Mitochondrial-type assembly of FeS centers in the hydrogenosomes of the amitochondriate eukaryote *Trichomonas vaginalis*

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Mitochondria are the site of assembly of FeS centers of mitochondrial and cytosolic FeS proteins. Various microaerophilic or anaerobic unicellular eukaryotes lack typical mitochondria (“amitochondriate” protists). In some of these organisms, a metabolically different organelle, the hydrogenosome, is found, which is thought to derive from the same proteobacterial ancestor as mitochondria. Here, we show that hydrogenosomes of *Trichomonas vaginalis*, a human genitourinary parasite, contain a key enzyme of FeS center biosynthesis, cysteine desulfurase (*TviscS*-2), which is phylogenetically related to its mitochondrial homologs. Hydrogenosomes catalyze the enzymatic assembly and insertion of FeS centers into apoproteins, as shown by the reconstruction of the apoform of [2Fe-2S]ferredoxin and the incorporation of 35S from labeled cysteine. Our results indicate that the biosynthesis of FeS proteins is performed by a homologous system in mitochondrial and amitochondriate eukaryotes and that this process is inherited from the proteobacterial ancestor of mitochondria.

The human genitourinary parasite, *Trichomonas vaginalis*, and other parabasalid protists display metabolic peculiarities. A striking deviation from “typical” eukaryotes is the lack of mitochondria and the presence of another organelle, the hydrogenosome (1–4). This double-membrane-bound organelle differs from mitochondria in its lack of DNA (5) and many metabolic characteristics (1, 6). Hydrogenosomes oxidize pyruvate or malate to acetate and, in the absence of alternative electron acceptors, to molecular hydrogen. Proteins of this pathway are pyruvate/ferredoxin oxidoreductase, [2Fe-2S]ferredoxin and [Fe]hydrogenase, all of which contain FeS centers. The oxidation of pyruvate and malate produces acetyl-CoA with the energy of the thioester bond used for substrate level phosphorylation of ADP to ATP (1). Hydrogenosomes do not contain pyruvate dehydrogenase complex, tricarboxylic acid cycle, cytochrome-mediated electron transport chain, cytochrome oxidase, or F1F0-ATPase, which are characteristic components of classical mitochondria. The origin of hydrogenosomes is much debated. The absence of DNA from parabasalid hydrogenosomes deprives us of the clues that an organellar genome could provide in resolving this question. Nevertheless, increasing biochemical and phylogenetic evidence supports the notion that hydrogenosomes derive from a common ancestor with typical mitochondria (2, 3, 7).

Similar to most mitochondrial proteins, hydrogenosomal proteins are coded by nuclear genes and posttranslationally translocated into the organelles (8, 9). Protein import is directed by short targeting sequences at the N terminus of the nascent proteins, and all examined soluble hydrogenosomal proteins possess such sequences (2). It is reasonable to expect that the maturation of the FeS proteins is completed within the hydrogenosomes by the insertion of FeS centers, as it is in mitochondria (10, 11); however, this process has not yet been studied, to our knowledge, in hydrogenosomes.

Proteins involved in FeS cluster assembly within mitochondria are homologs of bacterial proteins belonging to the iron–sulfur cluster (ISC) assembly system (12). Isc orthologs have been identified in all eukaryotes, and their products are generally detected in mitochondria (13–15); however, in some cell types, they are also found in the cytosol and the nucleus (15–18). In *Saccharomyces cerevisiae*, the mitochondrial ISC-like machinery plays an essential role in the maturation of mitochondrial and extramitochondrial FeS proteins (11). The role of the cytosolic and nuclear components remains to be clarified (15–18). In addition to the ISC system, two FeS biosynthetic systems occur in bacteria, the NIF (nitrogen fixation) system that is involved in the maturation of proteins of the nitrogen-fixing machinery (19) and the SUP (mobilization of sulfur) system (20). Homologs of *suf* genes occur also in plastid-bearing eukaryotes where they are involved in the biosynthesis of FeS proteins of plastids (21).

A model for FeS cluster formation in mitochondrion was proposed based on studies of yeast (22). The clusters are first transiently assembled on Isu1p and/or Isu2p proteins (homologs of bacterial *IscU* protein), which serve as a scaffold. S, as a protein associated persulfide, is provided from L-cysteine by pyridoxal-5-phosphate-dependent cysteine desulfurase Nfs1p (a homolog of bacterial *IscS* protein). The source of Fe is not known; however, frataxin has been suggested to be involved in Fe loading of the Isu proteins. The mitochondrial [2Fe-2S]ferredoxin Yah1p and NADH/ferredoxin reductase Arh1p were suggested to provide electrons for a critical step in the process. Subsequently, the assembled FeS clusters are transferred to the catalytic centers of FeS proteins with the possible participation of a mitochondrial chaperone system (22, 23).

The same mechanism is assumed to function in other eukaryotes (11). Detection of two genes in *T. vaginalis* coding for *Isc* homologs with putative hydrogenosomal import signals suggested that FeS-center assembly in hydrogenosomes might be homologous to the mitochondrial system (24). The clustering of the derived amino acid sequences with mitochondrial homologs in phylogenetic reconstructions strengthened this notion further (24, 25). Here, we show that one of these genes (*tviscS*-2) is expressed, translocated to hydrogenosomes, and involved in the biosynthesis of FeS proteins. Also, we show that in *T. vaginalis*, hydrogenosomes are the site of FeS center biosynthesis. These...
findings reveal a major functional similarity of typical mitochondria and parabasalid hydrogenosomes.

Materials and Methods

Organisms, Cultivation, and Cell Fractionation. T. vaginalis strain C-1:NIH (30001; American Type Culture Collection) was used throughout this study. The organisms were maintained in TYM medium with 10% heat-inactivated horse serum (pH 6.2) at 37°C. To study gene transcription under Fe-rich or Fe-restricted conditions, the medium was supplemented with 100 μM Fe-nitritotriacetate or 50 μM 2,2-dipyridyl. S. cerevisiae YPH499 (204679; American Type Culture Collection) was grown in a rich medium containing 1% yeast extract, 2% peptone, 0.01% adenine sulfate, and 2% raffinose. Preparation of subcellular (cytosolic, large granule, and hydrogenosomal) fractions of T. vaginalis is described in Supporting Materials and Methods, which is published as supporting information on the PNAS web site. Yeast mitochondria were obtained according to ref. 26.

RNA Synthesis in Permeabilized Cells and RT-PCR. Synthesis of nascent mRNA was assessed in lysolcithin-permeabilized trichomonal cells (27). The primers used for PCR amplification of specific DNA probes, as well as the conditions and primers used in RT-PCR, are given in Supporting Materials and Methods.

Analysis of 5′-UTRs. Clones containing complete genes coding for IscS-1 and IscS-2 were obtained by screening of a genomic DNA library, as described in ref. 24. The 5′-UTRs of the genes were sequenced by primer walking.

IscS Expression and Ab Generation. The complete IscS-2 ORF was inserted into the expression vector pQE30 (Qiagen, Valencia, CA). The 6×His-tagged recombinant protein was expressed in Escherichia coli and purified under denaturing conditions on Ni(II)-nitrilotriacetate resin according to the manufacturer’s protocol (Qiagen). A rabbit polyclonal Ab was raised against the sequence of TviscS-2-(HA)2 plasmid (Lampire Biological Laboratories, Pipersville, PA).

Selecteable Transformation of T. vaginalis. TviscS-2-(HA)2 plasmid was constructed as follows. TviscS-2 ORF was amplified by PCR and introduced into a plasmid with a hemagglutinin tag at the 3′ end, which carried a neomycin (neo) phosphotransferase cassette (28). T. vaginalis (∼2.5 × 106) were electroporated with 50 mg of plasmid DNA. Cells containing the plasmid were selected with 200 mg/ml of G418 (Invitrogen).

The [2Fe-2S] Ferredoxins. An expression vector containing the coding sequence of T. vaginalis ferredoxin (pET-3aTvFd) was kindly provided by M. S. Vidakovic and J. P. Germanas (University of Houston, Houston, TX). The vector was transfected into E. coli, and the expressed ferredoxin was purified as described in Supporting Materials and Methods. Spinach ferredoxin was purchased from Sigma. Apoferrodoxin were prepared by acidifying holoferrodoxins with 0.5 M HCl in the presence of 100 mM dithiothreitol for 10 min on ice. Subsequently, the protein solutions were neutralized to pH 7.5 by addition of 1 M Tris base.

Reconstitution of [FeS] Clusters. The standard reaction mixture contained 30–100 μg of organellar protein, 10 μg of apoferrodoxin, 20 mM dithiothreitol, 0.5% Triton X-100, 50 μM ferrous ascorbate, 25 μM L-cysteine, 10 μCl (1 Ci = 37 GBq) of 35S-L-cysteine, and 20 mM Hepes (pH 8.0). Dependance of the reaction on Fe availability was tested by addition of ferrous ascorbate or the Fe chelators 2,2 dipiridyl and EDTA to the reaction mixture before incubation. The reaction took place in an anaerobic jar containing palladium catalyst under an atmosphere of 95% N2/5% H2 for 60 min at 25°C and was terminated by adding 5 mM EDTA. In controls, no organellar extract was added. Unincorporated radioactivity was removed by gel filtration. The samples were separated on nondenaturing 15% polyacrylamide gels at 4°C. Radioactivity on the vacuum-dried gels was detected by phosphorimaging. In the time-course experiments, the bands corresponding to ferredoxin were cut out of the gel and rehydrated in 0.1 M HCl and radioactivity associated with ferredoxin was quantified by liquid scintillation counting.

SDS/PAGE and Western Blotting. Proteins were analyzed according to standard protocols, as described in Supporting Materials and Methods. The primary Abs were rabbit α-TviscS-2 polyclonal Ab (described above) or mouse α-penta-His mAb (Qiagen).

Immunofluorescent Microscopy. The hydrogenosomal proteins, TviscS-2 and malic enzyme were visualized in fixed T. vaginalis cells with mouse α-hemagglutinin mAb and/or rabbit α-malic enzyme polyclonal Ab (29). Details of the Abs and procedures are given in Supporting Materials and Methods.

Results

Transcription of TviscS-2. The sequence upstream of the ATG translation initiation codon of the TviscS-1 and TviscS-2 genes were obtained and examined for the only known promoter in trichomons: the initiator (30, 31). The genes were found to contain at least one initiator motif immediately upstream of the ATG (Fig. L4). These elements have been shown to be essential for transcription of trichomonad genes (31). Monitoring mRNA synthesis in permeabilized cells, we detected transcription of the TviscS-2, but not the TviscS-1, gene. Because Fe stimulates transcription of genes encoding various hydrogenosomal proteins in trichomons (32), mRNA synthesis was compared under Fe-rich and Fe-restricted conditions. Transcription of TviscS-2 increased ∼5-fold under Fe-restricted conditions, whereas transcription of gene coding for PFOR decreased as expected (Fig. 1B). Based on the inability to detect TviscS-1 gene expression (Fig. 1B and C), TviscS-2 was selected for further analyses.

Cellular Localization of TviscS. TviscS proteins were not detected in Commassie blue-stained gels of cellular fractions, indicating their relatively low abundance (Fig. 2A). Western blotting using rabbit polyclonal Ab against recombinant TviscS-2 detected a single band corresponding to TviscS-2 in the cell homogenate.
and in Percoll-purified hydrogenosomes (Fig. 2B). The apparent molecular mass of TviscS-2 (45 kDa) agreed with the molecular weight of 44,800, calculated for the mature protein deduced from the gene sequence. The mobility of purified expression product was diminished slightly compared with the mature hydrogenosomal protein because of the presence of the tag and the N-terminal presequence (Fig. 2B).

Subsequently, tviscS-2 was overexpressed with a C-terminal hemagglutinin tag in T. vaginalis transformants to study the cellular localization of the gene product. Cell fractionation and Western blot analysis showed the presence of recombinant TviscS-2 in the sedimentable fraction that contains the hydrogenosomes (Fig. 2C). By means of immunofluorescence microscopy, an α-hemagglutinin epitope on the recombinant TviscS-2 was used to localize the protein in numerous organelles surrounding the nuclei and cytoskeletal structures (Fig. 3). Double labeling for TviscS-2 and NADH-dependent malic enzyme, a marker enzyme for hydrogenosomes (29), showed their colocalization, demonstrating that TviscS-2 is localized to hydrogenosomes. Although in yeast and human cells iscS gene products are also targeted to nucleus (15, 18), TviscS-2 was not detectable in the nuclei of T. vaginalis transformants (Fig. 3). Accordingly, the nuclear targeting motif RRRPR, which has been verified experimentally in yeast Nfs1p (18), is highly conserved in all mitochondrial homologues but is absent from trichomonad and Giardia intestinalis IscSs (Fig. 4B). However, we cannot exclude the possibility that undetectable levels of the T. vaginalis protein are contained in the nucleus.

Hydrogenosome-Dependent Reconstitution of [FeS] Clusters. [FeS] cluster formation was studied by following the incorporation of 35S from labeled cysteine into apoferredoxin in a system containing [35S]cysteine, hydrogenosomal apoferredoxin, and lysate of purified hydrogenosomes under reducing conditions. The reaction was terminated by the addition of 5 mM EDTA to remove unincorporated Fe. Holoferredoxin was separated electrophoretically on polyacrylamide gels under native conditions and processed for autoradiography, and the radioactivity associated with it was quantified. Hydrogenosomal holoferredoxin migrated in native gels as a distinct rapidly migrating band, with its apoform migrating considerably slower (Fig. 5A), as shown for the mitochondrial homolog (33).

The [FeS] cluster assembly on ferredoxin was linear for 60 min (Fig. 5B and C). The rate of [FeS]ferredoxin formation was 0.3 pmol of S per milligram of hydrogenosomes per minute. Dependence on concentrations of hydrogenosomal proteins showed that the activity corresponded to an enzyme catalyzed process (Fig. 6A). Negligible association of 35S with ferredoxin was observed when hydrogenosomal extract was omitted from the reaction mixture. The reaction was inhibited almost completely when incubated on ice.

We also tested the dependence of hydrogenosome-mediated
chelator EDTA (500 μM) present in hydrogenosomes, or contaminating buffers, by the metal ferrous ascorbate was added. Removal of endogenous Fe from three independent experiments in duplicate.

**Specificity of the FeS-Forming Activity in Hydrogenosomes and Mitochondria.** We compared FeS formation in isolated hydrogenosomes and yeast mitochondria by using [2Fe-2S] apoferredoxin from T. vaginalis and spinach. Both hydrogenosomal and mitochondrial extracts catalyzed [FeS] cluster formation in T. vaginalis ferredoxin (Fig. 7), but only hydrogenosomal lyase catalyzed cluster formation inspinach ferredoxin. This finding indicates a specificity of [FeS] cluster assembly machinery present in the two different organelles.

**Lack of in Vivo Transcription of TviscS-1.** As mentioned previously, the 5′-UTR sequence of the tviscS-1 gene contains two putative initiator promoter elements upstream of the ATG codon, indicating that this gene might be transcribed in vivo (Fig. 1A). Surprisingly, no corresponding transcripts could be detected by monitoring mRNA synthesis in permeabilized cells from Fe-rich or Fe-depleted cultures of T. vaginalis (Fig. 1B). This result was confirmed by RT-PCR (Fig. 1C). Although both TviscS sequences possess 8-aa N-terminal extensions that resemble the presequences that target the proteins to hydrogenosomes, the TviscS-1 extension lacks a typical cleavage site (Fig. 4A). Moreover, the C-terminal conserved [FeS] cluster assembly on the availability of divalent Fe. Hydrogenosomes contain high amounts of Fe associated with FeS proteins and an Fe pool of unknown molecular character that may represent intrahydrogenosomal Fe storage (34). Not surprisingly, [FeS] cluster formation was observed even when no Fe was added to the reaction (Fig. 6B). However, the signal increased several-fold when ferrous ascorbate was added. Removal of endogenous Fe present in hydrogenosomes, or contaminating buffers, by the metal chelator EDTA (500 μM) or the ferrous Fe chelator 2,2-dipyridyl (100 μM) abrogated the formation of holoferrredoxin.

![Fig. 5. Reconstitution of [FeS] clusters in T. vaginalis apoferredoxin. (A) Different migration of the apoforms and holoforms of T. vaginalis ferredoxin on native gels. Recombinant T. vaginalis holoferrredoxin purified from E. coli (lane 1) was treated with 0.5 M HCl to remove [FeS] clusters (lane 2). (B) Time-dependence of the [FeS] cluster reconstitution catalyzed by hydrogenosomal lyase. Apoferrredoxin was incubated with [35S]-cysteine, Fe-ascorbate, and hydrogenosomal lyase and analyzed by native gel electrophoresis, followed by autoradiography. (C) The bands corresponding to holoferrredoxin were cut out of the gel, and the associated radioactivity quantified by scintillation counting.](image)

![Fig. 6. Dependence of [FeS] cluster reconstitution in apoferredoxin on the concentrations of the hydrogenosomal lyase and Fe. (A) T. vaginalis apoferredoxin was incubated by using standard conditions with various amounts of the hydrogenosomal lyase. (B) Reconstitution of [FeS] clusters in the presence of 500 μM EDTA; 25 and 100 μM ferrous Fe chelator 2,2-dipyridyl; 25 and 100 μM Fe-ascorbate, in the presence of 100 μg of hydrogenosomal lyase. Formation of holoferrredoxin was analyzed by native gel electrophoresis, followed by storage phosphorimaging autoradiography. Incorporation of [35S] into ferredoxin was expressed in arbitrary units. Bars indicate SD, calculated from three independent experiments in duplicate.](image)
was demonstrated based on [FeS] cluster reconstitution in recombinations (24, 25). clade with its mitochondrial homologs in phylogenetic reassembly, could be localized in the hydrogenosomes. This study provides functional evidence that the site of FeS-center assembly in the “amitochondriate” eukaryote, T. vaginalis is the hydrogenosome and that the system performing this process is very similar to that found in typical mitochondria of S. cerevisiae (11, 22). A key enzyme of this process, cysteine desulfurase (TvIscS), as well as the process of FeS-center assembly, could be localized in the hydrogenosomes. TvIscS-2 also has an putative N-terminal targeting sequence similar to that of other hydrogenosomal proteins and is found in a well supported clade with its mitochondrial homologs in phylogenetic reconstructions (24, 25).

Hydrogenosome-mediated formation of [FeS] clusters in vitro was demonstrated based on [FeS] cluster reconstitution in recombinant T. vaginalis apoferredoxin by using 35S-cysteine as a source of S under anaerobic conditions. Addition of ferrous Fe to the reaction mixture increased [FeS] cluster formation, whereas Fe chelators had an inhibitory effect. This observed in vitro assembly of [FeS] clusters extends our earlier observations that addition of 59Fe to live trichomonads results in fast accumulation of Fe in hydrogenosomes and its incorporation into [FeS] clusters of hydrogenosomal ferredoxin (34). Although further functional similarities between the hydrogenosomal and the mitochondrial system can be expected, the extent of these similarities remains to be established. One interesting difference is that the cysteine desulfurase of T. vaginalis appears to be localized only in the hydrogenosomes, in contrast to its homologs in yeast and vertebrates, which are also detected in the cytosol and nucleus (15, 18). This observation may be explained by the absence of a nuclear localization motif from the T. vaginalis protein. Interactions among components of the [FeS] cluster assembly machinery and with target FeS apoproteins might differ in hydrogenosomes and mitochondria. Indeed, hydrogenosomal lysates mediate [FeS] cluster reconstitution in spinach apoferrredoxin, whereas yeast mitochondria did not support this reaction.

The FeS biosynthetic machineries of parabasalid hydrogenosomes and typical mitochondria are functionally and evolutionarily related. This idea is in agreement with the notion that these two organelles are two metabolically different endpoints of the divergent evolution of the protomitochondrion that arose during eukaryogenesis (3, 7). Support for this notion has been adduced from similarities of the biogenesis of these two organelles (2, 9, 36) and from the phylogenetic relationships of some of their protein components (24, 37–39).

Hydrogenosomes of T. vaginalis and other parabasalids are, however, only one of the diverse organelles that are assumed to be descendants of the proteobacterial component of the ancestral eukaryote (4). A comparative study of protists reveals that double-membrane-bound organelles of possible shared ancestry are present in different species, ranging from typical mitochondria, hydrogenosomes (1), to mitosomes (cryptons), which are smaller structures with no demonstrated role in core carbohydrate metabolism (40, 41). The taxonomic distribution of these organelles indicates that they may have arisen independently in different lineages several times from the ancestral protomitochondrion (3, 4, 42).

The results of this study and our earlier work (24) raise the question whether the double-membrane-bound organelles of other eukaryotes without typical mitochondria are also involved in FeS biosynthesis. Although the data are scanty, the answer is probably yes. In all of the amitochondriate organisms studied so far, a gene encoding an IscS homolog has been recognized and found to be part of the clade formed by typical mitochondrial homologs (24, 25, 43). It should be stressed that the studied amitochondriate groups belong to separate taxonomic lineages. Additional observations support this conclusion. The diplomonad G. intestinalis deserves special mention. In this species, FeS biosynthesis and its two components have been shown to be compartmentalized, giving cytological evidence for the existence of a possible mitochondrion derivative in this group (41). In Cryptosporidium parvum, an apicomplexan without typical mitochondria, the genes encoding several proteins involved in [FeS] cluster assembly have been identified in its genome. All of them possess N-terminal presequences similar to mitochondrial targeting sequences, suggesting that they are localized in the relic mitochondria described (43, 44) in this organism. A further example is provided by the group of microsporidia, which are highly reduced fungi adopted to intracellular parasitism. These organisms were regarded for some time as the best example of the amitochondriate phenotype (45, 46). However, a possible mitochondrial remnant has been described also in the microsporidian Trachipleistophora hominis (47), and several genes of mitochondrial origin are present in the genome of another microsporidian Encephalitozoon cuniculi. Most of these proteins belong to the [FeS] cluster assembly machinery (48). It is tempting to speculate that [FeS] cluster assembly is the main function retained by mitochondrion-related organelles without known metabolic functions (relic mitochondria and mitosomes) (40, 43, 47, 48). The evolutionary reason for retaining such organelles could be the need to compartmentalize toxic ferrous ions and sulfide, which are required for FeS biogenesis. Moreover, amitochondriate organisms living under anaerobic or oxygen-poor environments have considerably higher nutritional requirements for Fe than aerobes (49, 50). In hydrogenosomes, we found 54 nmol of Fe per 1 mg of protein, which is a 10- to 100-fold higher concentration than in yeast mitochondria (34). Thus, compartmentation of Fe metabolism could be an essential strategy for these organisms.

In conclusion, our data indicate that hydrogenosomes of the amitochondriate protist T. vaginalis are the site of FeS biosynthesis, which is a typically mitochondrial process. These findings underscore the notion that mitochondria and hydrogenosomes are closely related organelles with a common ancestry. Additional data on other amitochondriate organisms indicate that their FeS biosynthesis is similar to that seen in mitochondria and parabasalid hydrogenosomes, suggesting that this process is a common property of all double-membrane-bound organelles that arose from this endosymbiotic event. Clearly, further data are needed to test this proposal.

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