions of a spinach FNR antibody with various plant species [64]. Our sequence comparisons showed, however, that the N-terminus of the mature FNR, which is not involved in the *in vitro* enzymatic activity [65], is less conserved than the rest of the enzyme. For example, even for our best alignment (giving highest overall scores in the various methods applied) the identity scores for the partial sequences are considerably lower than those for entire sequences (compare data in Tables I and II with values quoted above).

Proposal for a direct indication of gene transfer

Affording such a purpose it is necessary to remember the assumption that all plastids derive from photosynthetic prokaryotic ancestors more or less similar to cyanobacteria, although some different characteristics between plastids and cy-

Table II. Identity and structural similarity indices for the aligned N-terminal partial sequences of mature ferredoxin-NADP$^+$ oxidoreductase*.

Species		A	B	C	D	E	F
Cyanophora paradoxa	A:	−	73	71	32	39	39
Spirulina sp.	B:	91	−	68	27	39	41
Anabaena PCC 7119	C:	94	90	−	20	34	34
Spinacia oleracea	D:	71	66	69	−	73	63
Mesembryanthemum crystallinum	E:	77	72	74	91	−	63
Pisum sativum	F:	76	73	75	88	90	−

* Index calculation for alignment as in Table I; identity indices are shown in the right triangle; structural similarity indices based on the matrix in [58] are given in the left triangle (original scale span normalized in order to range from 0 to 1; underlined indices see text).

anobacteria exist. But this assumption does not necessarily include *per se* that plastids – as now generally accepted, but not really proven – derive exogenously from endocytobiotic cyanobacteria. According to the Compartmentation Hypothesis [2] the plastids and the other eukaryotic cell compartments developed endogenously by splitting up the genome followed by membrane surrounding (compartmentation). If the splitting is preceded by genome duplication, the compartments would contain equivalent genomes, both reducing by deletions during later evolutionary stages dependent on chance and constraints. Such scenario does not require a gene transfer.

In contrast to other "primary" plastids (see Introduction), morphological and biochemical characteristics of the *C. paradoxa* cyanoplasts undoubtly point to their origin from an original *endocytobiotic* cyanobacterium. That is the basic statement for the following considerations. For the evolutionary history of the *C. paradoxa* FNR, the first characterized nucleus encoded cyanoplast protein, we at least need to consider three possibilities of development as sketched in Fig. 1:

(I) the FNR evolved "from the very beginning" in the nuclear genome of *C. paradoxa* as a "eukaryotic" enzyme;

(II) the *C. paradoxa* FNR derives from a nuclear encoded protein previously destined for a foregoing, but deleted plastid (or for another cell compartment not furthermore discussed);

(III) the FNR derives from an *endocytobiotic* cyanobacterial ancestor.

With regard to possibility (I) (see also Fig. 1) the *Cyanophora* sequence should be distinct from the prokaryotic sequences, "non-cyanobacterial", and most similar to today's plastid sequences, supporting an endogenous origin [1, 2] of these cyanoplasts and analogously of the other primary plastids as postulated by the Compartmentation Hypothesis [1, 3, 66].

For the second possibility (II, Fig. 1) it must also be assumed that the *Cyanophora* sequence is more similar to the plastid sequences than to the cyanobacterial ones. Moreover, the following evolutionary steps should have happened: 1) The original host carrying a chloroplast-like cell organelle and encoding FNR on its nuclear DNA formed a symbiotic consortium together with an endocytobiotic cyanobacterium, a cyanelle (similar to some

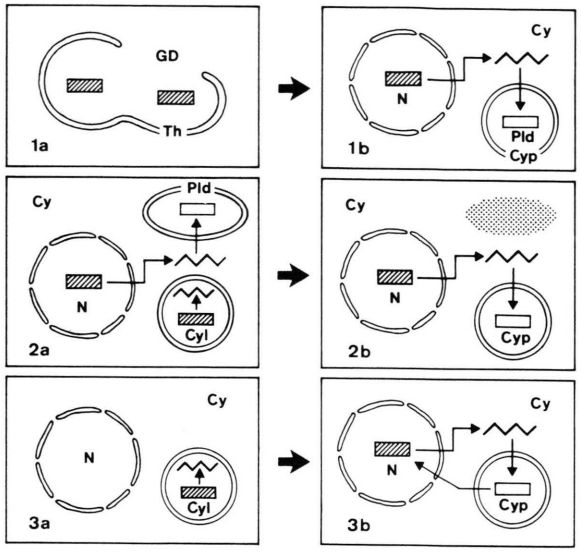

Fig. 1. Sketch of possibilities for cyanoplastidic FNR evolution. I. Endogenosome formation (gene duplication/segregation/compartmentation); II. exogenosome formation (gene product substitution); III. exogenosome formation (gene transfer, intertaxonic combination) (further explanation see text). ▨ : FNR gene active; ▭ : FNR gene transferred/deleted; ∿∿: FNR gene product; Cy: cytoplasma; Cyl: cyanelle; Cyp: cyanoplast; GD: gene duplication; N: nucleus; Pld: plastid; Th: thylakoid.

diatoms, *e.g.* the freshwater diatom *Rhopalodia gibba* [67]); 2) after loss of the cyanobacterial FNR and during coevolution of the symbiotic partners the host substituted the FNR gene product; 3) while the original chloroplast had been lost, the symbiotic partners developed into a new cell species with cyanoplasts instead of chloroplasts. This scenario, however, would demonstrate a protein substitution from host to guest shifting back the problem of FNR development (and gene transfer) to an earlier evolutionary event, namely again to the principal question of the plastid evolution.

In the third case (III) the FNR gene must have been transferred from the cyanobacterial endocytobiont to the host nucleus DNA. Consequently, the cyanoplast FNR amino acid sequence should have a greater similarity to cyanobacterial FNRs than to the chloroplast enzyme of green algae or higher plants.

Sequence comparison and conclusion

The pairwise comparison of N-terminal amino acid sequence of mature FNRs with the partial

sequence of the *C. paradoxa* FNR is shown in Table I. Since there are only a few sequences known, our interpretation must be regarded as preliminary. We note that various subsequences are either common to the eukaryotic FNRs, *e.g.* EAP(7–9), V(E/V)KHS(16–20), (E/D)E(24–25), and (V/T)GR(39–41), or the cyanobacterial FNRs, *e.g.* IGK(39–41) (see underlined coefficients in Table II). For the high overall similarity of *C. paradoxa* FNR with cyanobacterial counterparts as well as its lack of sharing the most prominent eukaryotic subsequences noted above we postulate that *C. paradoxa* FNR is of cyanobacterial origin. If this interpretation remains correct, the compiled data demonstrate a gene transfer (Fig. 1, III) as postulated by the Endosymbiosis Theory (*e.g.* [50]).

It is noteworthy that interspecies distances for the nucleus encoded FNR and the cyanoplast encoded ferredoxin of *C. paradoxa* [45, 46] indicate a similar evolutionary fate. In the bivariate plot corresponding distance matrix elements (Fig. 2) show a strong correlation, *e.g.* distances of both cyanoplast proteins are closer to the cyanobacterial counterparts than to the higher plant and slightly

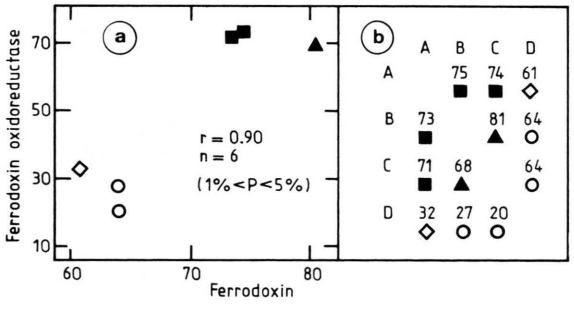

Fig. 2. Plot of distance matrix elements for FNR *versus* ferredoxin. (a) Bivariate plot for interspecies distances; (b) distance matrix values with corresponding label. Left triangle FNR (see Table II), right triangle ferredoxin (whole sequence comparison). A = *C. paradoxa;* B = *Spirulina* sp.; C = *Anabaena;* D = *S. oleracea.*

lower than those among cyanobacteria. This would not be expected, if cyanoplast proteins were subjected to different molecular clocks due to a different micro-environment since long (in relative terms). It seems more likely that the gene transfer has taken place relative recently, *e.g.* later than a similar event (if at all) in higher plants or green algae.

The gene transfer from the endocytobiont to the host nucleus converted the cyanelle of *C. paradoxa* to a more or less integrated cell organelle ("Demand of Cavalier-Smith") [68], to an exogenosome [69] termed cyanoplast [30, 31, 70]. The cyanoplast's loss of coding autonomy in turn changed the host's nuclear genome that finally a new cell type arose. According to our interpretation this event goes beyond a "stabilization of the symbiotic relationship" [51], but it presents a macro-mechanism [71] for the evolution of a new "species". The irreversible and successful gene transfer, a prerequisite for intertaxonic combination [16], is the last step in the symbiogenesis [72] of an exogenous derivable cell organelle. With respect to *C. paradoxa* this evolutionary macro-mechanism altered the original cyanome (until now unknown) to a metacyanome [32] and the organism as a whole to a new algal species, the modern *C. paradoxa.* Hence with respect to evolution of cyanoplasts our interpretation strongly supports the Endosymbiosis Theory. We are inclined to think that in an analogous mode a similar evolution could hold true for other phylogenetically primary chloroplasts. Forthcoming work on sequencing the full length protein (in collaboration with W. Löffelhardt, Wien) and comparisons with other prokaryotic and eukaryotic FNRs will put our conclusions on a trial stand.

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