Trichomonas vaginalis Hmp35, a Putative Pore-forming Hydrogenosomal Membrane Protein, Can Form a Complex in Yeast Mitochondria*

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Received for publication, April 17, 2003, and in revised form, May 20, 2003
Published, JBC Papers in Press, May 23, 2003, DOI 10.1074/jbc.M304032200

An abundant integral membrane protein, Hmp35, has been isolated from hydrogenosomes of Trichomonas vaginalis. This protein has no known homologue and exists as a stable 300-kDa complex, termed HMP35, in membranes of the hydrogenosome. By using blue native gel electrophoresis, we found the HMP35 complex to be stable in 2 M NaCl and up to 5 M urea. The endogenous HMP35 protein was largely protease-resistant. The protein has a predominantly β-sheet structure and predicted transmembrane domains that may form a pore. Interestingly, the protein has a high number of cysteine residues, some of which are arranged in motifs that resemble the RING finger, suggesting that they could be coordinating zinc or another divalent cation. Our data show that Hmp35 forms one intramolecular but no intermolecular disulfide bonds. We have isolated the HMP35 complex by expressing a His-tagged Hmp35 protein in vivo followed by purification with nickel-agarose beads. The purified 300-kDa complex consists of mostly Hmp35 with lesser amounts of 12-, 25–27-, and 32-kDa proteins. The stoichiometry of proteins in the complex indicates that Hmp35 exists as an oligomer. Hmp35 can be targeted heterologously into yeast mitochondria, despite the lack of homology with any yeast protein, demonstrating the compatibility of mitochondrial and hydrogenosomal protein translocation machineries.

Trichomonas vaginalis is a deep-branching protist that lacks archetypal eukaryotic “aerobic” organelles, specifically mitochondria and peroxisomes. This microaerophilic human-infective parasite carries out fermentative carbohydrate metabolism within hydrogenosomes. Hydrogenosomes are bounded by double membranes and produce ATP by substrate level phosphorylation (1). Hydrogenosomes are also found in certain chytrids, ciliates, and fungi, in lineages that are phylogenetically distant to the Parabasalian lineage to which chytrids, ciliates, and fungi belong (1–4). Currently, several lines of evidence support a common endosymbiotic ancestry for hydrogenosomes and mitochondria by examining the origin of their chaperonins, metabolic enzymes, and membrane proteins. Chaperonin genes, specifically heat shock protein (Hsp)170, cpm160, and Hsp10 (11–14), and the IscS enzyme, involved in FeS cluster formation (15) appear to have a mitochondrial origin. However, analyses of metabolic enzymes such as hydrogenase (16, 17), which is typically found in anaerobic bacteria, present a far more ambiguous picture. Phylogenetic analyses of another metabolic enzyme, pyruvate:ferredoxin oxidoreductase, revealed a clustering of all eukaryotic sequences known to date but failed to reveal a relationship to any particular eubacterial group (6). Only one trichomonad hydrogenosomal membrane protein, Hmp31, has been analyzed to date, and it was shown to be a distant homologue of the ADP/ATP carrier, a member of the mitochondrial carrier family (18).

We have continued to study hydrogenosomal membrane proteins to gain further information on the nature of the endosymbiont that gave rise to the trichomonad hydrogenosome, and to further examine the possibility that the same endosymbiont gave rise to both mitochondrial and trichomonad hydrogenosomes. Organellar membrane proteins may originate from pre-existing proteins from the endosymbiont that evolve to fulfill new functions in the emerging proto-organelle. For instance, the Toe75 protein pore translocase in chloroplasts has a homologue of unknown function in cyanobacteria that shows similar pore characteristics (19). Alternatively, “new” protein families may have emerged such as the mitochondrial carrier family proteins that translocate ATP and various solutes across the organellar membrane in mitochondria (20). Likewise, most of the protein translocases in mitochondria have no distinguish-

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* This work was supported by National Institutes of Health Grant AI 27857 and a Burroughs-Wellcome Scholar award in Molecular Parasitology (to P. J. J.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of National Institutes of Health Training Grant AI07323.

¶ Supported by the Deutsche Forschungsgemeinschaft.

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1 The abbreviations used are: Hsp, heat shock protein; MOPS, 4-morpholinepropanesulfonic acid; Tricine, N,N′-bis(hydroxy-1,1-bis(hydroxy-

methyl)ethyl)glycine; TLCK, N,N′-tosyl-L-lysine chloromethyl ketone; DTT, dithiothreitol; TCEP, Tris(carboxyethyl)phosphate hydrochloride; AMS, 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid; PMSF, phenylmethylsulfonyl fluoride; BN-PAGE, blue native polyacrylamide gel electrophoresis; IMS, intermembrane space.

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This paper is available online at http://www.jbc.org
able homologues in prokaryotes and appear to have evolved with organelles (21).

Here we describe a novel hydrogenosomal membrane protein, Hmp35, that has no known homologue in prokaryotes or eukaryotes to date. It is a relatively abundant membrane protein that exists as an integral membrane complex. The exceptional stability of the complex under stringent salt, urea, and protease treatment is similar to that of bacterial and organelar outer membrane protein pores. Although it has no primary sequence homology to those proteins, Hmp35 has a predominant predicted β-sheet structure similar to that found in the outer membrane proteins of Gram-negative bacteria, mitochondria, and plastids (22). Taken together, these data suggest a similar function for Hmp35. Finally, we demonstrate that despite the absence of a homologue in yeast or of a recognizable mitochondrial targeting sequence, the Hmp35 can be heterologously expressed in yeast and is targeted to yeast mitochondrial membranes in vivo.

EXPERIMENTAL PROCEDURES

Organisms and Strains—T. vaginalis T1 (gift from J. F. Alderete) and C1 (ATCC 30001) strains were used where indicated in this study. Saccharomyces cerevisiae MB2-22 wild-type strain (23) was used as a host for further analyses. T. vaginalis T1 (gift from J. F. Alderete) and C1 (ATCC 30001) strains were used where indicated in this study.

Isolation of Hydrogenosomes and Subfractionation—Hydrogenosomes were isolated from T. vaginalis as described previously (24). Isolated hydrogenosomes were stored at –80 °C in freezing buffer (250 mM sucrose, 10 mM MOPS-KOH, 0.5% bovine serum albumin, 10 μg/ml leupeptin, 50 μg/ml N-p-tosyl-L-lysine chloromethyl ketone (TLCK), pH 8). Hydrogenosomes were washed in 250 mM sucrose, 10 mM MOPS-KOH, pH 8 (SM) prior to any manipulation. Hydrogenosomes were subfractionated into integral membrane and soluble proteins by sodium carbonate extraction as described before (18). Sequencing of MP40 Proteins—Hydrogenosomal membrane proteins were size-separated by 15% SDS-PAGE and stained with Coomassie Blue dye R-250, and a gel slice containing 5 μg of the 35–40-kDa protein was excised, washed with 50% acetonitrile, and subjected to tryptic digest and peptide sequencing at the Harvard University Microchemistry Facility (Cambridge, MA).

Generation of Antiserum against Endogenous Membrane Proteins and Recombinant Hmp35—Polyclonal antisera against 35–40-kDa hydrogenosomal membrane proteins (MP40) were raised in rabbits by 40-kDa polyclonal antisera against 35–40-kDa tryptic digest and peptide sequencing at the Harvard University Microchemistry Facility (Cambridge, MA). Taken together, these data suggest a similar function for Hmp35. Finally, we demonstrate that despite the absence of a homologue in yeast or of a recognizable mitochondrial targeting sequence, the Hmp35 can be heterologously expressed in yeast and is targeted to yeast mitochondrial membranes in vivo.

| Primer | Sequence |
|--------|----------|
| MP40F  | 5′-CGGAGATCTGGCAGCAGATACATCCGTTGAACTGT-3′ |
| MP40R  | 5′-ACGCCTGCAGGTTTCCACCTGAGGATTG-3′ |
| MP40B  | 5′-CGGAGATCTCCATGGCAGAACAGACATCTC-3′ |
| MP40S  | 5′-CGCGGATCCCTAAAGACATTCGGAATG-3′ |
| Nmp35H | 5′-GTTCATATGGAAACAAGACATCGAACAC-3′ |
| BAmp35H| 5′-CGGAGATCTTTGGAGTGATGATTGGTTACCTCAGCGGAAAG-3′ |

Probe from the cDNA clone CD40.31, yielding a positive clone, MP40.1, with a 2-kb EcoRI fragment bearing the complete hmp35 open reading frame.

Plasmid Construction—A 969-bp fragment was generated by PCR from the genomic clone MP40.1 using the primers MP40F and MP40R (Table I) to introduce a 5′ BamHI and a 3′ SalI restriction enzyme site, respectively, for subsequent ligation into the vector pQE20 (Qiagen) to yield the expression construct pEP40.17 with an in-frame hemagglutinin tag situated at the N terminus of the hmp35 open reading frame.

For construction of the yeast transformation plasmid pRS133-hmp35, the genomic DNA clone MP40.1 was amplified with the PCR primers MP40F and MP40S (Table I) to generate the open reading frame of hmp35 with a 5′ BamHI and a 3′ SalI site, respectively, to allow cloning into the pRS313 vector.

For generation of the pHmp35H plasmid, we used the previously described (18) plasmid construct pHmp31-(HA)3 which carries a neo-mycin phosphotransferase (neo) cassette that allows selection of transformants in T. vaginalis (25). The hmp35 open reading frame was amplified from the genomic clone MP40.1 using the forward primer pHmp35H (Table I) to introduce an NdeI restriction site at the 5′ end of the PCR product and the reverse primer BAmp35H (Table I) to introduce a hexahistidine codon at the 3′ end, followed by a BamHI restriction site. Following restriction digest with NdeI and BamHI, the purified PCR product was introduced into the corresponding sites in the restricted pHmp31-(HA)3 plasmid.

Expression and Purification of His-Hmp35—The pEP40.17 plasmid was transformed into E. coli M15 (pREP4) cells and selected with 100 μg/ml ampicillin, 30 μg/ml kanamycin in LB media. A 500-ml culture was grown at 37 °C to A600 = 0.8 before induction with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 37 °C. Harvested cells were solubilized in 6 M guanidine hydrochloride, 1% Triton X-100, 0.1 M NaH2PO4, 10 mM Tris, pH 8, for 1 h at room temperature. The insoluble material was pelleted at 10,000 × g for 30 min at 4 °C, and the resulting lysate applied to a nickel-nitriolitrctic acid-agarose column. The column was washed four times with 8 M urea, 0.1 M NaH2PO4, 20 mM imidazole, 10 mM Tris, pH 6.3. Specifically bound proteins were eluted with 8 M urea, 0.1 M NaH2PO4, 0.25 M imidazole, 10 mM Tris, pH 4.5, and neutralized.

Modulation of Cysteine Residues with AMS—200 μg of isolated hydrogenosomes were initially boiled for 5 min in SM, 50 mM NaCl, 0.5% SDS and cooled to 37 °C. Controls with His-tagged Hmp35 recombinant protein (3 μg per assay) were processed in parallel. Each sample was then incubated for 1 h at 37 °C with or without 5 mM EDTA or 10 mM Tris [2-carboxyethyl] phosphine hydrochloride (TCEP), or 5 mM H2O2, or a mixture of these reagents as indicated. All samples were precipitated with 10% trichloroacetic acid, resuspended in 100 μl of alkylating solution (100 mM iodoacetamide, 100 mM Tris, 100 mM Tris, 10 mM EDTA, pH 9.5), and incubated for 5 min at 4 °C. The reaction was stopped by trichloroacetic acid precipitation, and all samples were resuspended in 50 μl of 10 mM TCEP, 100 mM Tris, 0.5% SDS, 10 mM EDTA, pH 9.5, and incubated for 1 h at 44 °C. Some samples were further treated with 4-acetamido-4-maleimidylstilbene-2,2-disulfonic acid (AMS) at a final concentration of 25 mM, and all samples were incubated for 90 min at 25 °C. Finally, all samples were trichloroacetic acid-acid precipitated and resuspended in reducing Laemmli sample buffer for 12% SDS-PAGE separation and Western analysis.

Blue Native Gel Electrophoresis of Organelles—Hydrogenosomes were solubilized at a concentration of 1 mg/ml for 30 min on ice in n-dodecyl maltoside or Triton X-100 at indicated concentrations in the presence of 20 mM MOPS, 0.2 M or 0.5 M NaCl, 1 mM MgCl2, 10% dodecylmaltoside, or 2 mM phenylmethylfluoride (PMPS), pH 8.0. Carbonate-extracted hydrogenosomal membranes were solubilized at a protein concentration of 0.1 mg/ml for 30 min on ice in 0.5% n-dodecyl maltoside or 0.5% Triton X-100 in the presence of 20 mM MOPS, 0.5 M NaCl, 1 mM MgCl2, 10% glycerol, 2 mM PMPS, pH 8.0. Insoluble material was removed by centrifugation at 100,000 × g for 15 min at 4 °C.
Denatured samples were generated by heating hydrogenosomes (1 mg/ml protein concentration) at 95 °C for 5 min. 0.5% SDS, 20 mM MOPS, 1 mM MgCl₂, 10% glycerol, 2 mM PMSF, pH 8.0. The solubilized proteins were analyzed by blue native electrophoresis on a 6–16% linear polyacrylamide gradient (26). Solubilization of mitochondria (2.5 mg/ml protein concentration) was performed in 0.5% digitonin as described previously (27).

Protease Treatment of Hydrogenosomes—Hydrogenosomes (200 µg of total protein) were incubated in SM buffer for 30 min at 37 °C in the presence of TCEP and/or 10 mM EDTA as indicated and treated with 0.25 mg/ml proteinase K (Roche Applied Science) for 30 min at 0 °C. The digestion was inhibited with 2 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min on ice. Samples were trichloroacetic acid-precipitated and resuspended in Laemmli sample buffer for SDS-PAGE. A similar protease treatment was performed on hydrogenosomes initially solubilized at 1 mg/ml protein concentration in 1% Triton X-100, 0.1 M NaCl, 20 mM MOPS, 10% glycerol, 2.5 mM MgCl₂, pH 8.0, and samples were processed for SDS-PAGE. Sodium carbonate-extracted hydrogenosomal membranes were resuspended at 0.1 mg/ml protein concentration in SM and treated with 0.1 mg/ml or 0.25 mg/ml proteinase K for 20 min on ice. Following inhibition with 2 mM PMSF, the membranes were recovered by centrifugation at 100,000 × g and resuspended in SM for trichloroacetic acid precipitation before resuspension in Laemmli sample buffer for SDS-PAGE.

Transformation of Yeast—The S. cerevisiae wild-type strain MB2-22 was transformed with the plasmid pH3S13-hmp35 under the manipulation of the histidine marker. Standard genetic techniques were used for growth and for transformation of yeast strains (28).

Selectable Transformation of T. vaginalis—T. vaginalis T1 cells were electroporated with 50 µg of pHmp35H as described previously (25), and transformants (35H) harboring the plasmid were selected with 200 µg/ml geneticin (Invitrogen).

Crude Fractionation of T. vaginalis 35H Transformants—Cells from cultures of the 35H transformants were broken in a cell disruptor (Energy Service Co.) and fractionated by centrifugation at 12,000 × g into a crude cytosolic fraction and a crude organellar fraction as described previously (18).

Isolation and Characterization of Hmp35, a Hydrogenosomal Membrane Protein—To pursue the identification of hydrogenosomal membrane proteins from T. vaginalis, we targeted a number of proteins in the 35–40-kDa range (Fig. 1A, lane 3, MP40) that are of intermediate abundance. The MP40 proteins were microsequenced on a small scale and isolated on a larger scale to raise rabbit polyclonal antibodies, anti-MP40. A positive cDNA clone, CD40.31, was isolated by screening an expression T. vaginalis cDNA library. Four of the peptide sequences obtained from the MP40 proteins exactly matched stretches in the translated CD40.31 sequence, confirming that a gene encoding one of the MP40 proteins had been cloned (Fig. 2A). This gene was named hmp35, for hydrogenosomal membrane protein 35. From Northern and Southern analyses performed with the CD40.31 insert (data not shown), the hmp35 gene appeared to be unique. A probe from the CD40.31 clone was used to screen a T. vaginalis genomic DNA library to isolate a clone, MP40.1, that bore the complete hmp35 gene sequence. The gene is flanked by a double initiators sequence 18 bases up-
stream of the initial methionine residue of the predicted protein, which is within the expected range for T. vaginalis genes (30). The conceptual translation of the hmp35 gene open reading frame is presented. Amino acid stretches matching peptide sequence obtained from sequencing the endogenous Hmp35 protein are underlined. Cysteine residues are highlighted by an asterisk and histidine residues by a black dot. B, hydrophilicity and transmembrane profiles of Hmp35.

To investigate the properties of the Hmp35 protein, we raised rabbit polyclonal antibodies (anti-Hmp35) against purified recombinant His-tagged Hmp35. Western analysis of sodium carbonate-extracted hydrogenosomal sample with these antibodies showed that the Hmp35 protein is found in the membrane fraction and not in the soluble fraction, thus confirming that Hmp35 is present in the hydrogenosomes exclusively as an integral membrane protein (Fig. 1B, lanes 2 and 3). The protein, with a calculated mass of 34.6 kDa, migrated at an apparent molecular mass of 39 kDa on SDS-PAGE (Fig. 1B).

Analysis of the Hmp35 Protein Sequence—The deduced amino acid composition of Hmp35 is shown in Fig. 2A. The predicted N-terminal sequence of Hmp35 bears no homology to the cleavable presequence (MAQPAAEQLIAT) that had been found previously (18) in the only other characterized hydrogenosomal membrane protein Hmp31. The Hmp35 protein has an unusually high percentage of charged amino acids (24%), with a correspondingly high proportion of lysine residues (11%), resulting in a basic hydrophilic protein (Fig. 2B, top panel) with a high isoelectric point (pI) of 9.4. In comparison, a yeast mitochondrial membrane protein, Tom40, has only 17% charged residues and has a pI of 5.2. However, the Hmp35 protein has discrete areas of hydrophobicity, which are predicted by the von Heijne transmembrane algorithm to form 4 or 5 potential transmembrane domains (Fig. 2B, both panels). No transmembrane helices were predicted from the TMPRED program. Secondary structure computation using the PHD program predicted the protein structural composition as 47% $\beta$-sheet, 3% $\alpha$-helical, and the rest mixed, suggesting that the protein has predominant $\beta$-sheet structure. Interestingly, Hmp35 has 14 cysteine residues, representing 4.4% of the total amino acid content, with nine of them clustered at the C-terminal third of the protein (Fig. 2A). Seven of these residues are found in close proximity to each other in the 221–265 amino acid region, in the conformation CX$_6$CC$_X$CXX$_{10}$CCXHX$_2$C, where C is a cysteine residue, H is a histidine residue, and X is any residue.

Hmp35 Is a Novel Protein—Data base searches performed using the Hmp35 protein sequence with the BLASTp and tBLASTn algorithms revealed no significant similarity to known proteins. However, by using the WU-BLAST 2.0 program (31), we found that the 154–318 region of Hmp35 had 46% similarity to a 167-amino acid region in a 35-kDa basic membrane lipoprotein, BmpC, from Borrelia burgdorferi, a bacterium of the spirochaete family. This protein is part of a family of outer membrane lipoproteins found in various bacteria.

To find if the residues in the cysteine-rich region in Hmp35 were part of a known motif, we searched the data base using the BLAST program with the short nearly exact matches algorithm using only the 221–265-amino acid region. The subregion from 221 to 231 (Fig. 2A) had 83% similarity, with all cysteine residues conserved, to a zinc binding region from the anaphase-promoting complex subunit 11 protein found in a number of eukaryotes. This short 84–140-amino acid cysteine-rich protein has a RING finger motif (32). The RING (for really interesting new gene) finger is a conserved cysteine-rich domain of 40–60 residues that binds two zinc atoms in a “cross-brace” manner (33). Two variants of this motif have been described, the C3HC4 and the C3H2C3 types, neither of which exactly match the Hmp35 cysteine-rich region, as computed by the ExPaSy PROSITE pattern and profile searches. However, the number and distribution of cysteine and histidine residues in the 221–265 region of Hmp35 are reminiscent of those patterns, and this region may represent a related but distinct motif that binds zinc.

Analysis of Cys Residues in Hmp35—To determine whether the cysteine residues in endogenous Hmp35 are engaged in intermolecular disulfide bonding, we analyzed the migration of Hmp35 by SDS-PAGE under non-reducing conditions. The test hydrogenosomal sample was treated with the alkylating agent N-ethylmaleimide before separation on SDS-PAGE to prevent artifactual oxidation of free cysteines during handling. We found that the migration of the Hmp35 protein was the same in the absence or presence of the reducing agent dithiothreitol (DTT), suggesting that the endogenous protein does not form intermolecular disulfide bridges with itself or with other proteins (Fig. 3A). As a control, Western analysis was performed using antisera against ferredoxin, a protein with three cysteine residues that coordinate iron (34).
determination of the number of disulfide bridges in soluble proteins (35, 36). The hydroxosomes were treated with a reducing (TCEP) or oxidizing agent ($\text{H}_2\text{O}_2$) or left untreated, and then free thiol groups were blocked with the alkylating agents N-ethylmaleimide or iodoacetamide. Finally, after a reduction step to break any induced or endogenous disulfide bridges, the proteins were treated with AMS to modify any resulting free cysteines. By calculating the difference in migration of the protein as determined by Western analysis after this blocking treatment, the number of AMS moieties added can be computed and hence the number of disulfide bridges determined. Because Hmp35 is an integral membrane protein, we performed the first step of denaturing hydroxosomes with SDS to ensure full accessibility of Hmp35 to the various reagents.

To test whether the alkylation and AMS procedures were efficient, we used reduced or oxidized recombinant His$_6$-Hmp35 under denaturing conditions. When the recombinant protein was initially reduced, then alkylated with iodoacetamide before further reduction and treatment with AMS, we found that the protein migrated at the same position as the unmodified protein at 40 kDa (Fig. 3B, lanes 1, 2, and 4), indicating that no free cysteines were available for modification by AMS. When the recombinant protein was initially oxidized prior to the alkylation, reduction and AMS treatment, the relative migration of the protein increased by 7 kDa (Fig. 3B, lanes 3 and 5), suggesting that 14 AMS molecules had been added. This result implies that all the cysteine residues in the recombinant protein had been efficiently modified by AMS, and hence have potential for inter/intramolecular disulfide bond formation (Fig. 3B, lanes 4 and 5). The presence of EDTA in either case did not affect the number of AMS molecules added, as might be expected because the recombinant protein had initially been completely denatured in 8 M urea, and no divalent cations were added to this in vitro assay.

We found that in SDS-denatured hydroxosomal protein, the AMS treatment increased the apparent molecular mass of the endogenous Hmp35 protein from 39 to 40 kDa (Fig. 3B, lanes 6 and 7). This 1-kDa increase would correspond to the addition of two AMS moieties to two cysteine residues. This means that one disulfide bridge is present, most likely intramolecularly, in Hmp35. When the proteins had been reduced prior to the trapping, the migration was unaffected as the endogenous disulfide bridge was reduced and the iodoacetamide reagent efficiently alkylated all the cysteine residues, blocking any reaction with AMS (Fig. 3B, lane 8). The initial oxidizing step did not induce any further disulfide bonds (Fig. 3B, lane 9), unlike in the case of the oxidized recombinant (Fig. 3B, lanes 4 and 5) where all the cysteine residues had been oxidized. This suggests that the endogenous protein is in a form or structure that is incapable of forming extra disulfide bonds even when denatured in SDS. However, when EDTA was included during the oxidizing treatment, the migration of the protein increased by a further 1 kDa (Fig. 3B, lanes 9 and 13). This suggests that a divalent cation may be bound by the endogenous Hmp35 protein, which prevents the induction of further disulfide bonds, unless it is chelated by EDTA during oxidation.

Hmp35 Forms Part of a High Molecular Weight Complex in Hydroxosomal Membranes—To assess whether the Hmp35 protein is in a stable protein complex in hydroxosomal membranes, we adapted the technique of BN-PAGE that has been widely used for analyses of protein complexes in mitochondria. Isolated hydroxosomes were solubilized in two non-ionic, non-denaturing detergents, n-dodecyl maltoside and Triton X-100, and the cleared lysates were subjected to BN-PAGE. Western analysis performed with anti-Hmp35 showed that the Hmp35 protein migrates mostly within a discrete band of ~300 kDa in both detergents (Fig. 4A), suggesting that it forms a stable complex in the hydroxosome. The slight difference in migration between the two detergents used could be due to different micelle sizes.

To investigate whether this complex is held together by ionic interactions, we tested the effect of sodium chloride on its

### Table: Cysteine-rich Hydrogenosomal Membrane Protein Hmp35

| Control   | DTT  | Hmp35 | DTT + Hmp35 |
|-----------|------|-------|-------------|
| NEM       | 220  |       | 220          |
| DTT       | 180  |       | 180          |
|           | 120  |       | 120          |
|           | 60   |       | 60           |
|           | 30   |       | 30           |
|           | 15   |       | 15           |
|           | 10   |       | 10           |

**Fig. 3. Analysis of cysteine residues in Hmp35.** A. Effect of DTT on the migration of endogenous Hmp35. 200 µg of isolated hydroxosomes were alkylated with 20 mM N-ethylmaleimide (NEM, lanes 1 and 2) for 15 min at room temperature and resuspended in Laemmli sample buffer with or without 50 mM dithiothreitol (DTT) at a final protein concentration of 1 µg/µl. As a control for artificial oxidation, hydroxosomes were mock-treated with ethanol (lanes 3 and 4) prior to resuspension at a final protein concentration of 1 µg/µl in Laemmli sample buffer with or without 50 mM DTT. 20 µg of protein was size-separated by 8–20% SDS-PAGE linear gradient, blotted onto PVDF, and immunodecorated with anti-Hmp35 or anti-ferredoxin (Fd) polyclonal antisera. Molecular mass markers are indicated in kDa. B. Modification of cysteine residues with AMS. Isolated hydroxosomes (200 µg of total protein) were denatured by boiling in 0.5% SDS and incubated for 1 h at 37 °C with or without 5 mM EDTA (chelating agent), or 10 mM TCEP (reducing agent), or 5 mM $\text{H}_2\text{O}_2$ (oxidizing agent), or a mixture of these reagents as indicated (lanes 7–13). As controls, 3 µg of purified recombinant His$_6$-Hmp35 protein were subjected to the same reducing or oxidizing treatment under denaturing conditions prior to AMS modification (lanes 2–5). After incubation at 37 °C, all samples were alkylated to block any free cysteines and then reduced. All samples except for those in lanes 1, 6, and 10 were treated with 25 mM AMS. The protein samples (40 µg per lane) were size-separated by 12% SDS-PAGE, transferred to PVDF, and immunodecorated with anti-Hmp35 antisera. Protein sizes were estimated using a calibration curve plotted according to the migration of known standards.
stability. The complex migrated at 300 kDa at all concentrations tested up to 2 M (Fig. 4B, lanes 1–6), without any monomer detected unless the hydrogenosomes were boiled in SDS prior to loading on the gel (Fig. 4B, lane 7). This resistance of the complex to salt shows that ionic interactions are not involved in maintaining its components together.

To determine what part of this complex consists of integral membrane proteins, we solubilized the membrane fraction of sodium carbonate-extracted hydrogenosomes and separated the stable complexes by BN-PAGE. Western analysis with anti-Hmp35 antibodies revealed that Hmp35 still migrated at 300 kDa in n-dodecyl maltoside and Triton X-100 (Fig. 4C). These
**FIG. 5.** **Endogenous native Hmp35 is protease-resistant.**

*Panel A.* Hmp35 in intact or non-denatured solubilized hydrogenosomes is protease-resistant. Hydrogenosomes were preincubated in isotonic SM buffer for 30 min at 37 °C with increasing concentrations of TCEP and with 10 mM EDTA as indicated (lanes 1–10). Half of each sample was treated with 0.25 mg/ml proteinase K (lanes 2, 4, 6, 8, and 10) for 30 min on ice, whereas the remaining half was mock-treated (lanes 1, 3, 5, 7, and 9). Two hydrogenosomal samples were denatured at 95 °C in 0.5% SDS with or without 10 mM EDTA and split into 2 aliquots prior to addition or not of proteinase K (lanes 11–14). Total hydrogenosomal protein solubilized in 1% Triton X-100 was preincubated for 30 min at 37 °C with increasing concentrations of TCEP and with 10 mM EDTA as indicated (lanes 15–24). Half of each sample was treated with 0.25 mg/ml proteinase K (lanes 16, 18, 20, 22, and 24) for 30 min on ice, whereas the remaining half was mock-treated (lanes 15, 17, 19, 21, and 23). Proteins (40 μg of protein per lane) were separated by 15% SDS-PAGE, transferred to PVDF, and detected with anti-Hmp35 antisera.

*Panel B.* The HMP35 complex is protease-resistant. Isolated hydrogenosomes (250 μg of total protein per 250 μl of buffer) were incubated in isotonic buffer with or without 0.25 mg/ml proteinase K (lanes 1, 2, 7, and 8). A second series was solubilized in 0.16% n-dodecyl maltoside in the presence or absence of 0.25 mg/ml proteinase K (lanes 3, 4, 9, and 10). A third series was boiled in 0.5% SDS for 5 min prior to
results show that the HMP35 complex consists essentially of proteins that are not sodium carbonate-extractable and are therefore integral membrane proteins.

We investigated the resistance of the HMP35 complex to urea to determine whether hydrogen bonds were involved in maintaining it. The complex was not completely disassembled until it was exposed to 6 mM urea (Fig. 4D, lane 7) but was maintained at a high molecular weight up to the 5 M urea treatment (Fig. 4D, lanes 1–6). The complex decreased in size by 12 kDa increments as the urea concentration was increased from 1 to 3 M, suggesting the loss of small proteins. When the urea concentration was increased from 3 to 4 M, a decrease of 32 kDa was noted, and a further 20 kDa as 5 M urea was used. These data suggest that the core of the complex is fairly stable in urea and that hydrogen bonds may be involved in anchoring small proteins to the core of the complex.

**Endogenous Native Hmp35 Protein Is Resistant to Protease**—We treated intact hydrogenosomes, solubilized hydrogenosomes, and isolated hydrogenosomal membranes, respectively, with proteinase K and tested the effect on Hmp35. When intact hydrogenosomes were exposed to 0.25 mg/ml proteinase K, the endogenous Hmp35 protein appeared to be unaffected (Fig. 5A, lanes 1–10). We tested the potential effect of EDTA, which would chelate any divalent cations that may create a stable domain within the protein, and also used the reducing agent TCEP, which would reduce the intramolecular disulfide bond within the endogenous Hmp35 protein (Fig. 3B). Neither of these reagents had any effect on the protease resistance of the Hmp35 protein in intact hydrogenosomes (Fig. 5A, lanes 4–10). To rule out the possibility that the protein may be inherently resistant to protease due to its primary or secondary structure, we treated SDS-denatured hydrogenosomes with proteinase K and found that under these conditions Hmp35 was completely digested (Fig. 5A, lanes 11–14). Taken together, these data show that the Hmp35 protein does not have any major protease-sensitive cytosol-exposed domain. When the protein was solubilized under non-denaturing conditions, it was still largely resistant to 0.25 mg/ml proteinase K (Fig. 5A, lanes 15–24). However, some of the Hmp35 protein was cleaved into fragments of 17 and 18 kDa (Fig. 5A, lanes 15–24) and may be protruding out of the complex core. Because these fragments are not seen in the assay performed on intact hydrogenosomes (Fig. 5A, lanes 1–4), it is likely that the protruding part of the complex is inside the hydrogenosome or within the membrane bilayer. As the reducing agent TCEP was added, these fragments got smaller until they disappeared at 50 mM TCEP (Fig. 5A, lanes 19–24). There is also a reduction in the amount of full-length Hmp35 detected where 20 and 50 mM TCEP were included in the assay (Fig. 5A, lanes 22 and 24). Hence, the intramolecular disulfide bond detected in endogenous Hmp35 (Fig. 3B) may have a small but detectable effect on the integrity of the complex and its resistance to protease. Thus, the Hmp35 protein may be forming an exceptionally stable structure that defies protease digestion. The efficiency of the proteinase K to digest solubilized hydrogenosomal proteins was verified by Western analysis of the samples in Fig. 5A with antibodies against the membrane protein Hmp31. This protein was found to be completely sensitive to the proteinase K when solubilized under non-denaturing conditions (data not shown (18)). The HMP35 complex was found to be unaffected by proteinase K in either intact hydrogenosomes (Fig. 5B, lanes 1 and 2) or solubilized non-denatured hydrogenosomal protein (Fig. 5B, lanes 3 and 4). Thus, the HMP35 complex in its integrity is protease-resistant. As a control for efficient digestion, we verified the effect of the protease on Hsp70, a soluble matrix protein, on intact hydrogenosomes, where it is completely proteolyzed (Fig. 5B, lanes 7 and 8), and on solubilized non-denatured hydrogenosomal protein, where it is completely digested (Fig. 5B, lanes 9 and 10). Finally, when isolated sodium carbonate-extracted hydrogenosomal membranes were treated with proteinase K, we saw no discernible effect on the Hmp35 protein (Fig. 5C), although the Hmp31 integral membrane protein was digested. This observation, together with data from Fig. 5A, indicates that the protruding part of the Hmp35 protein within the complex (Fig. 5A, lane 16) is membrane-embedded. The recombinant protein His<sub>6</sub>-Hmp35 was found to be sensitive to 0.1 mg/ml proteinase K even under non-denaturing conditions (data not shown).

**Purification of the HMP35 Complex**—In order to purify the HMP35 complex from hydrogenosomes, we created 35H, a *T. vaginalis* transformant that expresses a C-terminally hexahistidine-tagged Hmp35 protein. This fusion protein is expressed from an episomal plasmid, pHmp35H (Fig. 6A), which also contains the neomycin phosphotransferase gene that confers resistance to the drug geneticin and provides for selection (18, 25). The Hmp35-His<sub>6</sub> protein was efficiently targeted to the organelle pellet as shown by a crude cell fractionation and Western analysis with an anti-hexahistidine antibody (Fig. 6B, lanes 1–3). Within purified 35H hydrogenosomes, the tagged protein was exclusively found in the membrane fraction of a sodium carbonate extraction, showing that the tag did not affect its sub-localization (Fig. 6B, lanes 4–6).

To purify the HMP35 complex, we solubilized membranes from sonicated 35H and wild-type (wt) hydrogenosomes in 0.5% n-dodecyl maltoside in the presence of buffered 1 M NaCl and various protease inhibitors, and we incubated the resulting precleared lysate with nickel beads to isolate bound proteins. The wash buffers used, which included up to 4 M urea and 1 M NaCl, provided stringent conditions that would keep the complex largely intact (Fig. 4, A and D) but would clear non-specifically bound proteins from the beads. The beads were divided into 2 aliquots that were eluted differently. One aliquot was eluted with a buffered detergent solution that contains 4 M urea supplemented with 0.25 mM imidazole (Fig. 6C, lanes 2, 3, 6, and 7). Under these conditions, the HMP35 complex should maintain a size of at least 200 kDa (Fig. 4D, lane 5), and the imidazole should specifically release nickel-bound hexahistidine-tagged proteins from the beads. The second aliquot was treated for 30 min at room temperature with a buffered detergent solution that contains 6 M urea and no imidazole. Under these conditions, the HMP35 complex is unstable (Fig. 4D, lane 7) and thus would disassemble into its constituents. However, because no imidazole was included in the buffer, the Hmp35-His<sub>6</sub> protein would stay bound to the beads. The resulting eluates were applied to a blue native gel, which was destained to reveal any major protein complexes stained by Coomassie.
FIG. 6. Purification of the hydrogenosomal HMP35-His<sub>6</sub> complex. A, the pHmp35H plasmid construct for selectable transformation of T. vaginalis. The Hmp35-His<sub>6</sub> open reading frame is under transcriptional control of the flanking regions of the α-succinyl-CoA synthetase gene (5′ α-SCS FR and 3′ α-SCS FR). The neomycin phosphotransferase gene (neo), under the control of the flanking regions of the α-tubulin gene (5′ α-Tub and 3′ α-Tub), confers genetic resistance to the 35H transformant. B, the Hmp35-His<sub>6</sub> protein is targeted to hydrogenosomal membranes in 35H transformants. Panels depict protein samples separated on 12% SDS-PAGE and detected with a monoclonal anti-hexahistidine tag antibody. In the top panel, B indicates broken cells (lane 1); S indicates supernatant after a 12,000 × g spin (lane 2); P indicates crude hydrogenosomal pellet (lane 3). The fractions were loaded such that supernatant + crude hydrogenosomal pellet = broken cells. In the bottom
Blue (Fig. 6C, lanes 1–5) or transferred to PVDF for Western analysis with anti-hexahistidine antibody (Fig. 6C, lanes 6–9). A large 300–330-kDa band was detected specifically in the 4 M urea, 0.25 M imidazole eluate from 35H hydrogenosomes (Fig. 6C, lanes 3 and 7). No monomer-sized Hmp35-His9 protein or any species smaller than 300 kDa was detected by the anti-hexahistidine antibody. This suggests that the purified HMP35-His9 complex is the eluate of mostly high molecular weight. When the complex was eluted with 6 M urea, no protein complex was seen in the destained gel (Fig. 6C, lane 5) nor was any protein detected by the anti-hexahistidine antibody (Fig. 6C, lane 9). This confirmed that most of the Hmp35-His9 remained bound to the beads and that treatment with 6 M urea would not release an intact high molecular weight complex. The 4 M urea, 0.25 M imidazole eluates were separated by SDS-PAGE followed by silver staining to reveal the individual constituent proteins. The HMP35-His9 complex appeared to consist of a major 40-kDa protein, a 35- and 12-kDa protein of moderate intensity, and lesser proteins of 32 and of 25–27 kDa (Fig. 6D, lane 2). The band at 65-kDa is likely to be a non-specific protein because it also appears in the ut eluate (Fig. 6D, lane 1). The 6 M eluate from 35H hydrogenosomes reveals a similar pattern when analyzed on SDS-PAGE but has a reduced amount of the 40-kDa protein (Fig. 6D, lane 4). The 6 M eluate from ut hydrogenosomes does not contain these bands (Fig. 6D, lane 3), confirming that they are likely to originate from the HMP35-His9 complex. Western analysis of the SDS-PAGE-separated proteins from the eluates with anti-hexahistidine antibody and with anti-Hmp35 antisera confirmed that the major 40-kDa band contained Hmp35-His9 (Fig. 6E, both panels, lane 2). The latter antisera also detected the lesser 35-kDa band (Fig. 6D, lane 2, and Fig. 6E, bottom panel, lane 2), which could be a degradation product of Hmp35. The 40-kDa protein in the 6 M eluate did not react with the anti-histidine antibody (Fig. 6E, top panel, lane 4), confirming that these elution conditions did not release Hmp35-His9 protein from the beads. However, the 40-kDa protein was detected by the anti-Hmp35 antibody (Fig. 6E, bottom panel, lane 4) and is likely to be endogenous non-histidine-tagged Hmp35. Because this untagged protein was purified from a complex containing HMP35-His9, the present data show that at least two Hmp35 monomers can self-associate. Moreover, from the relative amounts of the individual proteins in the HMP35-His9 complex (Fig. 6D, lane 2), it appears that its major constituent is Hmp35 itself. Hmp35 Protein Can Be Expressed in Yeast and Targeted to Mitochondrial Membranes in Vivo—Given evidence for a common ancestry for hydrogenosomes and mitochondria, we tested whether Hmp35, which has no known homologue in yeast, might have a functional role in organelle fractionation were all distributed as expected (Fig. 7C, lane 9), suggesting that it is present between the two membranes. To determine whether Hmp35 is present as an integral membrane protein in h35 mitochondria, the latter were sodium carbonate-extracted. It was found that the Hmp35 protein was distributed in both the integral membrane fraction and the soluble fraction (Fig. 7C, lane 9). The other markers for this fractionation were all distributed as expected (Fig. 7C, lane 9), with the integral membrane proteins Tom20 and Tim54 in the pellet, and the soluble proteins cytochrome b2 and Cpn10 and the peripheral membrane protein Tim44 in the soluble fraction. Taken together, these localization experiments suggest that...
the Hmp35 protein is present in yeast mitochondria as both a peripheral and an integral membrane protein from the trans side of the outer membrane or the cis side of the inner membrane.

Hmp35 Forms a Stable Protein Complex in Mitochondria—To assess whether Hmp35 formed a high molecular weight complex in mitochondria, we performed BN-PAGE on digitonin-solubilized organelles from both wild-type and h35 yeast cells. The respective sizes of the TOM complex (Fig. 7D, lanes 3 and 4) at 400 kDa (41), the TIM23 complex (Fig. 7D, lanes 5 and 6) at 90 kDa (42), and of the TIM22 complex (Fig. 7D, lanes 7 and 8) at 250–300 kDa were similar in h35 mitochondria and in wild-type mitochondria, showing that the three major protein translocon complexes were unaffected in h35 mitochondria. Western analysis with anti-Hmp35 antisera showed that Hmp35 was present in a stable discrete complex of 300 kDa in h35 mitochondria (Fig. 7D, lanes 1 and 2). A smaller band was not detected by the antisera, suggesting that most of the Hmp35 protein is associating into this complex, despite the sodium carbonate extraction procedure showing that the protein was both peripherally and integrally attached to the membranes (Fig. 7C, lanes 8 and 9). Coimmunoprecipitation experiments performed with antibodies described above and against several other yeast mitochondrial membrane proteins known to be in large complexes were attempted, and all failed to immunoprecipitate Hmp35 (data not shown).
Cysteine-rich Hydrogenosomal Membrane Protein Hmp35

Fig. 8. Secondary structure prediction profile for Hmp35. The secondary structure for Hmp35 (GenBank™ accession number AY312361), S. cerevisiae Tom40 (GenBank™ accession number NP013930), and Mycobacterium smegmatis MspA (GenBank™ accession number CAB56052) was predicted by the Garnier-Osguthorpe-Robson method using the programs Peptide Structure and Plotstructure (GCG Wisconsin Package, Accelrys). T indicates turn; H indicates α-helix; and B indicates β-sheet. Hmp35 and Tom40 are predicted to form 14 β-sheets each, and MspA has 10 potential β-sheets. The β-sheets in Hmp35 and Tom40 are separated by turns such that some of them may be arranged as anti-parallel sheets to form a β-barrel that could act as a pore.

DISCUSSION

We have characterized Hmp35, a novel membrane protein from hydrogenosomes of T. vaginalis, that has no known homologues. This protein has a putative RING-like, zinc-binding, cysteine-rich motif and forms an intramolecular disulfide bond. We demonstrate the application of blue native gel electrophoresis for hydrogenosomal complex separation and show that the Hmp35 protein is part of an integral membrane complex of about 300 kDa. HMP35, which is resistant to high concentrations of protease, urea, and salt. We have purified this complex, which appears to consist mostly of oligomerized Hmp35. Despite the Hmp35 protein not having any homologues in yeast, we show that it can be expressed heterologously in vivo in yeast and is targeted efficiently to mitochondria where it is integrated into membranes and forms a 300-kDa complex.

Hmp35 is the second membrane protein that has been reported from trichomonad hydrogenosomes after Hmp31, a member of the mitochondrial carrier family (18). The N terminal sequence of Hmp35 has no sequence similarity to the N-terminal cleavable presequence defined in Hmp31, which had been shown to be functional for targeting a matrix protein to the hydrogenosome, yet unnecessary for targeting mature Hmp31 (18). Thus, it appears that the Hmp31 N-terminal presequence is not conserved among membrane proteins as in the case for the N-terminal presequence on matrix-destined proteins (43).

Hmp35 is the first protein from hydrogenosomes to be described that has a putative zinc finger motif. Although most zinc finger protein motifs have been defined in transcription factors, there are examples of these motifs found in organelar membrane proteins. For example, subunit Vb of the cytochrome c oxidase complex, which is found in the inner mitochondrial membrane, has three conserved cysteine residues that coordinate one zinc atom (44). Other notable examples are the peroxins, which are integral membrane protein translocases in peroxisomes. The peroxins Pex2 (45), Pex10 (46), and Pex12 (47) are cysteine-rich proteins that have most of the cysteine residues clustered at the C-terminal end in a RING motif. The RING domain in Pex12 was found to be essential for its function in peroxisome biogenesis by interaction with another peroxin, Pex5, which conducts matrix protein import (47). These peroxins have no primary sequence similarity with each other, but the RING finger motif is conserved in a similar position. The arrangement of the seven cysteine residues at the C-terminal end of Hmp35 raises a parallel to these peroxins, although they are not arranged in a defined RING motif.

The iodoacetamide/AMS trapping experiment allowed us to determine that 2 cysteines out of the 14 in Hmp35 were engaged in intramolecular disulfide bonding in the endogenous protein. Proteins targeted to hydrogenosomes are post-translationally imported after synthesis in the cytosol (48). The cytosol is a reducing environment, such that precursor Hmp35 in the cytosol is likely to be in the reduced form. This implies the existence of a redox system in hydrogenosomes that is capable of oxidizing residues after import. In prokaryotes, the most studied disulfide bond formation is the Dsb system that consists of oxidation/isomerization proteins to form and regulate disulfide bonds (49). In mitochondria, it has been proposed that protein-disulfide isomerase (50) in the outer membrane could act similarly. Our finding that the inclusion of EDTA during the oxidation step of the trapping assay allows an extra disulfide bond to be formed suggests that divalent cations may be coordinated to the Hmp35 protein, presumably through some of the numerous cysteines. We were unable to force formation of extra disulfide bridges by oxidation of the endogenous protein in the absence of EDTA, although oxidation of most if not all of the cysteine residues was possible on the recombinant protein. This suggests that the cysteine-rich Hmp35 protein is unlikely to serve as defense against oxidative stress.

We found that Hmp35 was part of a high molecular mass protein complex of around 300 kDa, the first to be described in hydrogenosomes. The HMP35 complex stability in up to 5 M urea and 2 M salt is comparable with that of known pore proteins. For instance, in yeast mitochondria, the general im-
port pore, which contains the core proteins Tom22 and Tom40 in a 400-kDa complex, was shown to be resistant up to 1.5 M NaCl and 6 M urea and to sodium carbonate extraction (51). The general import pore forms the core of the outer membrane translocase that allows proteins from the cytosol to enter mitochondria. It has been proposed that the stability of the general import pore has evolved to withstand the forces involved in protein translocation into the matrix of mitochondria (51). The remarkable stability demonstrated for HMP35 suggests that this complex plays a role in hydrogenosomal translocation.

The endogenous non-denatured Hmp35 protein and HMP35 complex were found to be remarkably protease-resistant, whereas the recombinant non-denatured protein was completely protease-sensitive. It is possible that the resistance arises from a stable structure or a tight conformation between the individual components of the endogenous complex. Alternatively, the Hmp35 protein could be protected by another protein as in the case of bacterial secretins, which are protease-resistant through protection by pilot proteins (52). One point to note is that the Hmp35 protein expressed heterologously in mitochondria was protease-sensitive, although it was in a complex and membrane-inserted. This implies that there may be a chaperone or other helper protein, present in hydrogenosomes but missing in mitochondria, that confers protease resistance upon the Hmp35 protein. Recently, the major porin from Mycobacteria, MspA, has been described as one of the most stable transmembrane proteins (53). This porin exists as a tetramer that is stable in heat up to 90 °C in 2% SDS and in 7.6 M urea and at all pH values. In addition, the purified tetramer was found to be protease-resistant over extended periods and at high concentrations. It has been proposed that the extreme stability of this tetramer is due to the predominantly β-structure of the MspA protein, which may form a β-barrel within the tetramer to form the pore. Likewise, Tom40, the major constituent of the urea/salt/carbonate-resistant mitochondrial general import pore (51) is also predicted to have a β-barrel structure that forms a voltage-gated ion channel through which proteins are imported into mitochondria (22). Interestingly, Hmp35 is also predicted to be a predominantly β-structured protein with a 47% β-sheet content, which is comparable with the 45% predicted and the 48–52% determined experimentally for MspA (53). The Toc75 protein translocase found in chloroplasts likewise exists as a β-barrel (54). Furthermore, the β-barrel structure is found in all outer membrane proteins in Gram-negative bacteria and a large number of outer membrane proteins in organelles of symbiotic origin, mitochondria, and chloroplasts. In contrast, membrane proteins in plasma or endoplasmic reticulum membranes are α-helical (55). As illustrated in Fig. 8, the secondary structure prediction profile for Hmp35 strongly resembles that of known pore-forming proteins. These data, together with the complex stability, indicate that the Hmp35 protein complex has a pore-forming function either as a protein translocase or as a solute carrier. Further experimentation addressing this issue awaits the development of new biochemical techniques for the hydrogenosome, such as the capacity to distinguish between the membranes, and the generation of productive protein translocation intermediates.

We had shown previously that a trichomonad hydrogenosomal membrane protein, Hmp31, could be targeted in organello into isolated yeast mitochondria. The Hmp31 protein has up to 52% similarity to yeast mitochondrial carrier family proteins and therefore was likely to have conserved membrane-targeting signals recognized by the mitochondrial protein translocation machinery (18, 56). Recently, a related mitochondrial carrier family protein, the hydrogenosomal ADP/ATP carrier from the fungus Neocallimastix, which has >90% similarity with its yeast counterparts, was successfully imported in vivo into yeast mitochondria and restored an ADP/ATP carrier-deficient strain (8). In the present study, we show that a hydrogenosomal protein with no homology to any reported protein from yeast was expressed stably in yeast and was targeted to its mitochondria. It is unlikely that the Hmp35 protein was loosely associated to mitochondria because sodium carbonate extraction showed that at least half of it was integrated into the membrane. Interestingly, we found that the Hmp35 protein is present in a 300-kDa complex in mitochondria, which is of similar size to the endogenous HMP35 complex. The inability to coimmunoprecipitate this complex with mitochondrial proteins could be due to Hmp35 possibly being able to form a pore or complex by itself.

The successful import of hydrogenosomal Hmp35 into yeast mitochondria reiterates the compatibility between the protein translocation machineries of the two organelles which we and others have reported before (18, 58). Our finding implies conservation of signals at three levels as follows: recognition by the cytosolic chaperone during protein synthesis in the cytosol, recognition by the TOM receptor for import into mitochondria, and finally, conserved membrane insertion signals (57). This finding has important evolutionary implications as it suggests that hydrogenosomal Hmp35 was present in mitochondrionates and subsequently lost, although the protein import machinery of mitochondria can still recognize its targeting signals.

Trichomonad hydrogenosomal proteins reported to date have been shown to be homologous to either aerobic or anaerobic prokaryotic proteins or to mitochondria-derived proteins. Hmp35, on the other hand, has no known homologue in any kingdom even though a wide range of prokaryotes, Archaea and Metazoa, have now been sequenced. This raises the possibility that this abundant membrane protein arose during the creation of the trichomonad hydrogenosome. As genome sequence data becomes available for chytrids, fungi and ciliates that contain hydrogenosomes, it will be important to determine whether Hmp35 homologues exist in these lineages. The ubiquity of Hmp35 in hydrogenosome-containing lineages would imply a single origin for this organelle, although its presence solely in trichomonad hydrogenosomes would support polyphyletic origins.

Acknowledgments—We thank Danielle Leuenberger, Sean Curran, and Miriam Makabi for help with experiments and our colleagues in the lab for helpful discussions and critical comments on the manuscript.

REFERENCES

1. Muller, M. (1993) J. Gen. Microbiol. 139, 2879–2889
2. Fenichel, T., and Finlay, B. J. (1995) Ecology and Evolution in Anoxic Worlds, Oxford University Press, Oxford, UK
3. Martin, W., and Muller, M. (1998) Nature 392, 37–41
4. Biagioni, G. A., Finlay, B. J., and Lloyd, D. (1997) FEMS Microbiol. Lett. 155, 133–140
5. Dyal, S. D., and Johnson, P. J. (2000) Curr. Opin. Microbiol. 3, 404–411
6. Embley, T. M., Van Der Giezen, M., Horner, D. S., Dyal, P. L., and Foster, P. (2003) Philos. Trans. R. Soc. Lond. Biol. Sci. 358, 191–203
7. Annesarova, A., Vonzenz, R., van Alen, T., van Hoek, A., Bozma, B., Vogels, G., Veenhuis, M., and Hackstein, J. H. P. (1998) Nature 396, 527–529
8. van der Giezen, M., Slotboom, D. J., Horner, D. S., Dyal, P. L., Harding, M., Xue, G. P., Embley, T. M., and Kunji, E. R. (2002) EMBO J. 21, 572–579
9. Clemens, D. L., and Johnson, P. J. (2000) Mol. Biochem. Parasitol. 106, 307–313
10. Gray, M. W., Burger, G., and Lang, B. F. (1999) Science 283, 1476–1481
11. Bui, E. T. N., Bradley, P. J., and Johnson, P. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9651–9656
12. Gernot, A., Philippe, H., and Le Guyader, H. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14614–14617
13. Horner, D. S., Hirt, R. P., Kivlington, S., Lloyd, D., and Embly, T. M. (1996) Philos. Trans. R. Soc. Lond. Biol. Sci. 358, 1053–1059
14. Biagioni, G. A., Felice, P., and Doolittle, W. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 14618–14622
15. Tachezy, J., Sanchez, L. B., and Muller, M. (2001) Mol. Biol. Evol. 18, 1919–1928
16. Bui, E. T., and Johnson, P. J. (1996) Mol. Biochem. Parasitol. 76, 305–310
17. Horner, D. S., Heil, B., Happe, T., and Embly, T. M. (2002) Trends Biochem. Sci. 27, 148–155
