β -Succinyl-Coenzyme A Synthetase from *Trichomonas vaginalis* Is a Soluble Hydrogenosomal Protein with an Amino-Terminal Sequence That Resembles Mitochondrial Presequences

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We describe studies directed toward understanding the biogenesis and origin of the hydrogenosome, an unusual organelle found exclusively in certain anaerobic eukaryotes that lack mitochondria. Hydrogenosomes are involved in fermentative carbohydrate metabolism and are proposed to have arisen through conversion of mitochondria or via endosymbiosis with an anaerobic bacterium. We cloned a gene encoding the beta subunit of the hydrogenosomal protein succinyl-coenzyme A synthetase (β -SCS) and isolated the protein from *Trichomonas vaginalis*. The *T. vaginalis* β -SCS gene encodes a protein with a calculated molecular mass of 43,980 Da that has 43% amino acid identity (65% similarity) with β -SCS from *Escherichia coli*. The trichomonad protein partitions into the soluble fraction of hydrogenosomes treated with sodium carbonate at high pH, consistent with a matrix localization within the organelle. The protein is encoded by a multigene family composed of at least three members. Amino-terminal sequencing of β -SCS purified from *T. vaginalis* hydrogenosomes shows that the mature protein lacks the first nine amino acids encoded in the gene. This apparent amino-terminal leader sequence is strikingly similar to that of another hydrogenosomal protein and to mitochondrial presequences.

Trichomonas vaginalis is an ancient eukaryote that lacks mitochondria (32, 39). This anaerobic protozoan parasite does, however, contain an unusual organelle called the hydrogenosome (32). Hydrogenosomes are bound by a double membrane and do not appear to contain DNA. These organelles compose >5% of the cell volume and play a key role in carbohydrate metabolism in trichomonads. They are the site of fermentative oxidation of pyruvate, accompanied by substrate-level formation of ATP. Thus, hydrogenosomes could be considered the anaerobic equivalent of mitochondria.

The hydrogenosomes originally described were from *T. vaginalis* and other trichomonad protozoa (31, 32). Hydrogenosomes have also been found in a number of free-living anaerobic ciliates (12, 13) as well as in ciliates and a fungus that inhabit the rumens of ungulates (32). These organelles share biochemical properties with anaerobic bacteria and mitochondria. The presence of a double membrane makes it likely that hydrogenosomes arose from an endosymbiont. Two plausible hypotheses for the origin of hydrogenosomes are that they arose by conversion of mitochondria (8, 13) or through an independent endosymbiosis of an anaerobic bacterium (31, 32, 47).

Comparison of hydrogenosomal constituents with those of other organelles and of prokaryotes should clarify the nature of hydrogenosomes. To this end, we have begun to characterize the protein constituents of the hydrogenosome of *T. vaginalis*. We report an analysis of the beta subunit of the hydrogenosomal protein succinyl-coenzyme A synthetase (β -SCS). Succinyl-coenzyme A synthetase (SCS) is typically located in mitochondria of eukaryotic cells and catalyzes the formation of ATP via substrate-level phosphorylation (5). In

trichomonad hydrogenosomes, SCS performs the same function (40). We isolated and analyzed a gene that encodes this protein as well as the protein from *T. vaginalis*. The trichomonad protein partitions into the soluble fraction of the hydrogenosome, consistent with its localization in the matrix of the organelle. A comparison of the amino terminus predicted by the gene sequence with the amino terminus determined for the purified protein indicates that β -SCS is synthesized with a 9-amino-acid N-terminal extension that is absent from the mature protein found in hydrogenosomes. Interestingly, the N-terminal extension of β -SCS has significant homology with the N terminus of the only other hydrogenosomal protein sequenced to date (24). Moreover, there is striking similarity between the N-terminal sequence of β -SCS and the N-terminal leader sequences that are responsible for targeting mitochondrial proteins to the organelle.

MATERIALS AND METHODS

Parasites. *T. vaginalis* C1 (ATCC 30001) was cultured and harvested as previously described (17).

Recombinant DNA methods. A *T. vaginalis* cDNA library was prepared in lambda gt11, and the library was screened with polyclonal antisera as previously described (24). cDNA-A and cDNA-B were subcloned into pUC19 and sequenced on both strands by the method of Sanger et al. (38) with a Sequenase kit (U.S. Biochemical).

A full-length cDNA clone (cDNA-B') was generated by using an anchored polymerase chain reaction technique described previously (30). A synthetic oligonucleotide primer (45 bp) complementary to the 3' untranslated region of cDNA-B was used for first-strand synthesis by reverse transcriptase and precipitated with spermine (9). Polymerase chain reaction products were cloned into pBluescript and

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sequenced. Genomic clone pSCS-53 (gene B) was generated by subcloning a 2.0-kb *Eco*RI fragment derived from a lambda EMBL3 clone into pBluescript. The lambda EMBL3 clone was isolated from a *T. vaginalis* genomic library as described previously (24).

Nucleic acid isolation and analysis. High-molecular-weight DNA and total RNA were isolated by a modified guanidinium thiocyanate procedure described previously (44). Southern blots and Northern RNA blots were prepared and hybridized with 32 P-labelled β -SCS probes as previously described (24). S1 nuclease analysis was done as described previously (4).

In vitro transcription and translation. A construct containing gene B was linearized 3' of the termination codon by digestion with restriction endonuclease *Sma*I. Capped RNA transcripts were synthesized in vitro with an mCAP transcription kit as recommended by the manufacturer (Stratagene) and translated in a wheat germ extract system (Promega) with [35 S]cysteine as a label.

Generation of fusion protein and antiserum. A plasmid encoding a β -SCS-TrpE fusion protein was constructed by cloning cDNA-A, a 276-bp *Eco*RI fragment, into the bacterial TrpE fusion protein vector pATH20 (10). Expression of the plasmid was induced in *Escherichia coli* DH5 α transformants, and the fusion protein was extracted and purified on a sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gel. Gel fragments containing approximately 0.5 mg of fusion protein were homogenized with Freund complete adjuvant (Difco Laboratories) and used to immunize rabbits. The animals were given booster injections three times with 0.1 mg of fusion protein in Freund incomplete adjuvant at 3- to 4-week intervals.

Immunoprecipitation, immunoblot analysis, and SDS-PAGE. Immunoprecipitations were conducted by standard procedures. Briefly, an enriched fraction of *T. vaginalis* hydrogenosomes was incubated with the β -SCS antiserum in an immunoprecipitation buffer (10 mM Tris [pH 7.4], 1% Nonidet P-40, 2 mM EDTA, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride). Immunocomplexes were adsorbed to protein A-Sepharose (Pharmacia), washed with immunoprecipitation buffer, and resuspended in sample buffer. Proteins were separated by discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (26) and electroblotted onto nitrocellulose (41). Immunoblot analysis was performed with standard protocols and appropriate dilutions of antisera. Antigen-antibody complexes were detected by reaction with 125 I-labelled protein A.

Purification of hydrogenosomes. *T. vaginalis* cells were cultured and harvested as previously described (17). Cell pellets were washed two times and resuspended in SMD (0.25 M sucrose, 10 mM morpholine propane sulfonic acid [pH 7.2], 10 mM dithiothreitol). Hydrogenosomes were purified as described previously for purification of glycosomes (33) with few modifications. Cells were lysed in a cell disrupter (Stansted) and centrifuged at $1,000 \times g$ for 10 min at 4°C. The supernatant was mixed with an equal volume of 90% Percoll in SMD and centrifuged at 30,000 rpm for 1 h in a vertical VC53 rotor (Beckman). The lower band containing the hydrogenosomes was collected and washed twice with 10 volumes of SMD and resuspended in SMD.

Subfractionation of hydrogenosome membranes and soluble proteins by sodium carbonate treatment. Membranes and soluble proteins in 1-ml samples of purified hydrogenosomes were separated as described previously (14). The membrane pellet was washed and resuspended in 0.2 volume of sample buffer. Soluble proteins contained in the supernatant were

FIG. 1. Immunoblot analysis of *T. vaginalis* cellular lysates reacted with antihydrogenosomal antiserum. *T. vaginalis* lysates were centrifuged at $750 \times g$, and 30 μ g of this postnuclear supernatant was size separated by 5 to 15% polyacrylamide gradient SDS-PAGE. Lane A was stained with Coomassie brilliant blue. Lane B was transferred to nitrocellulose and reacted with the antihydrogenosomal serum. Antigen-antibody complexes were detected by using 125 I-labelled protein A. Sizes are given in kilodaltons.

precipitated by the addition of trichloroacetic acid as described previously (3). Pellets were washed twice with ice-cold diethyl ether and resuspended in 1 volume of sample buffer.

Protein purification and NH₂-terminal sequencing. An immunoaffinity column was prepared as described previously (18) with 4 ml of antiserum per ml of protein A-Sepharose. Then 10 mg of a hydrogenosome-enriched preparation diluted in ice-cold lysis buffer containing protease inhibitors (50 mM Tris [pH 7.5], 1% Nonidet P-40, 100 μ g of leupeptin per ml, and 400 μ g of *N*- α -tosyl-L-lysine chloromethyl ketone per ml) was passed through the column and washed with 10 column volumes of lysis buffer (10 mM phosphate buffer [pH 6.8], 0.25 M NaCl, 50 mM Tris [pH 7.5], 20 mM EDTA) and 10 mM phosphate buffer (pH 6.8). Protein was eluted from the column with 0.1 M glycine at pHs ranging from 5.0 to 1.9. All chromatography was performed at 4°C. Fractions containing the β -SCS protein were pooled, concentrated by ultrafiltration at 4°C with Centricon 30 (Amicon), and subjected to SDS-12.5% PAGE. The gel was electroblotted to polyvinylidene difluoride (Bio-Rad) in 0.5 $\times$ Towbin reagent for 12 h at 20 V (20). The band of interest was identified by staining with Coomassie blue, excised, and subjected to microsequence analysis by Edman degradation.

Nucleotide sequence accession number. The DNA sequence of gene B presented in Fig. 2 has been deposited with GenBank under accession number M97553.

RESULTS

Isolation of cDNAs encoding hydrogenosomal proteins. To isolate genes encoding hydrogenosomal proteins of *T. vaginalis*, we screened a lambda gt11 cDNA expression library with a polyclonal antiserum raised against *T. vaginalis* hydrogenosomes. Immunoblot analysis shows that this antiserum recognizes several dominant proteins as well as many less abundant proteins (Fig. 1). Several cDNA clones were isolated from the expression library, based on their reactivity with the antihydrogenosomal serum. The sequences of

two of these clones, cDNA-A and cDNA-B, show that they are derived from similar, nonidentical genes. A comparison of the predicted amino acid sequences of these cDNAs reveals 6 amino acid differences in a 61-amino-acid overlap (Fig. 2). Of the few nucleotide changes observed (12%), the majority are in the third position of the codon. In contrast to the coding regions, the short 3' untranslated regions of cDNA-A and cDNA-B are totally divergent. Thus, these cDNAs appear to be derived from two nonallelic genes that encode similar hydrogenosomal proteins.

Using a unique 45-bp sequence in the 3' untranslated region of cDNA-B (nucleotides 1228 through 1273, Fig. 2) as a primer in an anchored polymerase chain reaction (30), we generated a full-length cDNA called cDNA-B'. This cDNA was then used to isolate the *T. vaginalis* gene (gene B) by screening a genomic clone library. The sequences of this gene (Fig. 2) and its corresponding full-length cDNA (cDNA-B'; data not shown) are identical, showing that the gene lacks introns.

Gene identification. Sequence analysis of gene B reveals an open reading frame of 1,224 bp encoding a polypeptide of 407 amino acids (Fig. 3). The calculated molecular mass of this protein is 43,980 Da. Computer analysis of the predicted protein (34) reveals a 43% identity to the 41,390-kDa β -SCS from *E. coli* (Fig. 3) (7). Taking conservative amino acid changes into account, these two proteins have a 65% primary sequence similarity. This sequence identity is statistically significant ($z = 108$) when evaluated by the RDF program (28). The RDF program compares the deduced amino acid sequence of the *T. vaginalis* β -SCS gene with those of *E. coli* β -SCS and randomly permuted versions of *E. coli* β -SCS; similarity values greater than 10 ($z > 10$) are considered biologically significant.

Only one internal gap, comprising 8 amino acids, must be introduced to optimize the alignment of the *T. vaginalis* and *E. coli* β -SCS proteins (Fig. 3). The only other differences in alignment are found at the termini of the proteins. The predicted sequence of the *T. vaginalis* β -SCS has 2 additional amino acids at the C terminus and 9 additional amino acids at the amino terminus.

Gene copy number and mRNA characterization. Southern blot analysis of *T. vaginalis* genomic DNA shows that β -SCS is encoded by a multigene family, in agreement with the isolation of the two nonidentical cDNAs. The number of bands hybridizing to the β -SCS gene probe indicates the presence of at least three gene copies (Fig. 4A). The genes give rise to a single RNA band of approximately 1,300 nucleotides, as detected by Northern RNA blot analysis (Fig. 4B).

We used S1 nuclease protection to map the 5' end of the β -SCS transcript. With a 400-nucleotide fragment encompassing the 5' end of the gene (Fig. 5A), we observed dominant bands ranging in size from 159 to 163 nucleotides in protection assays. Whether these multiple bands are the result of breathing near the end of the duplex or represent variable 5' ends all clustered within a few nucleotides is unclear. In either case, these data map the start of the mRNA approximately 15 nucleotides upstream of the initiation codon (Fig. 5B). This maps within a few nucleotides of the 5' end of the apparent full-length cDNA (cDNA-B') produced by primer extension (data not shown). It is of interest that the only other characterized *T. vaginalis* transcript (24) and most of the transcripts examined from two other anaerobic protists, *Giardia lamblia* (25) and *Entamoeba histolytica* (11), contain unusually short 5' untranslated regions.

We also mapped the 3' end of the β -SCS transcript by using RNA protection assays. This results in a cluster of bands approximately 190 nucleotides in size and a smaller band of approximately 160 nucleotides (Fig. 5C). The smaller band maps within an A+T-rich region and is probably the result of breathing of the duplex. The A+T-rich nature of the 3' flanking regions could likewise explain the multiple higher-molecular-weight bands. These data indicate that the 3' end of the transcript derived from β -SCS gene B is approximately 65 nucleotides downstream of the stop codon. In contrast, the 3' untranslated region of cDNA-A is shorter, with a poly(A) tail occurring 20 nucleotides downstream of the stop codon (Fig. 2).

Since nothing is known regarding the regulation of gene transcription in this ancient eukaryote, it is notable that the sequences upstream of the apparent start of transcription of the β -SCS gene lack typical transcription regulation motifs, such as TATA or CCAAT boxes (Fig. 2). Analysis of the 3' flanking region, however, does show that the poly(A) addition sites of the two cDNAs are flanked by sequences (underlined in Fig. 2) similar to the polyadenylation consensus sequence AATAAA found in genes of higher eukaryotes (36).

Isolation, localization, and amino-terminal sequence analysis of the 43-kDa β -SCS protein. The comparison of the amino acid sequences derived from the β -SCS genes of *T. vaginalis* and *E. coli* show that there are 9 amino acids at the N terminus of the *T. vaginalis* protein that are absent in the *E. coli* protein (Fig. 3). By analogy with the sequences shown for nucleus-encoded mitochondrial proteins (20, 43) and the only other hydrogenosomal protein examined to date (24), these data suggest that *T. vaginalis* β -SCS is synthesized with a 9-amino-acid leader sequence that is proteolytically removed from the mature protein found in the organelle.

To test this hypothesis, we isolated β -SCS from *T. vaginalis* hydrogenosomes and sequenced the amino terminus. *T. vaginalis* β -SCS cDNA-A (Fig. 2) was fused to the TrpE protein in a pATH20 vector (10). The fusion protein was then expressed in bacteria, gel purified, and used to immunize rabbits. The resulting antiserum immunoprecipitates an in vitro translation product of 43 kDa generated from transcripts of the cloned full-length cDNA-B' (data not shown). Moreover, this antiserum immunoprecipitates a 43-kDa protein present in *T. vaginalis* hydrogenosome-enriched preparations, consistent with the size of the in vitro translation product of the β -SCS gene (Fig. 6).

We used the β -SCS antiserum to isolate β -SCS from hydrogenosome-enriched fractions of *T. vaginalis* by immunoaffinity chromatography. To prevent protein degradation, cysteine protease inhibitors (29) were included in the preparation of *T. vaginalis* cell extracts loaded on the chromatography column. This procedure provided an enrichment of a 43-kDa protein that was electroblotted onto a polyvinylidene difluoride filter and subjected to amino-terminal sequence analysis. This analysis showed that the first 11 amino acid residues of the immunoaffinity-purified β -SCS unambiguously match amino acids 10 through 21 predicted from the sequence of the gene (Fig. 7). We also determined the amino acid composition of the affinity-purified protein. The results agree with the composition predicted by the gene sequence, further confirming the identity of the sequenced protein as β -SCS (data not shown). These data demonstrate that the isolated protein is the product of the gene we examined or a gene that encodes an identical sequence at the mature amino terminus. Homogeneity of the amino-terminal sequence of

FIG. 2. Comparison of the nucleotide sequences and deduced amino acid sequences of gene B and cDNA-A. The nucleotide sequence of gene B is numbered 5' to 3' starting with the ATG triplet that encodes the initial methionine. The deduced amino acid sequence is shown above the nucleotide sequence. The partial cDNA-A nucleotide sequence is below gene B. The DNA sequence of cDNA-B is identical to that of gene B over the corresponding region (nucleotides 924 through 1264). Nucleotides that are identical between gene B and cDNA-A are denoted by asterisks. Amino acid differences resulting from nucleotide changes are indicated below the cDNA-A sequence. Arrows mark the beginnings and ends of the transcripts as defined by RNA protection analysis. The nontranscribed sequence is shown in lowercase letters. Possible polyadenylation motifs in the 3' flanking region are underlined.

FIG. 3. Comparison of the deduced amino acid sequences of β -SCSs from *T. vaginalis* and *E. coli*. The Protein Identification Resource of the National Biomedical Research Foundation was searched for sequences showing similarity to that of the *T. vaginalis* β -SCS, and amino acids were aligned for maximum similarity by using the FASTA program (34). Identical residues are boxed, and conservative changes are denoted by asterisks. Dashes indicate gaps.

the protein and identity with the sequence predicted by the gene argue that the product of this gene is represented by the purified protein that was subjected to amino-terminal sequence analysis.

These data indicate that the β -SCS found in hydrogenosomes has been posttranslationally processed to remove the first 9 amino acids at its amino terminus. The fact that processing of this 44-kDa protein removes less than 2 kDa has precluded a definitive resolution of the unprocessed and processed forms of the protein by SDS-PAGE analysis (our unpublished data).

Interestingly, this apparent nonapeptide leader sequence on β -SCS is very similar to the amino-terminal extension of the only other hydrogenosomal protein sequenced to date (Fig. 7) (24). Moreover, the hydrogenosomal amino-terminal leader sequences have a number of properties in common with the leader sequences of nucleus-encoded proteins that are targeted to the matrix of the mitochondria.

To determine the location of β -SCS in hydrogenosomes, we subjected purified hydrogenosomes to ice-cold 100 mM sodium carbonate (pH 11.5) treatment and then high-speed centrifugation to fractionate soluble proteins and integral membrane proteins (14). Analysis of the fractions for the presence of β -SCS with SDS-PAGE and immunoblotting

shows that this protein is contained within the soluble fraction of the organelle (Fig. 8). These data are consistent with a matrix localization of β -SCS. We cannot exclude its presence in the intermembrane space of hydrogenosomes, since all nonintegral membrane proteins would be predicted to partition into the soluble fraction. However, by analogy with the location of the mitochondrial β -SCS in the matrix of this organelle, the data shown in Fig. 8 support a matrix location for this enzyme in hydrogenosomes.

DISCUSSION

We identified a gene encoding a hydrogenosomal protein of *T. vaginalis* by screening a cDNA expression library with antisera raised against purified hydrogenosomes. This gene encodes β -SCS, based on a 65% sequence similarity (43% identity) with *E. coli* β -SCS (7). The reactivity of *T. vaginalis* β -SCS with antihydrogenosomal sera is in agreement with metabolic studies on hydrogenosomes. SCS activity has been localized to hydrogenosomes, where it appears to catalyze substrate-level production of ATP by converting succinyl-coenzyme A and ADP to succinate and coenzyme A (22, 40).

SCS falls into two groups according to size and subunit

FIG. 4. Analysis of total genomic DNA and total RNA. (A) Southern blot analysis of DNA. DNA samples were digested with *Bgl*II (lane 1), *Cla*I (lane 2), and *Hind*III (lane 3). Fragments were size fractionated in agarose, transferred to nitrocellulose, and hybridized with a 32 P-labelled β -SCS cDNA-B probe. Size markers are in kilobases. (B) Northern blot analysis of RNA. Total RNA was prepared, size fractionated in agarose-formaldehyde gels, transferred, and hybridized as described for panel A. The sizes are given in nucleotides.

composition (5, 46). The large SCS complex, found in *E. coli*, is an $\alpha_2\beta_2$ tetramer with an apparent molecular mass of approximately 140 kDa. The small SCS complex, found in mammalian mitochondria and gram-positive bacteria, is an $\alpha\beta$ dimer with an apparent molecular mass of approximately 70 kDa. Recently, the *T. vaginalis* SCS holoenzyme was reported to have an apparent molecular mass of approximately 140 kDa (22). Whether this protein is composed of an $\alpha_2\beta_2$ tetramer is yet to be determined.

β -SCS is encoded in *T. vaginalis* by a multigene family composed of at least three genes. We have demonstrated that at least two of the genes are transcribed. Whether the proteins encoded by multiple β -SCS genes are functionally equivalent or possess differing specificities remains to be determined. It is possible that they have different nucleotide specificities (ATP or GTP) or that they play a role in ketone metabolism, as is known for certain mammalian and protozoan SCSs (22, 23, 45).

We isolated *T. vaginalis* β -SCS and determined its location in hydrogenosomes as well as the N-terminal amino acid sequence of the protein. β -SCS partitions into the soluble fraction of hydrogenosomes, consistent with its location within the matrix of the organelle. Interestingly, comparison of the determined amino-terminal sequence with that deduced from the gene shows that the purified protein lacks the first 9 amino acids that are encoded in the gene. Although we cannot rigorously exclude proteolytic degradation of *T. vaginalis* β -SCS during purification, a number of lines of evidence argue against this. The protein migrates as a distinct band on SDS-PAGE gels. Furthermore, the amino acid sequence analysis identified the first 11 residues with no ambiguities, ruling out random degradation or heterogeneity at the amino terminus. Finally, the protein predicted by the *T. vaginalis* β -SCS gene contains an extra 9 amino acids at

FIG. 5. Mapping the ends of the *T. vaginalis* β -SCS gene transcript by using RNase protection assays. (A) Restriction map of the genomic subclone pSCS-53. The box represents the β -SCS coding region. Fragments 1 and 2 were used to map the ends of the transcript. (B) S1 nuclease mapping of the 5' end. DNA fragment 1 was 32 P end labelled and annealed to total RNA. Lanes: 1, fragment 1 only; 2, fragment 1 plus S1 nuclease; 3, fragment 1 plus yeast tRNA and S1 nuclease; 4, fragment 1 plus *T. vaginalis* RNA and S1 nuclease; 5, Maxam-Gilbert A/G sequence ladder of fragment 1; 6, Maxam-Gilbert T/C ladder of fragment 1. The coding strand sequence is shown with an arrow marking the end of the transcript. (C) S1 nuclease mapping of the 3' end. Lanes 1 through 4 contain the same samples as those in panel B, except that fragment 2 was 32 P labelled and used in annealing reactions before nuclease digestion. Sizes are given in nucleotides.

its amino terminus when compared with β -SCS in *E. coli* (7) and the mature protein found in pig heart mitochondria (2). Thus, the *T. vaginalis* β -SCS appears to contain a 9-amino-acid leader sequence that is proteolytically removed upon maturation of the protein.

Given the location of β -SCS in the soluble fraction of the hydrogenosome, our results suggest that the 9-amino-acid extension acts as a signal sequence for targeting and translocating the protein into the matrix of this organelle. We have shown that β -SCS and ferredoxin, another hydrogenosomal protein, are synthesized in the cytosol and posttranslationally translocated into hydrogenosomes (27). In this regard, hydrogenosomes are similar to mitochondria (1), chloroplasts (16), glycosomes (19), and peroxisomes (15), i.e., organelles whose protein constituents contain targeting signals that act posttranslationally to direct them into the correct subcellular compartment.

The similarity of the β -SCS leader sequence and the

FIG. 6. Immunoprecipitation and blot analysis of *T. vaginalis* β -SCS. The in vitro transcription product of β -SCS gene B was translated in vitro (lane 1). The antiserum raised against the β -SCS-TrpE fusion protein was used in immunoprecipitation reactions containing a hydrogenosome-enriched fraction of *T. vaginalis* cell lysates (lane 2). Proteins were separated by SDS-PAGE on gradient 5 to 20% polyacrylamide gels, electroblotted to nitrocellulose, probed with the anti- β -SCS serum, and reacted with 125 I-labelled protein A.

apparent leader sequence of the only other hydrogenosomal protein yet examined, ferredoxin (24), is striking (Fig. 7). Both are unusually short compared with amino-terminal presequences that are known to direct proteins into other organelles (43). Also, the first three residues of the apparent leader sequences of β -SCS and ferredoxin are identical (Met-Leu-Ser), and both contain an Arg at position -2 relative to the predicted cleavage site. Interestingly, 40% of the known mitochondrial presequences that target proteins to the matrix of mitochondria begin with Met-Leu, and 16% begin with Met-Leu-Ser. In contrast, presequences that are known to target proteins to chloroplasts almost invariably begin with Met-Ala (43). Also, a majority of mitochondrial presequences contain an Arg at either position -2 or position -10 relative to the cleavage site (20, 43). Finally,

A

<i>T.v.</i> β -SCS	F	N	I	L	E	W	Q	S	K	E	I	-	-	-
mito β -SCS	L	N	L	Q	E	Y	Q	S	K	K	L	-	-	-
<i>E.c.</i> β -SCS	M	N	L	H	E	Y	Q	A	K	Q	L	-	-	-

B

<i>T.v.</i> β -SCS	M	L	S	S	S	F	A	R	N	-	-	-
<i>T.v.</i> Fd	M	L	S	Q	V	C	R	F	-	-	-	-

FIG. 7. (A) Comparison of the mature amino termini of β -SCS proteins from different organisms. The amino-terminal sequences of mature β -SCSs isolated from the hydrogenosome of *T. vaginalis* (*T.v.*), pig heart mitochondria (mito) (2), and *E. coli* (*E.c.*) (7) are shown at the top. Residues that are identical in all three proteins are boxed, and those conserved are designated with an asterisk. (B) Comparison of the amino-terminal leader sequences of *T. vaginalis* hydrogenosomal proteins. The leader sequences of hydrogenosomal proteins β -SCS and ferredoxin (Fd) (24) are aligned to maximize similarity. Identical residues are boxed.

FIG. 8. Separation and immunoblot analysis of hydrogenosomal membrane and soluble fraction proteins. Purified hydrogenosomes (1 ml) were subfractionated by a sodium carbonate procedure. The membrane fraction was resuspended in 0.2 volume of sample buffer, and the soluble protein fraction was resuspended in 1 volume of sample buffer. Equivalent volumes of hydrogenosomes, membrane and soluble fractions were separated by SDS-15% PAGE and stained with Coomassie brilliant blue (A) or electroblotted and probed with anti- β -SCS serum (B). Lanes: 1, 5 μ g of *T. vaginalis* total lysate; 2, purified hydrogenosomes; 3, soluble protein fraction; 4, membrane protein fraction. Size markers are in kilodaltons.

plotting the 9 amino-terminal amino acids of β -SCS on an α -helical wheel shows that it can form an amphiphilic α -helix, with one side of the helix containing four of five polar or charged residues (residues 4, 5, 8, and 9), and the other side containing three of four nonpolar residues (residues 2, 6, and 7). Potential for formation of amphiphilic α -helices is also a characteristic of the leader sequence of hydrogenosomal ferredoxin (24, 35) and mitochondrial presequences (42). Amphiphilicity appears to be necessary for mitochondrial presequence function, but helical structure may not be essential (37).

In contrast to the similarity of hydrogenosomal and mitochondrial leader sequences, it should be noted that mitochondrial leader sequences are usually longer than the apparent hydrogenosomal presequences identified here (20). Nevertheless, nucleus-encoded mitochondrial proteins have been reported to contain presequences as short as 13 amino acids (48). Experiments are under way to test whether the hydrogenosomal presequences identified here can act to direct proteins into hydrogenosomes. It will also be of interest to determine whether these presequences can direct hydrogenosomal proteins into mitochondria in a heterologous system.

It is intriguing that SCS, which is confined to mitochondria in most eukaryotes (5, 6), is present in hydrogenosomes in *T. vaginalis*, an anaerobic eukaryote that lacks mitochondria. This and the similarity between the amino-terminal leader sequences of hydrogenosomal and mitochondrial proteins support the idea that hydrogenosomes are derived from mitochondria or from a common progenitor organelle. It is also noteworthy that hydrogenosomal ferredoxin is more similar to ferredoxins found in mitochondria and aerobic bacteria than to those of anaerobic bacteria (24). On the other hand, the presence of pyruvate ferredoxin oxidoreductase and hydrogenase, two enzymes that are often found in anaerobic prokaryotes and never in mitochondria, in hydrogenosomes (31, 32) is consistent with the idea that hydrogenosomes are derived from endosymbiosis with an anaerobic bacterium. In this event, the shared properties of

hydrogenosomes and mitochondria would be the result of convergent evolution. Additional studies on hydrogenosomes from *T. vaginalis* and other organisms should provide further insight into the origin and biogenesis of this unusual organelle.

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