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Biogenesis of Respiratory Complex I

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Proteins specifically involved in the biogenesis of respiratory complex I in eukaryotes have been characterized. The complex I intermediate associated proteins CIA30 and CIA84 are tightly bound to an assembly intermediate of the membrane arm. Like chaperones, they are involved in multiple rounds of membrane arm assembly without being part of the mature structure. Two biosynthetic subunits of eukaryotic complex I have been characterized. The acyl carrier subunit is needed for proper assembly of the peripheral arm as well as the membrane arm of complex I. It may interact with enzymes of a mitochondrial fatty acid synthetase. The 39/40-kDa subunit appears to be an isomerase with a tightly bound NADPH. It is related to a protein family of reductases/isomerases. Both subunits have been discussed to be involved in the synthesis of a postulated, novel, high-potential redox group.

KEY WORDS: NADH:ubiquinone oxidoreductase; complex I; CIA proteins; mitochondrial acyl carrier proteins; reductases/isomerases; redox group X.

INTRODUCTION

Respiratory complex I is a ubiquitous enzyme of bacterial and mitochondrial electron transfer chains. Prokaryotic and eukaryotic complexes differ in size but show essentially the same shape and arrangement of prosthetic groups (Guénebaut *et al.*, 1998). A minimal set of subunits comprises seven peripheral subunits forming binding sites for NADH, FMN and up to nine iron–sulfur clusters, and seven membrane standing subunits (Friedrich *et al.*, 1995). The function of the latter is unknown except for one subunit, which is supposed to be involved in quinone binding (Friedrich *et al.*, 1990). The subunits are arranged in an L-shaped structure. The peripheral arm is located perpendicular to the membrane, where the membrane arm is embedded in it (Hofhaus *et al.*, 1991). In prokaryotes, complex I, with the minimal set of 14 subunits, is formed, while in eukaryotes the number of subunits is increased significantly by accessory subunits (Walker, 1992). Little is known about their function. Although the accessory subunits are mostly conserved among different eukaryotes, most of the sequences do not reveal any special features. Only two accessory subunits have drawn special attention. The 39/40-kDa subunit binds NADPH and is evolutionary related to a family of reductases/isomerases (Fearnley and Walker, 1992; Schulte *et al.*, 1998). The acyl carrier subunit is a prokaryotic type of acyl carrier protein with a phosphopantetheine group (Runswick *et al.*, 1991; Sackmann *et al.*, 1991). Both subunits have been implicated in the biogenesis of complex I and are described in detail in this review.

Biogenesis of complex I in eukaryotes involves the interplay of proteins coded in two different genomes. The seven "minimal" membrane standing subunits are encoded by mitochondrial genes. In most eukaryotes, all other subunits are encoded in the nucleus. Nuclear-coded subunits are synthesized in the cytosol and are then imported into the matrix of the mitochondrion. Some of the nuclear subunits are synthesized with an N-terminal presequence, which directs the import and is cleaved off in the matrix (Videira *et al.*, 1990a). Import of subunits without a cleavable presequence is most likely also directed by the N-terminus of the subunit (Videira *et al.*, 1990b). However, systematic import studies with complex I subunits have not been performed. In the mitochondrial matrix or in the inner membrane the imported nuclear subunits and the mitochondrially synthesized subunits join to form complex I. The assembly process has been studied in detail in the fungus *Neurospora crassa* (Schulte *et al.*, 1994). As described below, assembly intermediates have been characterized and proteins involved in the assembly process

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have been identified. Many aspects of the assembly still remain enigmatic. Prosthetic groups are most likely inserted in analogy to other systems (*e.g.*, formation of iron–sulfur clusters is supposed to be catalyzed by ferrochelatases, while the flavin might not need any protein factor for insertion). To what extend subunits of complex I are further modified is unknown. Myristoylation of a membrane arm subunit has been recently reported (Plesofsky *et al.*, 2000). Quite a number of genes have been identified by genetic screens suggested to be assembly factors of respiratory complexes. However, a general picture of the assembly process combining the various data is still missing. Drawing conclusions from analogous systems has its limitations since the assembly of the other respiratory complexes is not well understood. Genetic screens to search for assembly proteins of complex I have, so far not been successful.

The chapters of this review on the biosynthesis of complex I are like single pieces of a jigsaw puzzle. They are supposed to be parts of one picture, but do not yet fit together. More details of the process need to be elucidated to obtain the missing links.

THE REDOX GROUP X

Before the assembly of complex I is described, in this article, special attention is given to a novel redox group recently detected in complex I, in addition to the flavin and the iron–sulfur clusters. The molecular nature of this redox group is still unknown. It is postulated solely on the basis of UV/vis and FT–IR spectroscopic data of complex I isolated from *N. crassa* and *Escherichia coli* (Schulte *et al.*, 1998; Friedrich *et al.*, 2000). This review summarizes the experimental data indicating the presence of this redox group in complex I. In addition, its relation to biosynthetic subunits of complex I is also discussed.

The UV/vis spectrum of complex I is characterized by a broad absorption between 300 and 500 nm, which results from the overlap of absorptions caused by the various redox groups. Reduction of the redox groups leads to a small decrease of the absorption in this region. Details become more obvious in the reduced minus oxidized difference spectrum, which shows several overlapping negative bands with maxima at about 450, 425, and 330 nm and positive bands at 300 and 260 nm (Schulte *et al.*, 1998). Resolution of the overlapping spectra is possible by comparison of complex I in different redox states. These can be obtained by either kinetic or thermodynamic means. Reduction of complex I by NADH is very rapid and different redox states of the complex cannot be resolved even by stopped-flow measurements (Bakker and Albracht, 1986). Oxidation of reduced complex I is, however, in part slow enough to be time resolved. Oxidation of NADH-reduced complex I by either NAD^+ or oxygen occurs in two distinguishable phases. The low-potential redox groups of complex I, namely, FMN and the Fe–S clusters N1, N3, and N4, are oxidized very rapidly. This leads to the disappearance of the respective signals in the EPR spectrum and an increase of the absorption around 450 nm in the UV/vis spectrum. During the following slower phase of oxidation, Fe–S clusters N6a and N6b are oxidized (Rasmussen *et al.*, 2001; see also T. Friedrich, this issue). This phase is not accompanied by any changes in the EPR spectrum, but is characterized by the disappearance of two negative peaks at 425 and 325 nm in the UV/vis spectrum. Further oxidation of complex I is very slow. Even after several minutes, the complex remains in a partly reduced state. The UV/vis redox difference spectrum of complex I in this state (after subtraction of the spectrum of oxidized complex I) is characterized by a negative peak at 430 nm and a positive peak at 300 nm (both) of about equal height (Schulte *et al.*, 1998). Fe–S cluster N2 contributes little to this difference spectrum. EPR spectroscopy shows the slow oxidation of the cluster N2 in the course of several minutes. During this time, the UV/vis spectrum shows only minor changes. Therefore, the difference spectrum of the partly reduced complex I has to be attributed to a novel redox group. As long as the nature of this redox group is unknown, we call it redox group X.

By applying different redox potentials to complex I, the redox difference spectra of the various redox groups can be resolved thermodynamically. Employing NADH as a mediator, the redox potential of the redox couples lactate/pyruvate or hydroxybutyrate/ketoglutarate can be biochemically applied to complex I (Schulte *et al.*, 1998, 1999). A broad range of redox potentials can be applied in an electrochemical cell (Friedrich *et al.*, 2000). Thus, complex I can be analyzed in different redox states. At redox potentials above −100 mV, it is not possible to detect the known redox groups in a reduced state. The UV/vis spectrum of complex I at −100 mV is the same as obtained with partly reoxidized complex I, as described above. From electrochemical titration, a midpoint potential of –80 mV can be deduced for redox group X. Therefore, it fills the gap between the redox potentials of cluster N2 and ubiquinone. That redox group X is involved in electron transfer from cluster N2 to ubiquinone is further supported by the fact that its reduction is extremely slow in mutants lacking cluster N2 (M. Duarte, 2000).

THE ASSEMBLY PATHWAY

Complex I is assembled stepwise from different building blocks. The current model of the assembly

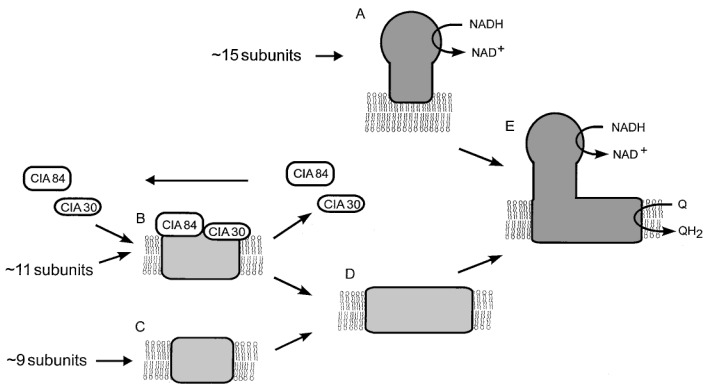

Fig. 1. Assembly pathway of complex I. Four assembly intermediates of complex I have been identified and are proposed to join stepwise to form mature complex I. The CIA proteins are associated with the large membrane arm intermediate. They are set free in the assembly process to participate in the formation of another intermediate. (A) Peripheral arm; (B) large membrane arm intermediate; (C) small membrane arm intermediate; (D) membrane arm; (E) mature complex I.

pathway is based on pulse-chase labeling of assembly intermediates in wild-type *N. crassa* and the characterization of incompletely assembled subcomplexes in *N. crassa* mutants (Tuschen *et al.*, 1990, Nehls *et al.*, 1992, Küffner *et al.*, 1998; Schulte and Weiss, 1999) (Fig. 1). After pulse labeling of wild-type *N. crassa*, it takes about 1 h for all the labeled subunits to appear in the mature complex (Videira and Werner, 1989). Transiently labeled assembly intermediates can be immunoprecipitated from solubilized, size-fractionated membranes after shorter periods of incorporation of radioactive label. In this manner, it was revealed that the peripheral arm and the membrane arm are independently formed. Both were shown to be assembly intermediates by their transient labeling (Tuschen *et al.*, 1990). When formation of the membrane arm is retarded by chloramphenicol, which inhibits synthesis of the mitochondrially encoded subunits, the excess peripheral arm is accumulated in the mitochondria (Friedrich *et al.*, 1989). Likewise, the membrane arm is accumulated in *N. crassa* grown under manganese limitation, which specifically affects formation of the peripheral arm (Schmidt *et al.*, 1992).

A complete block in the assembly pathway is caused by the lack of single subunits in mutants obtained by gene disruption (Nehls *et al.*, 1992; Duarte *et al.*, 1995). Loss of subunits of the peripheral arm gives rise to different phenotypes. In some mutants a complex I lacking only that single subunit is assembled. If, however, the peripheral arm lacking a certain subunit is unstable, the membrane arm is accumulated (Schulte *et al.*, 1994). Incompletely assembled parts of the peripheral arm have not been identified. The only subunit of the peripheral arm affecting the assembly of the membrane arm is the acyl carrier subunit. Loss of this subunit not only prevents formation of the peripheral arm, but impairs assembly of the membrane arm as well (Schneider *et al.*, 1995).

Mutants lacking a subunit of the membrane arm accumulate the peripheral arm and two different assembly intermediates of the membrane arm (Nehls *et al.*, 1992). The so-called large and small membrane arm intermediates are complementary to each other with regard to subunit composition. The small membrane arm intermediate is composed of two mitochondrially coded subunits and three nuclear encoded subunits. The large membrane arm assembly intermediate comprises five mitochondrially coded subunits and six nuclear-coded subunits. In addition, this intermediate is associated with two proteins not found in mature complex I. These are called complex I intermediate associated proteins CIA84 and CIA30, according to their location and molecular mass (Küffner *et al.*, 1998).

THE CIA PROTEINS

We have cloned the genes of the CIA proteins from *N. crassa*. The deduced amino acid sequences reveal globular proteins preceded by a typical import sequence of 12 amino acids for CIA30 and 24 amino acids for CIA84 (Küffner *et al.*, 1998). Sequences homologous to CIA30 are found in the genomes of animals and plants (Fig. 2). However, no homology is found to known proteins. Sequence identity between different CIA proteins ranges between 21 and 38%. Similarity searches also reveal a relationship to three hypothetical proteins deduced

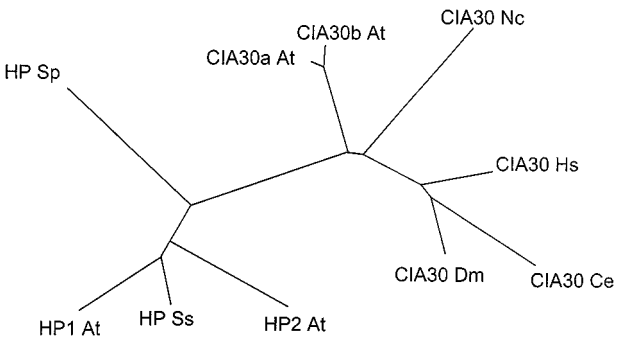

Fig. 2. Phylogenetic relationship of protein sequences related to CIA30. Sequences related to the CIA30 protein from *N. crassa* (CIA30 Nc) were retrieved from GenBank by similarity search (PSI-BLAST). Except for CIA30 Nc, all identified proteins are hypothetical. Sequences were aligned by Clustal W (Thompson *et al.*, 1994) and a tree was built by Phylip (Felsenstein, 1989). The closest relatives to CIA30 Nc were termed CIA30 tentatively. At, *A. thaliana*; Ce, *C. elegans*; Dm, *D. melanogaster*; Hs, *Homo sapiens*; Sp, *S. pombe*; Ss, *Synechocystis* sp. Accession numbers are: CIA30a At, AAF27148; CIA30b At, AAF97305; CIA30 Ce, CAB01129 (38.8 kDa protein C50B8.3); CIA30 Dm, AAF56928.1; CIA30 Hs, AF151823 (CGI-65); HP Sp, T39222; HP1 At, T04873; HP2 At, AAG09086; HP Ss, S76701.

from genomic sequences of *Arabidopsis thaliana* and *Synechocystis* sp. These proteins are considerably larger than the CIA proteins due to an additional N-terminal domain, which holds a typical NAD(P)H binding site. They belong to a reductase/isomerase family of proteins. Another member of this family is the 39/40-kDa subunit of complex I (see below). The CIA30 proteins show no significant sequence similarity to other members of the reductase/isomerase family. Distantly related to the CIA30 proteins is a hypothetical protein deduced from the genome of *Schizosaccharomyces pombe* (Fig. 2). No similar protein sequence is found in the genome of *Saccharomyces cerevisiae.* Both yeasts lack complex I (Nosek and Fukuhara, 1994; Büschges *et al.*, 1994) and, therefore, apparently do not need CIA proteins. Homologs to CIA84 are not readily identifiable in the genomes of animals and plants.

Neurospora crassa mutants were constructed by replacing the wild-type *cia* genes with defective copies. The phenotype of both mutants resembles a defect in a gene coding for a subunit of the large membrane arm intermediate. Complex I assembly is blocked. The peripheral arm and the small membrane arm intermediate accumulate (Küffner *et al.*, 1998).

The CIA proteins are tightly and specifically bound to the large membrane arm intermediate. Antisera raised against the two proteins immunoprecipitate the entire intermediate. No other assembly intermediates or mature complex I or any other mitochondrial proteins are coprecipitated. Characterization of the *cia* mutants does not indicate any other target for the CIA proteins as well. Complex I is the only respiratory complex affected in the mutants. The CIA proteins are found bound to the assembly intermediate and also as free unbound proteins. In wild type, roughly equal amounts of CIA proteins are present in both states. In a mutant lacking a subunit of the small membrane arm, intermediate assembly of the entire membrane arm is blocked and the large intermediate accumulates. This results in a shift of most of the CIA proteins from the free to the bound form. Similarly, exclusively free CIA proteins are found in mutants, which can not assemble a stable large membrane arm intermediate.

In pulse-labeling experiments, the labeling of the CIA proteins differs significantly from the labeling kinetics of complex I subunits. Added radioactive amino acids are incorporated into newly synthesized subunits, which together form assembly intermediates and, finally, mature complex I in the course of assembly. Radioactive amino acids are rapidly metabolized in the cells. Therefore, after the pulse phase, only nonradioactive subunits are synthesized. As the assembly intermediates consisting of labeled subunits form in the assembly process, they are replaced by nonlabeled intermediates resulting in a transient labeling. However, the CIA proteins bound to the intermediate are permanently labeled, which shows that the CIA proteins cycle continuously between the bound and the free state and thus participate in many assemblies (Küffner *et al.*, 1998).

The exact function of the CIA proteins is not yet known. Both proteins are essential and most likely specific for complex I assembly. Loss of either CIA protein leads to a complete block of the assembly. As they are transiently associated with an assembly intermediate and are not part of the mature structure, the CIA proteins would fit the definition of chaperones (Ellis and van der Vlies, 1991). As depicted in Fig. 1, the CIA proteins are integrated into the large membrane arm intermediate during an early stage of complex I assembly. They are set free as the assembly process proceeds in the formation of the membrane arm to become involved in the assembly of the next membrane arm. They might be needed to keep the large membrane arm intermediate in an assembly-competent state preventing aggregation or misfolding. However, a specific function in the maturation of the membrane arm could be envisioned as well.

Homologs to the CIA proteins of *N. crassa* have been identified so far only in eukaryotes. Although significantly smaller, the prokaryotic complex is considered to be structurally and functionally closely related to the eukaryotic complex (Friedrich *et al.*, 1995; Guénebaut *et al.*, 1998). Nevertheless, prokaryotes either employ different

proteins in the assembly of complex I or differences in the assembly pathway make CIA protein obsolete in prokaryotes. Since all subunits of the prokaryotic complex are expressed in the same compartment, in contrast to the eukaryotic complex I, differences in the assembly pathway could be conceivable.

THE 39/40-kDa SUBUNIT

Eukaryotic complex I has two well-defined binding sites for NAD(P)H. One binding site is provided by the 51-kDa subunit. It is the NADH oxidation site functioning as the electron input device. This binding site is present in prokaryotic complex I as well. The other binding site is confined to eukaryotic complex I. It is provided by the 39-kDa (40 kDa in *N. crassa*) subunit, which is a so-called accessory subunit. Other subunits of complex I have been labeled with radioactive NAD and analogs thereof (Yamaguchi *et al.*, 2000). The significance of these interactions are still unresolved. The 39-kDa subunit of bovine complex I is labeled only by [^{32}P]NADPH. The labeling required a partial relaxation of the structure by high concentrations of detergent. It was also shown that this subunit showed increased resistance to degradation by trypsin in the presence of NADPH, but not NAD(H) (Yamaguchi *et al.*, 1998). Isolated complex I of *N. crassa* holds a tightly bound NADPH. It is shielded from the other redox groups and it is not involved in respiratory electron transfer. In an *N. crassa* mutant lacking the 40-kDa subunit, a complex I without this subunit is assembled, which also lacks the bound NADPH. A mutant complex lacking the 51-kDa subunit still binds NADPH. It was concluded, therefore, that NADPH is bound to the nucleotide-binding site of the 40-kDa subunit (Schulte *et al.*, 1999).

The primary structure of the 39/40-kDa subunit reveals a typical adenine-binding pocket indicative of an NAD(P)H-binding site (Wierenga *et al.*, 1985; Fearnley and Walker, 1992). Nine to ten of 11 conserved sequence positions, including three prominent glycines, are found near the N-terminus. Among the large number of NAD(P)H-binding proteins, the 39/40-kDa subunit has its closest relatives among a reductase/isomerase family of proteins (Baker *et al.*, 1999; Schulte *et al.*, 1999). The members of this family share not only common sequence features (Baker and Blasco, 1992), but also similarities in the three-dimensional structure (Holm *et al.*, 1994). Known members include hydroxysteroid dehydrogenases, cholesterol dehydrogenases, dihydroflavonol reductases, nucleotide sugar epimerases, the 39/40-kDa subunits of the mitochondrial complex I, isoflavone reductases, and closely related putative proteins encoded in plants and cyanobacteria. On a wider scale, the reductase/isomerase family is part of the extended family of short-chain dehydrogenases/reductases SDR (Jörnvall *et al.*, 1995). The reductase/isomerase family comprises different biosynthetic enzymes participating in very divergent pathways. Shared by all enzymes is the use of NAD(H) or NADP(H) as cofactor. Most enzymes convert a keto group into a hydroxyl group or vice versa. In the epimerases, the intermediary redox reactions lead to a change in the configuration of the substrate. Isoflavone reductases catalyze the reduction of a double bond. Substrates are mostly polycyclic compounds, which have been synthesized from precursors bound to coenzyme A.

Although the NADPH bound by the 39/40-kDa subunit is not involved in respiratory electron transfer, lack of this subunit leads to inactivation of the respiratory activity of complex I. The complex I isolated from the *N. crassa* mutant lacking this subunit shows the same affinity for NADH and the same NADH/ferricyanide redox activity as the complex I isolated from wild type. The mutant complex is, however, unable to transfer electrons from NADH to quinones. All Fe–S clusters of mutant complex I are readily reducible by stoichiometric amounts of NADH. Not detectable, however, is the redox group X (Schulte *et al.*, 1999). It is not possible to obtain its typical UV/vis difference spectrum even by reduction with dithionite. Apparently this redox group is either absent in the mutant complex or has grossly altered redox properties, thus causing the block in electron transfer.

Being part of the reductase/isomerase family of biosynthetic enzymes is suggestive of an involvement of the 39/40-kDa subunit in the biosynthesis of the postulated redox group. The tight binding of the NADPH would indicate an isomerase activity involving a reduction of the substrate followed by its immediate reoxidation. Thus, no exchange of the cofactor would be necessary. The isomerization might take place once in the biosynthesis of redox group X or repeatedly after the synthesis of the group has been completed to keep it in an active state. In either case, the lack of the 39/40-kDa subunit would result in an inactive or unstable form of the redox group, thus causing a block in electron transfer activity.

FUNCTION OF MITOCHONDRIAL ACYL CARRIER PROTEINS

Mitochondria, like plastids, contain enzymes of a prokaryotic-type fatty acid synthetase (Schneider *et al.*, 1997). These include a mitochondrial ACP (mtACP), a mitochondrial condensing enzyme (CEM), and mitochondrial oxoacyl ACP reductase and acetyl CoA carboxylase. While the plastidal fatty acid synthetase is responsible for general fatty acid synthesis in plants, a cytosolic fatty acid

synthetase synthesizes fatty acids in animals and fungi. The mitochondrial fatty acid synthetase (mtFAS) is not involved in bulk fatty acid synthesis. Mitochondria import phospholipids from the endoplasmic reticulum and long-chain fatty acids for cardiolipin synthesis are imported from the cytosol (Daum, 1985; Voelker, 1991). A major function of mtFAS is the synthesis of octanoic acid as a precursor for lipoic acid. Disruption of either the *mtACP* gene or the *CEM* gene in *S. cerevisiae* resulted in a pleiotropic respiratory-deficient *pet* phenotype. The mutants are unable to grow on nonfermentable substrates and the mitochondria are essentially devoid of cytochromes. In addition, both mutants are devoid of lipoic acid (Harington *et al.*, 1993; Brody *et al.*, 1997).

With respect to the mtACP, *S. cerevisiae* and other yeasts represent a special case, because in other eukaryotes the mtACP is a subunit of complex I (Runswick *et al.*, 1991; Sackmann *et al.*, 1991). Fermentative yeasts lack a respiratory complex I, but still need a mtACP. Disruption of the *mtacp* gene in *N. crassa* results in complex I deficiency (see below), but has no effect on lipoic acid synthesis (J. Hennemann, 1999). In contrast, CEM appears to be an essential protein in *N. crassa*. Disruption of the *cem* gene in *N. crassa* did not yield viable, homokaryotic progeny (F. Bürger, 2000). How can these confusing data be assembled. *Neurospora crassa* might be able to import octanoic acid into the mitochondria and, therefore, would not need a mtACP for lipoic acid synthesis. The need for CEM in *N. crassa*, however, resembles the *pet* phenotype of the respective yeast mutant. While deficiency of lipoic acid leads to a *pet* phenotype in the facultative anaerobic yeast, such a deficiency would be lethal in obligate aerobic filamentous fungi. Assuming the mtFAS is needed for lipoic acid synthesis in *N. crassa* as well as in *S. cerevisiae*, the presence of more than one mtACP has to be postulated for *N. crassa*.

Data supporting the presence of multiple mtACPs in most eukaryotes can be drawn from sequence as well as biochemical data. Starting with known mtACPs, homologous sequences were searched in available genomic data bases. Several eukaryotic genomes have been completely sequenced and, therefore, should reveal the counterparts of mtACPs in these organisms. As expected, in the genomes of the yeasts *S. cerevisiae* and *S. pombe* only one *mtACP* gene is present as these organisms are devoid of complex I. The genome of *Caenorhabditis elegans* holds two putative *acp* genes. The deduced amino acid sequences of both show typical features of an N-terminal mitochondrial import sequence and strong similarity to other mtACPs (Fig. 3). Search for *mtacp* genes in *A. thaliana* has to take into account the presence of the evolutionary related plastidal ACPs. Several ACPs were found in the genome

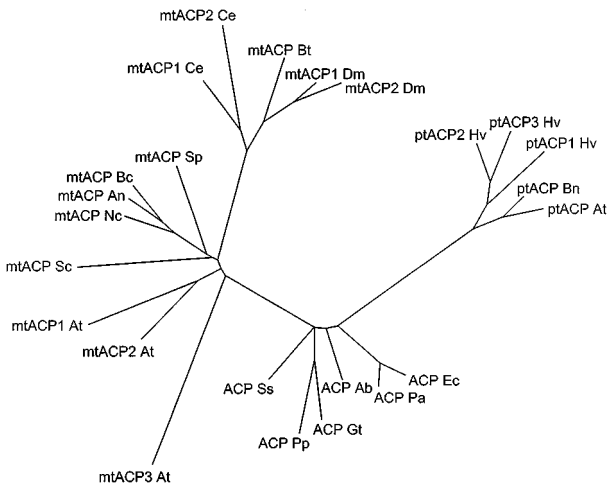

Fig. 3. Phylogenetic relationships of acyl carrier proteins (mtACPs). As described in the legend to Fig. 2, a tree was built based on the sequences of mtACPs and putative mtACPs derived from genomic sequence. For comparison, the sequences of a few arbitrarily chosen plastidal (ptACP) and prokaryotic acyl carrier proteins (ACP) were included in the analysis. Bt, *Bos taurus*; Sc, *S. cerevisiae*; An, *A. niger*; Bc, *Botrydis cinereus*; Bn, *Brassica napus*; Ec, *E. coli*; Hv, *Hordeum vulgare*; other abbreviations as in legend to Fig. 3. Accession numbers are: ptACP1 Hv, AAA32923; ptACP2 Hv, AAA32921; ptACP3 Hv, AAA32924; ptACP Bn, CAA31519; ptACP At, L14814; ACP Ec, AAC74178; ACP Pa, AAB94392; ACP Ab, CAA57794; ACP Gt, AAC35700; ACP Pp, AAC08166; ACP Ss, BAA17312; mtACP3 At, AAC27139; mtACP2 At, AAC27464; mtACP1 At, AAB96840; mtACP Sc, CAA82036; mtACP Nc, P11943; mtACP Bc, AL116367; mtACP Sp, CAA93348; mtACP1 Ce, CAB60498; mtACP2 Ce, AAB94237; mtACP1 Dm, AAF47480; mtACP2 Dm, AAF47479; mtACP Bt, CAA43970.

of *A. thaliana*. Three of these are closely related to other mtACPs. In the genome of *Drosophila melanogaster*, only one sequence of a mtACP is found. However, expression of this gene leads to two different mtACPs due to differential splicing (Ragone *et al.*, 1999). So far only one *mtacp* gene is known from *N. crassa*, *Aspergillus niger*, *Botrytis cinerea*, bovine, and man. As the genomes of these organisms are not yet completely available, the number of mtACPs is still unknown in these cases. A second mtACP in mitochondria of *N. crassa* has been shown by labeling with radioactive pantothenate (J. Hennemann, 1999). Because only small amounts of this mtACP are present in *N. crassa* mitochondria compared to the complex I ACP and because both proteins comigrate in SDS–PAGE, the two mtACPs cannot be distinguished in wild type. However, in the *N. crassa* mutant lacking the gene for the complex I ACP, the other mtACP can still be labeled by pantothenate. Combined, these data show that at least two mtACPs exist in most eukaryotes. One mtACP, most likely dispersed freely in the matrix, is needed for mitochondrial lipoic acid synthesis. Another mtACP is integrated as a subunit

into complex I. The yeasts, having no complex I, lack the latter mtACP.

While a clear cut function can be attributed to the matrix mtACP, the function of the complex I ACP is still enigmatic. It is clear that this subunit is not bound to complex I accidentally in need of a membrane anchor, but that it is essential for complex I. Loss of the subunit in the *N. crassa* deletion mutant has a major impact on the assembly of complex I. Without the ACP, the peripheral arm of complex I cannot be assembled at all. Although it is one of the smallest subunits, this might be caused by a structural instability. However, assembly of the membrane arm is affected as well (Schneider *et al.*, 1995). As mentioned above, this is very unusual for a subunit of the peripheral arm. The exact defect in the assembly of the membrane arm is difficult to define. It is not a complete block at a certain stage of assembly, but seems to lead to a decreased stability of the assembled membrane arm. The large and the small assembly intermediate are both accumulated in the mutant mitochondria. Both intermediates join to some extent, to form the entire membrane arm. Compared to other mutants lacking a peripheral arm, only small amounts of membrane arm are accumulated. An excess of mitochondrially encoded subunits is found aggregated after solubulization of mutant mitochondrial membranes. The pantothenate of the mitochondrial ACP from *N. crassa* is predominantly loaded with hydroxymyristate and shorter hydroxy acids, e.g., hydroxylaureate (Mikolajczyk and Brody, 1990; M. Massow, 1996). A single acyl group yielding a mass difference of 302 between acylated and deacylated ACP has been reported for the ACP from bovine complex I (Runswick *et al.*, 1991).

It is tempting, of course, to speculate about an interplay between the "biosynthetic" subunits of complex I, the 39/40-kDa subunit, and the ACP. Many relatives of the 39/40-kDa subunit act on acyl compounds bound to a phosphopantetheine. It is, therefore, conceivable to assume that the substrate of the 39/40-kDa subunit is bound to the phosphopantetheine of the ACP. As discussed above, the 39/40-kDa subunit might be involved in the biosynthesis or maintenance of the redox group X. That would imply an involvement of the ACP in this process as well. The redox group X as electron donor for ubiquinone should be located in or close to the membrane arm. Thus, by its involvement in the formation of redox group X, the ACP would have an impact on membrane arm assembly as well. Thus far, these speculations have not triggered experimental approaches proving or disproving the connection between the "biosynthetic" subunits and redox group X. The uncertainty about whether the molecular nature of the redox group X has especially hampered experimental progress. It is, for example, still unclear how the short-chain hydroxylated fatty acids found bound to isolated ACP would be linked to the function of this subunit. It has been reported that ND5, a mitochondrially coded subunit of complex I, is myristoylated (Plesofsky *et al.*, 2000). Whether complex I ACP is involved in this modification is unknown. The subunits were labeled *in vivo* by added radioactive myristic acid, a process which does not involve fatty acid synthesis, and therefore would not need an ACP.

Much hope is currently placed on the elucidation of the high-resolution three-dimensional structure of complex I. With so many question marks concerning the function of the membrane arm and the mechanism of proton translocation in complex I, the structure is desperately needed and will certainly provide new impetus to the entire field, as well as the progress in understanding the biogenesis of complex I.

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