

Protein Import into Hydrogenosomes of *Trichomonas vaginalis* Involves both N-Terminal and Internal Targeting Signals: a Case Study of Thioredoxin Reductases^{∇†}

Marek Mentel,[‡] Verena Zimorski, Patrick Haferkamp,[§] William Martin, and Katrin Henze*

Institute of Botany III, University of Duesseldorf, D-40225 Duesseldorf, Germany

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The parabasal flagellate *Trichomonas vaginalis* harbors mitochondrion-related and H₂-producing organelles of anaerobic ATP synthesis, called hydrogenosomes, which harbor oxygen-sensitive enzymes essential to its pyruvate metabolism. In the human urogenital tract, however, *T. vaginalis* is regularly exposed to low oxygen concentrations and therefore must possess antioxidant systems protecting the organellar environment against the detrimental effects of molecular oxygen and reactive oxygen species. We have identified two closely related hydrogenosomal thioredoxin reductases (TrxRs), the hitherto-missing component of a thioredoxin-linked hydrogenosomal antioxidant system. One of the two hydrogenosomal targeting signals, TrxRh1, carried an N-terminal extension resembling known hydrogenosomal targeting signals. Expression of hemagglutinin-tagged TrxRh1 in transfected *T. vaginalis* cells revealed that its N-terminal extension was necessary to import the protein into the organelles. The second hydrogenosomal TrxR isoform, TrxRh2, had no N-terminal targeting signal but was nonetheless efficiently targeted to hydrogenosomes. N-terminal presequences from hydrogenosomal proteins with known processing sites, i.e., the alpha subunit of succinyl coenzyme A synthetase (SCS α) and pyruvate:ferredoxin oxidoreductase A, were investigated for their ability to direct mature TrxRh1 to hydrogenosomes. Neither presequence directed TrxRh1 to hydrogenosomes, indicating that neither extension is, by itself, sufficient for hydrogenosomal targeting. Moreover, SCS α lacking its N-terminal extension was efficiently imported into hydrogenosomes, indicating that this extension is not required for import of this major hydrogenosomal protein. The finding that some hydrogenosomal enzymes require N-terminal signals for import but that in others the N-terminal extension is not necessary for targeting indicates the presence of additional targeting signals within the mature subunits of several hydrogenosome-localized proteins.

The parabasal flagellate *Trichomonas vaginalis* is the causative agent of the most prevalent nonviral sexually transmitted disease in humans (34) and possesses hydrogenosomes, which are anaerobic forms of mitochondria (30, 34). Hydrogenosomes, like the mitochondria of aerobic eukaryotes, are involved in energy metabolism, but ATP is synthesized through substrate-level phosphorylation instead of oxidative phosphorylation (35). Although this fermentative energy metabolism in *T. vaginalis* hydrogenosomes relies on the highly oxygen-sensitive enzymes pyruvate:ferredoxin oxidoreductase (PFO) (53) and [Fe]-hydrogenase (39), *T. vaginalis* is frequently exposed to oxygen concentrations of up to 60 μ M in its natural habitat on the vaginal surface (52). Furthermore, exposure to low oxygen concentrations on the order of 0.25 μ M yields its maximum growth rates (40), owing to the ability of the organism to more rapidly establish a redox balance with NAD(P)H-oxidases. These enzymes have high activities and rapidly reduce O₂ to

water, thereby affording the organism anaerobic growth conditions (31, 50), whereby at O₂ concentrations in the range of 50 to 250 μ M, 5 to 10% of the O₂ consumed can be converted into H₂O₂ (9). Although NAD(P)H-oxidases (diaphorases) afford O₂-sensitive enzymes in *Trichomonas* protection against O₂ (48), they do not provide protection against reactive oxygen species such as the superoxide radical and hydrogen peroxide (H₂O₂). Detoxification of superoxide radicals in trichomonads is performed by iron-containing superoxide dismutase which is active in both the hydrogenosomal and cytosolic compartments (31, 25, 42). On the other hand, the analysis of thiols in whole cells of *T. vaginalis* indicated the absence of glutathione, and activities of peroxide-reducing enzymes, such as glutathione- and heme-dependent peroxidases, were not detectable (15). Catalase was shown to be present only in *Tritrichomonas foetus* and not in *T. vaginalis* (39). Such findings led to the suggestion that the lack of peroxide-reducing enzymes was the reason for the sensitivity of *T. vaginalis* to oxygen concentrations above physiological levels (15).

More recently, a thioredoxin-linked antioxidant system consisting of thiol peroxidase, thioredoxin, and thioredoxin reductase (TrxR) was identified, which is localized in the cytosol (10). The importance of this system as one of the most significant antioxidant defense mechanisms in *T. vaginalis* was shown by the upregulation of thioredoxin- and thioredoxin peroxidase-encoding genes in response to oxidative stress inducing environmental changes. Thioredoxin peroxidase belongs to a recently discovered thiol peroxidase family of proteins named

* Corresponding author. Mailing address: Institute of Botany III, Heinrich-Heine University of Duesseldorf, Universitaetsstrasse 1, 40225 Duesseldorf, Germany. Phone: 49-211-81-11378. Fax: 49-211-81-13554. E-mail: katrin.henze@uni-duesseldorf.de.

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[‡] Present address: Department of Biochemistry, Faculty of Natural Sciences, Comenius University, Mlynská dolina CH-1, 842 15 Bratislava, Slovakia.

[§] Present address: Molekulare Enzymtechnologie, Universität Duisburg-Essen, Campus Duisburg, Geibelstr. 41, 47057 Duisburg, Germany.

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peroxiredoxins, which use conserved cysteines at their active sites for catalysis (45). Peroxiredoxins are able to reduce hydrogen peroxide and alkyl peroxides as well as peroxynitrite with electrons provided by physiological thiols such as thioredoxin (19, 45). The third redox partner in the system, which serves as a reductant to oxidized thioredoxin, is low-molecular-weight TrxR. Components of a functionally related antioxidant protection system were recently found in the hydrogenosomes of *T. vaginalis*, where hydrogenosomal proteome screening identified two novel proteins with strong sequence similarity to two prokaryotic peroxidases, rubrerythrin and peroxiredoxin thiol peroxidase (41). A thioredoxin with an N-terminal extension showing strong similarity to hydrogenosomal targeting presequences was also found in the same study. Thus, two out of three components of the cytosolic thioredoxin-linked antioxidant system (10) were shown to also be present in the hydrogenosome (41), but the hydrogenosomal electron donor for thioredoxin was missing.

Here we report the identification of two hydrogenosomal TrxR isoforms, the hitherto-missing component of the hydrogenosomal thioredoxin-linked peroxidase antioxidant system, from *T. vaginalis*. The intracellular distribution of different TrxR isoforms observed in this study, together with the divergent N termini of these isoforms, led us to investigate the identity and importance of the targeting signals that direct hydrogenosomal TrxRs into the organelle in more detail. The hydrogenosomes of *T. vaginalis* lack a genome, and thus all hydrogenosomal proteins are encoded in the nuclear genome and must be imported into the organelle. This import seems to rely on translocation complexes similar to, but probably less complex than, those in mitochondria. No homologs to components of the protein import complex of the mitochondrial outer membrane (TOM) have so far been identified (8), but proteins homologous to Tim23 and Tim17, core components of the mitochondrial inner membrane translocase complex (TIM), as well as the protein import motor Pam18 of the mitochondrial matrix, are present in hydrogenosomes (12). Many hydrogenosome-targeted proteins carry short, cleavable, N-terminal extensions reminiscent of mitochondrial targeting presequences (5, 21, 27). These presequences carry recognizable cleavage sites which are processed by a hydrogenosomal processing peptidase, a homolog to mitochondrial processing peptidases (6).

Investigation of N-terminal presequences previously thought to be required to import proteins into the hydrogenosome in transfected *T. vaginalis* cells expressing hemagglutinin-tagged constructs in this study uncovered an unexpected plasticity among hydrogenosomal targeting signals in this pathogen.

MATERIALS AND METHODS

Strains and cultivation. *T. vaginalis* strain T1 (J.-H. Tai, Institute of Biomedical Sciences, Taipei, Taiwan) was grown in TYM medium at 37°C as described previously (33). *Escherichia coli* XL1-Blue MRF' $\{\Delta(mcrA)183 \Delta(mcrCB-hsdSMR-mrr)173 \text{ endA1 supE44 thi-1 recA1 gyrA96 relA1 lac [F' proAB lac}^{\lambda}\text{ZAM15 Tn10 (Tet}^{\text{r}}\text{)]}\}$ was used for cloning of genes of interest, and it was grown in LB medium containing ampicillin (100 $\mu\text{g/ml}$; Roth) at 37°C when transformed with the ampicillin resistance-bearing plasmid pTagVag2.

Cloning, N-terminal deletion, and transient expression of TrxR isoforms. Open reading frames of all five *TRXR* genes without the stop codon were amplified by PCR using genomic DNA of *T. vaginalis* as the template and sequence-specific primers for each gene (Table 1). N-terminally deleted genes were amplified from cloned full-length genes. Forward and reverse primers contained NdeI and BamHI restriction sites, respectively, for cloning the genes

TABLE 1. Oligonucleotides used in this study

Primer designation	Nucleotide sequence
TrxRh2 for.....	CTGACGCATATGTCGGGTGACATTGATTGGA CTAAAGC
TrxRh2 rev.....	GACGTGGATCCGTCGGTAAATATTCTCAGC
TrxR L92 for.....	CTGACGCATATGCTTCCAATTTTACTTCCTTTT TTGC
TrxR L92 rev.....	GACGTGGATCCTTCTTGATTTTCTTCTGC GTCG
TrxR k96 for.....	CTGACGCATATGTCTGCTCAAGCATTCTGA TCTC
TrxR k96 rev.....	GACGTGGATCCGTCAGTACTGAGATATCTCT CAGC
TrxR L88 for.....	CTGACGCATATGATGATATTTTGTTTTTTGT TTTTTGG
TrxR L88 rev-new.....	GACGTGGATCCTCCTGTAATGTTTTTGACAAC
TrxRh1 for.....	CTGACGCATATGTTTTCTATTATCTTTTTCT CTAG
TrxRh1 rev.....	GACGTGGATCCAATAGCTTTCACTTGTGATAT ATAC
TrxRh1 Δ 10 fwd.....	CTGACGCATATGACTCTACCGACGATGACG
TrxRh1 Δ 36 fwd.....	CTGACGCATATGTGGAGTAAAGCGCCATT ATAT
TrxRh1prePFO A.....	CTGACGCATATGCTCCGAGCTTCTACTCTAC CGACGATGACGATGATGAAATG
TrxRh1preSCS α	CTGACGCATATGCTCTCTCTTCCTTCGAGCG CAACTACTCTACCGACGATGAC GATGATG AAATG
TrxRh2 Δ 21 for.....	CTGACGCATATGGGTGGCAGCACAGCAGCTC
SCS α for.....	CTGACGCATATGCTCTCTCTTCCTTCG GCGC
SCS α rev.....	GACGTGGATCCGATCTTGCCCATTCCTTT CATC
SCS α Δ 8 for.....	CTGACGCATATGCTCCACCAACCACTCCTTT CATCG

into the pTagVag2 expression vector (20). The only exception was TrxR₈₈₂₈₉; because of an NdeI cutting site starting at position 710, only the first 708 nucleotides of the full-length 1,002-nucleotide-long open reading frame were amplified. After the BamHI restriction site, a dihemagglutinin tag (HA tag) was added in frame, followed by a stop codon. *T. vaginalis* T1 cells were electroporated (11, 28) with cloned constructs, and transformants were selected in TYM medium containing G418 (100 $\mu\text{g/ml}$; Roth).

Cell fractionation and protein concentration determination. Isolation of hydrogenosomes was based on the method described by Bradley et al. (5) with modifications (41). Whole-cell lysate (supernatant) was collected after grinding of the cells and the removal of unlysed cells, glass bead remains, membranes, and nuclei by centrifugation at 500 $\times g$ for 10 min at 4°C. The cytosolic fraction (supernatant) was obtained by subsequent centrifugation of the whole-cell lysate at 8,000 $\times g$ for 15 min at 4°C. Protein concentrations were determined with the Bradford assay kit (Bio-Rad) according to the manufacturer's instructions.

Enzyme assays. The activity of malic enzyme was assayed by monitoring the reduction of NADP⁺ as described previously (26). Malate dehydrogenase activity was assayed in the direction of malate formation by observing the oxidation of NADH (46).

Western blot analysis. Samples (20 μg of proteins) for Western blot analysis were run on 12% resolving gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and blotted onto nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences). Blots were washed (three times for 10 min each) in TBS (20 mM Tris-HCl [pH 7.5], 150 mM NaCl) and blocked for 1 hour in TBS containing 3% (wt/vol) bovine serum albumin. The blots were then incubated for 1 hour at room temperature, with subsequent overnight incubation at 4°C with mouse anti-HA antibodies (dilution 1:5,000; Sigma). Blots were washed as before and incubated with anti-mouse immunoglobulin G (heavy plus light chains)-horseradish peroxidase conjugate (ImmunoPure goat; dilution, 1:10,000; Pierce)

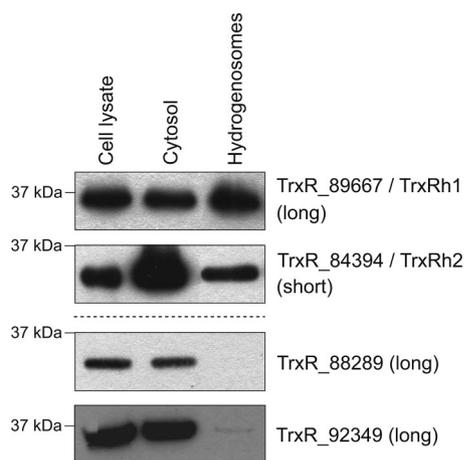


FIG. 2. Cellular localization of *T. vaginalis* TrxRs. Three cellular/subcellular fractions of *T. vaginalis* expressing HA-tagged TrxR isoforms were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent Western blotting. The tagged TrxRs were detected with mouse anti-HA antibodies.

the N terminus and either started with the typical Met-Leu motif or were rich in Ser at the N terminus (Fig. 1) (5). *T. vaginalis* T1 cells were transformed with HA-tagged constructs of the four remaining TrxR genes in the vector pTagVag2. Three cellular/subcellular fractions, i.e., whole-cell lysate, cytosol (without hydrogenosomes), and hydrogenosomes, of the recombinant strains were subjected to Western blot analysis with antibodies against HA and analyzed for the presence of the HA tag (Fig. 2). As expected, the majority of TrxR_89667, henceforth designated TrxRh1, was imported into the hydrogenosomes, but a substantial amount of the protein remained in the cytosol. This result was reproducible in four individual experiments, indicating inefficient hydrogenosomal import and a putative dual localization of this protein. The marker enzyme controls, malic enzyme (hydrogenosomal marker) and malate dehydrogenase (cytosolic marker) (Fig. 3), showed that cross contamination between cytosolic and hydrogenosomal fractions was minute, such that leakage from hydrogenosomes during cell fractionation is insufficient to account for the cytosolic signal observed for TrxRh1.

The coding region of TrxR_84394 possesses, in comparison to TrxRc, an N-terminal extension of only seven amino acids with no resemblance to typical hydrogenosomal targeting sequences (Fig. 1). TrxR_84394 was also found in both subcellular fractions, but the bulk of TrxR_84394 HA-tagged protein remained in the cytosol and only a minor quantity was reproducibly directed to the hydrogenosomes (Fig. 2). From these results we concluded that either the short N-terminal extension of TrxR_84394, henceforth designated TrxRh2, had a weak targeting capacity or the protein had an internal signal that was able to direct the protein into the hydrogenosomes, albeit with low efficiency.

TrxR_92349 and TrxR_88289, the other two isoforms with N-terminal extensions (Fig. 1), were not imported into the hydrogenosomes (Fig. 2), indicating that these proteins possess neither N-terminal nor internal hydrogenosomal targeting signals.

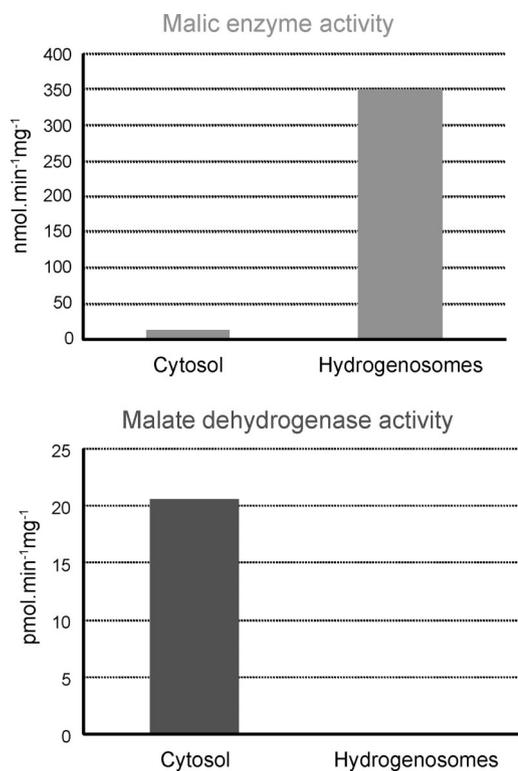


FIG. 3. Activities of marker enzymes in subcellular fractions of *T. vaginalis*. Activities of malate dehydrogenase (cytosolic marker) and malic enzyme (hydrogenosomal marker) in the cytosol and hydrogenosomes of *T. vaginalis* expressing HA-tagged TrxR_89667 (TrxRh1) are shown.

TrxRh1 without an N-terminal extension stays in the cytosol. TrxRh1 was the only one of three TrxRs with substantial N-terminal extensions (Fig. 1) that was, at least partially, imported into the hydrogenosomes (Fig. 2). To test whether this presequence was involved in targeting TrxRh1 to hydrogenosomes of *T. vaginalis*, we examined the N terminus of TrxRh1 in more detail in transfected cells. As the first 10 amino acids had only vague similarity to previously recognized hydrogenosomal targeting signals (8, 18), we first removed the N-terminal 36-amino-acid extension relative to the cytosolic isoform TrxRc. This construct, TrxRh1- Δ 36, remained exclusively in cytosol (Fig. 4a), indicating that this extension contains information that is necessary for organellar targeting. Hydrogenosomal targeting sequences are thought to be short, i.e., on the order of 10 amino acids, with Leu or Ser at the second position (8, 18) and with an Arg residue typically located at position -2 or -3 relative to the putative processing site of the N-terminal extensions (18). The first 11 amino acids of TrxRh1 lack Leu or Ser at position 2 but possess an Arg residue at position 9 (Fig. 1). This sequence was deleted in construct TrxRh1- Δ 10, which also was not imported (Fig. 4a). This indicates that, though lacking some typical features, the N-terminal extension MFSI IFFSRFS is required for hydrogenosomal targeting of TrxRh1 and that its absence is not compensated for by an internal signal. It cannot be excluded, though, that an internal signal is necessary but not sufficient for import in the absence of that 11-amino-acid motif. As TrxRh2 was partially imported into

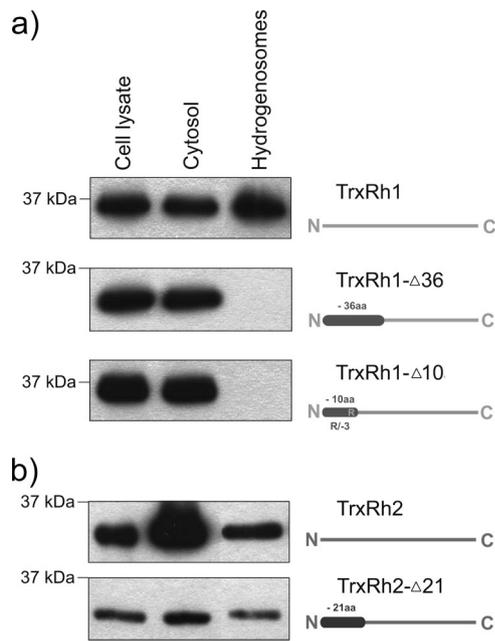


FIG. 4. Identification of hydrogenosomal targeting presequences of TrxRh1 and TrxRh2. (a) Subcellular localization of HA-tagged full-length TrxRh1 compared to HA-tagged N-terminally deleted TrxRh1- Δ 36 and TrxRh1- Δ 10. The arginine (R) position in the hydrogenosomal targeting signal of TrxRh1 is indicated. (b) Subcellular localization of HA-tagged full-length TrxRh2 compared to HA-tagged N-terminally deleted TrxRh2- Δ 21. Deletions are not drawn in proportion.

the hydrogenosomes, though having only a very short N-terminal extension without any resemblance to known hydrogenosomal targeting sequences, we examined the influence of its N terminus upon import. Since there was no obvious putative processing site in the first 34 amino acids of the N terminus of TrxR_84394, we removed the first 21 amino acids, which corresponded to approximately twice the characteristic presequence length of hydrogenosomal proteins in *T. vaginalis* and contained the first strongly conserved region of all five TrxR isoforms (Fig. 1). We were surprised to find that the distribution of TrxRh2- Δ 21 between cytosol and hydrogenosomes did not differ markedly from that of the wild-type protein in that a minor amount of the protein still localized to the hydrogenosomes (Fig. 4b). Thus, in contrast to TrxRh1, which is not imported without the first 10 amino acids after methionine, TrxRh2 must contain an internal targeting signal that acts independently of an N-terminal targeting sequence. That the HA tag itself does not confer hydrogenosomal targeting is shown by TrxR_88289 and TrxR_92349, which remain cytosolic despite the presence of the HA tag (Fig. 2).

Localization of TrxRh1 with typical, processed hydrogenosomal targeting presequences. After identification of a hydrogenosomal targeting sequence (albeit an inefficient one) in TrxRh1, we investigated the localization of this protein under the influence of a presequence from a protein known from biochemical studies to be localized exclusively in the hydrogenosomes. For this purpose, the presequence MLRSF of PFO A (locus tag TVAG_198110) was chosen, because complete hydrogenosomal localization of the protein and the processing

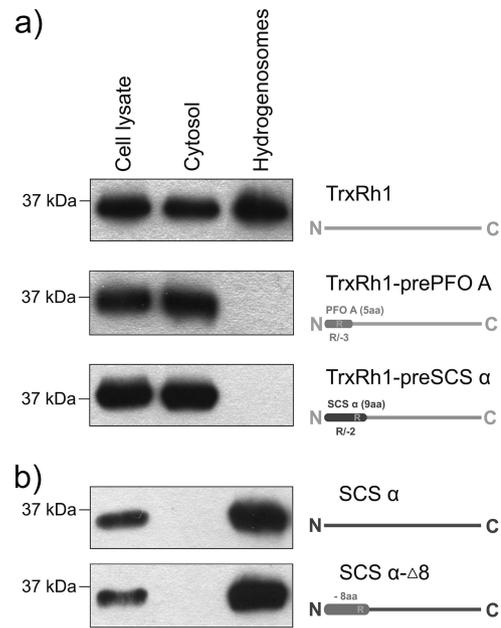


FIG. 5. Localization of TrxRh1 with N-terminal presequences of hydrogenosomal proteins PFO A and SCS α . (a) Subcellular localization of HA-tagged full-length TrxRh1 compared to two HA-tagged constructs of TrxRh1 with the presequences of TrxRh1-prePFO A and TrxRh1-preSCS α . (b) Subcellular localization of HA-tagged full-length SCS α and SCS α - Δ 8. R, arginine at position -2 relative to the processing site.

site of the N-terminal extension are known from earlier work (21). If this targeting signal is necessary and sufficient for import, it should guide TrxRh1 completely to the organelles. Surprisingly, TrxRh1 with its own presequence MFSIIFSRFS exchanged for the presequence MLRSF of PFO A (TrxRh1-prePFO A) stayed entirely in the cytosol (Fig. 5a), indicating that it is not sufficient for import. As the presequence of PFO A is extremely short, i.e., only half of the length of the TrxRh1 presequence, the experiment was repeated using a different, previously characterized presequence with approximately the same length as that in TrxRh1. For this, we used the presequence MLSSSFERN of the alpha subunit of succinyl coenzyme A synthetase (SCS α) (locus tag TVAG_165340) because, as for PFO A, SCS α is localized to hydrogenosomes and the processing site is known (28). Again, this presumably "typical" presequence was also not sufficient to direct construct TrxRh1-preSCS α to hydrogenosomes (Fig. 5a). This indicates that the PFO A and SCS α presequences, though demonstrably processed upon import of their cognate precursor proteins, do not contain by themselves all information that is required to direct proteins to hydrogenosomes in *T. vaginalis*, and hence additional information for localization must be present in the sequences of the imported subunits.

The processed N-terminal presequence of SCS α is not necessary for hydrogenosomal targeting. The observations described above called into question the specificity and role of N-terminal sequences in *T. vaginalis* hydrogenosomal targeting and pointed to the presence of signals contained within the sequence of the mature subunit. To explore this possibility further, we removed the N-terminal presequence of SCS α at its

known processing site (SCS α - Δ 8) and found that the mature HA-tagged subunit is still exclusively localized in the hydrogenosomes (Fig. 5b). We conclude that the presequence of SCS α , although processed (28), is not necessary to target the protein to the hydrogenosomes and that the mature SCS α subunit must have an internal targeting signal. Furthermore, the N-terminal sequence of SCS α is also not sufficient for targeting, because it does not substitute for the N terminus of TrxRh1, which is required for import (Fig. 5a). Hence the presequence of SCS α , though cleaved upon import, does not by itself contain any signals that specify import into the *T. vaginalis* hydrogenosome.

DISCUSSION

Peroxiredoxins, a recently discovered family of thiol peroxidases (24), are low-efficiency peroxidases that use thiols as electron donors. They are responsible for antioxidant defense in a variety of organisms, including bacteria, yeasts, and trypanosomatids (19). Considering the lack of catalase (39) and glutathione (15) together with the absence of activities of peroxide-reducing enzymes (e.g., glutathione-dependent peroxidase) in *T. vaginalis*, it was expected that this human parasite should possess alternative means to deal with hydrogen peroxides and alkyl peroxides. Coombs et al. (10) identified a divergent thioredoxin-linked peroxidase antioxidant system as the likely candidate for the principal antioxidant defense mechanism in the cytosol of *T. vaginalis*. Shortly thereafter, an analogous thiol-dependent antioxidant defense system was detected in hydrogenosomes of *T. vaginalis* (41). Along with peroxidase thiol peroxidase, its reductant thioredoxin with an N-terminal extension strongly resembling presumed hydrogenosomal targeting presequences was found in the hydrogenosomal proteome (41). This predicted the presence of an electron donor to thioredoxin in hydrogenosomes.

The conventional electron donor for thioredoxin in thioredoxin-linked redox systems is TrxR (37). TrxRs found in eukaryotes fall into two distinct groups: (i) high-molecular-mass TrxR isoforms (~55 kDa) and (ii) low-molecular-mass TrxR isoforms (~35 kDa) (1). These two distinct forms have different structures and catalytic mechanisms and show a mutually exclusive distribution: high-molecular-mass TrxRs are found in animals and apicomplexan protists, while most of the other eukaryotic taxa possess the presumably ancestral low-molecular-mass TrxR isoform (17). The *T. vaginalis* TrxR identified by Coombs et al. (10) clearly belongs to the latter class. We found five open reading frames corresponding to homologs of low-molecular-mass TrxRs in the *T. vaginalis* genome. Though they were very similar to one another, some differences were evident. Only three *T. vaginalis* TrxR isoforms, including that identified by Coombs et al. (10), possessed the fully conserved active-site amino acid sequence CATC (Fig. 1), and three had N-terminal extensions that could possibly serve as hydrogenosomal targeting signals. However, only two of these extensions remotely resembled known presequences of hydrogenosomal proteins. The basic amino acid arginine occupies either position -2 or position -3 from the predicted cleavage site in all presequences of putative hydrogenosomal precursor proteins inferred in the *T. vaginalis* genome sequencing project (8). The N-terminal extension of TrxR_92349, though having Arg at a suitable distance (position 12) from the start, possessed no Ser

within first 13 amino acids (Fig. 1), and the gene product was not imported into hydrogenosomes (Fig. 2). On the other hand, in spite of its atypical N-terminal extension sequence (Fig. 1), the majority of TrxRh1 clearly localized to hydrogenosomes. This observation was in contrast to the exclusively cytosolic localization of the other two TrxR isoforms with N-terminal extensions relative to TrxRc (Fig. 2). However, TrxRh1 did not localize exclusively to hydrogenosomes, with some protein remaining in the cytosol. Despite the unusual Phe at position 2 of the N terminus instead of the typical Leu or Ser, deletion of the first 10 amino acids after methionine in TrxRh1 showed that sequence to be involved in hydrogenosomal targeting (Fig. 4a). Because of its dual localization, we asked whether recombinant TrxRh1 protein carrying a hydrogenosomal presequence from an exclusively hydrogenosome-localized protein instead of its own presequence would be directed entirely to the organelle. PFO A and SCS α presequences did not target TrxRh1 to hydrogenosomes (Fig. 5a). Moreover, SCS α with its presequence deleted is quantitatively targeted to hydrogenosomes (Fig. 5b). This indicates that the presequences of hydrogenosomal enzymes PFO A and SCS α , though cleaved after protein import (21, 27), are not sufficient, and in the case of SCS α are not necessary, for hydrogenosomal localization of these proteins. Instead, yet-uncharacterized internal targeting signals in these proteins would appear to be involved in their organellar targeting. Similarly, TrxRh2 and TrxRh2- Δ 21 both were targeted, at least partially, to the hydrogenosomes as well (Fig. 4b), indicating the presence of an internal, albeit ineffective, targeting signal in this protein, too.

Information about the targeting of proteins to *T. vaginalis* hydrogenosomes is still scarce, with only about a dozen cases reported where the N terminus of the hydrogenosomal protein was compared to the sequence inferred from the mRNA (5). Expression in *Trichomonas* allows us examine targeting and the role of presequences in greater detail. Our results reveal that two previously characterized cleaved presequences, those in PFO A and in SCS α , do not carry all (or perhaps any) information required for targeting and that internal targeting sequences are present in the case of SCS α and in TrxRh2. It is possible that internal targeting signals are common among hydrogenosomal proteins, because rubrerythrin and thiol peroxidase, for instance, also do not contain apparent N-terminal transit peptides (41). An internal targeting signal had previously been proposed for hydrogenosomal membrane protein Hmp31 (14), a homolog of mitochondrial carrier family proteins which possess internal an organelle targeting signal(s) also in mitochondria (44). Similar to SCS α , Hmp31 also contains an N-terminal extension that is processed but not necessary for targeting. In contrast, the unconventional presequence of TrxRh1 is necessary for import (Fig. 4a), as is the N-terminal extension of hydrogenosomal ferredoxin, as shown in *in vitro* import studies (5). The present results clearly indicate that there are internal signals involved in the import of some hydrogenosomal proteins, but the nature of those signals has yet to be specified.

In that sense, hydrogenosomal import mirrors to some extent the situation in mitochondria. Hydrogenosomes and mitochondria are homologous organelles which descend from the same eubacterial endosymbiont (32, 47), and their membrane targeting and protein import mechanisms are conserved (5, 6,

14, 50). The import of matrix proteins to mitochondria is generally dependent upon cleavable, N-terminal presequences, while the import of inner membrane proteins tends to rely upon multiple internal signals (4, 38, 43), but numerous exceptions regarding the distribution and requirements of targeting signals have been described. Examples are (i) N-terminal presequences that do not act as a targeting signal but facilitate the solubility of preprotein, as it seems to determine the folding state (54); (ii) N-terminal presequences that are not essential for import and assembly of the protein (55); (iii) the coexistence of internal and N-terminal targeting signals, whereby the N-terminal cleavable extension is responsible for protein import (36); and (iv) a C-terminal cleavable mitochondrial targeting signal, as was reported for DNA helicase Hmi1p (29). Though the first three examples represent mitochondrial carrier family proteins which are expected to have multiple internal signals, nonconventional import pathways were also reported for matrix proteins, specifically for the nuclearly encoded mitochondrial transcription factor Mtf1p (2). Its translocation appears to be independent of a cleavable N-terminal presequence, and Mtf1p seems to be capable of using alternative import signals present in different regions of the protein (3).

The present results suggest that protein targeting to hydrogenosomes in some cases involves topogenic signals beyond those provided by N-terminal extensions. Given that two such examples were found among three hydrogenosomal proteins tested here, it is possible that this property is more common among hydrogenosomal proteins than among proteins of typical mitochondria. Despite the common ancestry of mitochondria and hydrogenosomes, some differences in their protein import might be expected. The electrochemical gradient generated by proton pumping in mitochondria that is required for protein translocation by TIM22 and TIM23 complexes (13, 38) is generally thought to be missing in hydrogenosomes, which lack cytochromes and membrane-associated electron transport (35). Although there have been reports that trichomonad hydrogenosomes develop transmembrane potential (22), the mechanisms by which this occurs are still obscure. Import of ferredoxin into isolated trichomonad hydrogenosomes was found to be inhibited by the protonophore *m*-chlorophenylhydrazine, albeit at concentrations roughly 10-fold higher than those that inhibit mitochondrial protein import (5). Trichomonad hydrogenosomes have been shown to efficiently import some proteins of *Giardia* mitosomes that possess N-terminal extensions (12). The findings reported here indicate, however, that N-terminal extensions are not always required for import into *Trichomonas* hydrogenosomes and thus raise new questions concerning the prevalence of this type of import and the mechanisms involved.

Neighbor-Net sequence similarity analysis was conducted, comparing the five TrxRs from *T. vaginalis* with selected bacterial and eukaryotic TrxRs (see Fig. S1 in the supplemental material). All five isoforms clearly cluster together, indicating that they represent paralogs which arose through gene duplication in the *T. vaginalis* lineage. First, all three long versions diverged progressively, including hydrogenosomally localized TrxR₈₉₆₆₇. Gene duplication giving rise to the two short paralogs obviously occurred last. Notably, eukaryotic TrxRs do not cluster together. TrxRs from *T. vaginalis* are divergent from and more similar to proteobacterial homologs than those of other eukaryotes. The Neighbor-Net topology indicates in-

terdomain gene transfer, though it is not possible to resolve whether it was horizontal gene transfer or endosymbiotic gene transfer, after the acquisition of mitochondrial predecessor.

Intriguingly, TrxRs in *T. vaginalis*, although all descendants through duplication from a single protein and still highly conserved, are localized to different intracellular compartments, and two of them have developed diverse hydrogenosomal targeting mechanisms. Thus, TrxRs appear to be a suitable system to explore the characteristics of hydrogenosomal protein targeting signals in more detail.

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REFERENCES

- Arcott, L. D., S. Gromer, R. H. Schirmer, K. Becker, and C. H. Williams, Jr. 1997. The mechanism of thioredoxin reductase from human placenta is similar to the mechanisms of lipoamide dehydrogenase and glutathione reductase and is distinct from the mechanism of thioredoxin reductase from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **94**:3621–3626.
- Biswas, T. K., and G. S. Getz. 2002. Import of yeast mitochondrial transcription factor (Mtf1p) via a nonconventional pathway. *J. Biol. Chem.* **277**:45704–45714.
- Biswas, T. K., and G. S. Getz. 2004. Requirement of different mitochondrial targeting sequences of the yeast mitochondrial transcription factor Mtf1p when synthesized in alternative translation systems. *Biochem. J.* **383**:383–391.
- Bolender, N., A. Sickmann, R. Wagner, C. Meisinger, and N. Pfanner. 2008. Multiple pathways for sorting mitochondrial precursor proteins. *EMBO Rep.* **9**:42–49.
- Bradley, P. J., C. J. Lahti, E. Plümper, and P. J. Johnson. 1997. Targeting and translocation of proteins into the hydrogenosome of the protist *Trichomonas*: similarities with mitochondrial protein import. *EMBO J.* **16**:3484–3493.
- Brown, M. T., H. M. Goldstone, F. Bastida-Corcuera, M. G. Delgadillo-Correa, A. G. McArthur, and P. J. Johnson. 2007. A functionally divergent hydrogenosomal peptidase with protomitochondrial ancestry. *Mol. Microbiol.* **64**:1154–1163.
- Bryant, D., and V. Moulton. 2004. Neighbor-Net: an agglomerative method for the construction of phylogenetic networks. *Mol. Biol. Evol.* **21**:255–265.
- Carlton, J. M., R. P. Hirt, J. C. Silva, A. L. Delcher, M. Schatz, Q. Zhao, J. R. Wortman, S. L. Bidwell, U. C. Alsmark, S. Besteiro, and P. J. Johnson. 2007. Draft genome sequence of the sexually transmitted pathogen *Trichomonas vaginalis*. *Science* **315**:207–212.
- Chapman, A., D. J. Linstead, and D. Lloyd. 1999. Hydrogen peroxide is a product of oxygen consumption by *Trichomonas vaginalis*. *J. Biosci.* **24**:339–344.
- Coombs, G. H., G. D. Westrop, P. Suchan, G. Puzova, R. P. Hirt, T. M. Embley, J. C. Mottram, and S. Müller. 2004. The amitochondriate eukaryote *Trichomonas vaginalis* contains a divergent thioredoxin-linked peroxiredoxin antioxidant system. *J. Biol. Chem.* **279**:5249–5256.
- Delgadillo, M. G., D. R. Liston, K. Niazi, and P. J. Johnson. 1997. Transient and selectable transformation of the parasitic protozoan *Trichomonas vaginalis*. *Proc. Natl. Acad. Sci. USA* **94**:4716–4720.
- Dolezal, P., O. Smíd, P. Rada, Z. Zubáková, D. Bursac, R. Suták, J. Nebešárová, T. Lithgow, and J. Tachezy. 2005. *Giardia* mitosomes and trichomonad hydrogenosomes share a common mode of protein targeting. *Proc. Natl. Acad. Sci. USA* **102**:10924–10929.
- Dolezal, P., V. Likic, J. Tachezy, and T. Lithgow. 2006. Evolution of the molecular machines for protein import into mitochondria. *Science* **313**:314–318.
- Dyall, S. D., C. M. Koehler, M. G. Delgadillo-Correa, P. J. Bradley, E. Plümper, D. Leuenberger, C. W. Turck, and P. J. Johnson. 2000. Presence of a member of the mitochondrial carrier family in hydrogenosomes: conservation of membrane-targeting pathways between hydrogenosomes and mitochondria. *Mol. Cell. Biol.* **20**:2488–2497.
- Ellis, J. E., N. Yarlett, D. Cole, M. J. Humphreys, and D. Lloyd. 1994. Antioxidant defences in the microaerophilic protozoan *Trichomonas vaginalis*: comparison of metronidazole-resistant and sensitive strains. *Microbiology* **140**:2489–2494.
- Reference deleted.
- Hirt, R. P., S. Müller, T. M. Embley, and G. H. Coombs. 2002. The diversity and evolution of thioredoxin reductase: new perspectives. *Trends Parasitol.* **18**:302–308.

18. Hirt, R. P., C. J. Noel, T. Sicheritz-Ponten, J. Tachezy, and P. L. Fiori. 2007. *Trichomonas vaginalis* surface proteins: a view from the genome. *Trends Parasitol.* **23**:540–547.
19. Hofmann, B., H. J. Hecht, and L. Flohé. 2002. Peroxiredoxins. *Biol. Chem.* **383**:347–364.
20. Hrdy, I., R. P. Hirt, P. Dolezal, L. Bardónová, P. G. Foster, J. Tachezy, and T. M. Embley. 2004. *Trichomonas* hydrogenosomes contain the NADH dehydrogenase module of mitochondrial complex I. *Nature* **432**:618–622.
21. Hrdy, I., and M. Müller. 1995. Primary structure and eubacterial relationships of the pyruvate:ferredoxin oxidoreductase of the amitochondriate eukaryote *Trichomonas vaginalis*. *J. Mol. Evol.* **41**:388–396.
22. Humphreys, M., J. Ralphs, and D. Lloyd. 1998. Confocal laser scanning microscopy of trichomonads: hydrogenosomes store calcium and show a membrane potential. *Eur. J. Protistol.* **34**: 356–362.
23. Huson, D. H., and D. Bryant. 2006. Application of phylogenetic networks in evolutionary studies. *Mol. Biol. Evol.* **23**:254–267.
24. Kim, K., S. G. Rhee, and E. R. Stadtman. 1985. Nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron. *J. Biol. Chem.* **260**:15394–15397.
25. Kitchener, K. R., S. R. Meshnick, A. S. Fairfield, and C. C. Wang. 1984. An iron-containing superoxide dismutase in *Trichomonas foetus*. *Mol. Biochem. Parasitol.* **12**:95–99.
26. Kremer, D. R., H. E. Nienhuis-Kuiper, C. J. Timmer, and T. A. Hansen. 1989. Catabolism of malate and related dicarboxylic acids in various *Desulfovibrio* strains and the involvement of an oxygen-labile NADPH dehydrogenase. *Arch. Microbiol.* **151**:34–39.
27. Lahti, C. J., P. J. Bradley, and P. J. Johnson. 1994. Molecular characterization of the alpha-subunit of *Trichomonas vaginalis* hydrogenosomal succinyl CoA synthetase. *Mol. Biochem. Parasitol.* **66**:309–318.
28. Land, K. M., M. G. Delgado-Correa, J. Tachezy, S. Vanacova, C. L. Hsieh, R. Sutak, and P. J. Johnson. 2004. Targeted gene replacement of a ferredoxin gene in *Trichomonas vaginalis* does not lead to metronidazole resistance. *Mol. Microbiol.* **51**:115–122.
29. Lee, C. M., J. Sedman, W. Neupert, and R. A. Stuart. 1999. The DNA helicase, Hmi1p, is transported into mitochondria by a C-terminal cleavable targeting signal. *J. Biol. Chem.* **274**:20937–20942.
30. Lindmark, D. G., and M. Müller. 1973. Hydrogenosome, a cytoplasmic organelle of the anaerobic flagellate *Trichomonas foetus*, and its role in pyruvate metabolism. *J. Biol. Chem.* **248**:7724–7728.
31. Lindmark, D. G., and M. Müller. 1974. Superoxide dismutase in the anaerobic flagellates *Trichomonas foetus* and *Monocercomonas* sp. *J. Biol. Chem.* **249**:4634–4637.
32. Martin, W., and M. Müller. (ed.). 2007. *Origin of mitochondria and hydrogenosomes*. Springer, Heidelberg, Germany.
33. Mertens, E., and M. Müller. 1990. Glucokinase and fructokinase of *Trichomonas vaginalis* and *Trichomonas foetus*. *J. Protozool.* **37**:384–388.
34. Miller, M., Y. Liao, A. M. Gomez, C. A. Gaydos, and D. D'Mellow. 2008. Factors associated with the prevalence and incidence of *Trichomonas vaginalis* infection among African American women in New York city who use drugs. *J. Infect. Dis.* **197**:503–509.
35. Müller, M. 1993. The hydrogenosome. *J. Gen. Microbiol.* **139**:2879–2889.
36. Murcha, M. W., A. H. Millar, and J. Whelan. 2005. The N-terminal cleavable extension of plant carrier proteins is responsible for efficient insertion into the inner mitochondrial membrane. *J. Mol. Biol.* **351**:16–25.
37. Mustachich, D., and G. Powis. 2000. Thioredoxin reductase. *Biochem. J.* **346**:1–8.
38. Neupert, W., and J. M. Herrmann. 2007. Translocation of proteins into mitochondria. *Annu. Rev. Biochem.* **76**:723–749.
39. Page-Sharp, M., C. A. Behm, and G. D. Smith. 1996. *Trichomonas foetus* and *Trichomonas vaginalis*: the pattern of inactivation of hydrogenase activity by oxygen and activities of catalase and ascorbate peroxidase. *Microbiology* **142**:207–211.
40. Paget, T. A., and D. Lloyd. 1990. *Trichomonas vaginalis* requires traces of oxygen and high concentrations of carbon dioxide for optimal growth. *Mol. Biochem. Parasitol.* **41**:65–72.
41. Pütz, S., G. Gellius-Dietrich, M. Piotrowski, and K. Henze. 2005. Rubrerythrin and peroxiredoxin: two novel putative peroxidases in the hydrogenosomes of the microaerophilic protozoan *Trichomonas vaginalis*. *Mol. Biochem. Parasitol.* **142**:212–223.
42. Rasoloson, D., E. Tomkova, R. Cammack, J. Kulda, and J. Tachezy. 2001. Metronidazole-resistant strains of *Trichomonas vaginalis* display increased susceptibility to oxygen. *Parasitology* **123**:45–56.
43. Rehling, P., K. Brandner, and N. Pfanner. 2004. Mitochondrial import and the twin-pore translocase. *Nat. Rev. Mol. Cell Biol.* **5**:519–530.
44. Rehling, P., N. Pfanner, and C. Meisinger. 2003. Insertion of hydrophobic membrane proteins into the inner mitochondrial membrane—a guided tour. *J. Mol. Biol.* **326**:639–657.
45. Rhee, S. G., H. Z. Chae, and K. Kim. 2005. Peroxiredoxins: a historical overview and speculative preview of novel mechanisms and emerging concepts in cell signaling. *Free Radic. Biol. Med.* **38**:1543–1552.
46. Stams, A. J. M., D. R. Kremer, K. Nicolay, G. H. Weenk, and T. A. Hansen. 1984. Pathway of propionate formation in *Desulfobulbus propionicus*. *Arch. Microbiol.* **139**:167–173.
47. Tachezy, J. (ed.). 2008. *Hydrogenosomes and mitochondria of anaerobic eukaryotes*. Springer, Heidelberg, Germany.
48. Tanabe, M. 1979. NADH oxidase activity in *Trichomonas vaginalis*. *Exp. Parasitol.* **48**:135–143.
49. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**:4876–4882.
50. van der Giezen, M., D. J. Slotboom, D. S. Horner, P. L. Dyal, M. Harding, G. P. Xue, T. M. Embley, and E. R. Kunji. 2002. Conserved properties of hydrogenosomal and mitochondrial ADP/ATP carriers: a common origin for both organelles. *EMBO J.* **21**:572–579.
51. Reference deleted.
52. Wagner, G., and R. Levin. 1978. Oxygen tension of the vaginal surface during sexual stimulation in the human. *Fertil. Steril.* **30**:50–53.
53. Williams, K. P., P. F. Leadlay, and P. N. Lowe. 1990. Inhibition of pyruvate:ferredoxin oxidoreductase from *Trichomonas vaginalis* by pyruvate and its analogues. Comparison with the pyruvate decarboxylase component of the pyruvate dehydrogenase complex. *Biochem. J.* **268**:69–75.
54. Zara, V., A. Ferramosca, I. Palmisano, F. Palmieri, and J. Rassow. 2003. Biogenesis of rat mitochondrial citrate carrier (CIC): the N-terminal presequence facilitates the solubility of the preprotein but does not act as a targeting signal. *J. Mol. Biol.* **325**:399–408.
55. Zara, V., F. Palmieri, K. Mahlke, and N. Pfanner. 1992. The cleavable presequence is not essential for import and assembly of the phosphate carrier of mammalian mitochondria but enhances the specificity and efficiency of import. *J. Biol. Chem.* **267**:12077–12081.