Acetate:Succinate CoA-transferase in the Hydrogenosomes of *Trichomonas vaginalis*

**IDENTIFICATION AND CHARACTERIZATION**

Received for publication, March 23, 2007, and in revised form, October 25, 2007. Published, JBC Papers in Press, November 16, 2007, DOI 10.1074/jbc.M702528200

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Acetate:succinate CoA-transferases (ASCT) are acetate-producing enzymes in hydrogenosomes, anaerobically functioning mitochondria and in the aerobically functioning mitochondria of trypanosomatids. Although acetate is produced in the hydrogenosomes of a number of anaerobic microbial eukaryotes such as *Trichomonas vaginalis*, no acetate producing enzyme has ever been identified in these organelles. Acetate production is the last unidentified enzymatic reaction of hydrogenosomal carbohydrate metabolism. We identified a gene encoding an enzyme for acetate production in the genome of the hydrogenosome-containing protozoan parasite *T. vaginalis*. This gene shows high similarity to *Saccharomyces cerevisiae* acetyl-CoA hydrolase and *Clostridium kluyveri* succinyl-CoA:CoA-transferase. Here we demonstrate that this protein is expressed and is present in the hydrogenosomes where it functions as the *T. vaginalis* acetate:succinate CoA-transferase (*Tv*ASCT). Heterologous expression of *Tv*ASCT in CHO cells resulted in the expression of an active ASCT. Furthermore, homologous overexpression of the *Tv*ASCT gene in *T. vaginalis* resulted in an equivalent increase in ASCT activity. It was shown that the CoA transferase activity is succinate-dependent. These results demonstrate that this acetyl-CoA hydrolase/transferase homolog functions as the hydrogenosomal ASCT of *T. vaginalis*. This is the first hydrogenosomal acetate-producing enzyme to be identified. Interestingly, *Tv*ASCT does not share any similarity with the mitochondrial ASCT from *Trypanosoma brucei*, the only other eukaryotic succinate-dependent acetyl-CoA-transferase identified so far. The trichomonad enzyme clearly belongs to a distinct class of acetate:succinate CoA-transferases. Apparently, two completely different enzymes for succinate-dependent acetate production have evolved independently in ATP-generating organelles.

*Trichomonas vaginalis* is the most prevalent sexually transmitted parasite with annually over 100 million cases worldwide. This parasite is an aerotolerant anaerobe that lacks mitochondria, the characteristic energy producing organelle of most eukaryotic organisms. However, trichomonads do contain other double membrane-bound organelles (1, 2) called hydrogenosomes (3), which are evolutionarily related to mitochondria (4–9). These hydrogenosomes convert pyruvate into acetate and carbon dioxide, resulting in hydrogen formation and ATP production (10–12).

Upon entry into the hydrogenosome, pyruvate is oxidatively decarboxylated by pyruvate:ferredoxin oxidoreductase (PFO), EC 1.2.7.1, yielding carbon dioxide and acetyl-CoA. The electrons released in the oxidation of pyruvate by PFO are transferred to ferredoxin and subsequently to protons, producing hydrogen gas. The acetyl-CoA formed by PFO is converted into acetate by an acetate:succinate CoA-transferase (ASCT), which transfers the CoA-group from acetyl-CoA to succinate. The succinyl-CoA derived from this reaction is then converted back into succinate by succinyl-CoA synthetase (SCS, EC 6.2.1.4), thereby producing ATP (12). ATP production in hydrogenosomes occurs exclusively via substrate level phosphorylation, using this ASCT/SCS cycle. ASCT is the only enzyme involved in *T. vaginalis* hydrogenosomal carbohydrate metabolism that has never been identified. In fact, acetate is not only produced in the hydrogenosomes of the parabasalid *T. vaginalis*, but also in the hydrogenosomes of certain ciliates (e.g. *Nyctotherus ovalis*), amoeboflagellates (e.g. *Psalteriomonas lanterna*) and in chytridiomycete fungi (e.g. *Neocallimastix*) (3, 12–14). However, the enzyme responsible for acetate formation has never been identified in any hydrogenosomal organism.

In addition to hydrogenosomes, ASCT activity is also known to be present in the anaerobically functioning mitochondria of metazoa that produce acetate, such as those of the parasitic helminths *Fasciola hepatica* and *Ascaris suum* (15, 16). Moreover, ASCT activity has been identified in the strictly aerobically functioning mitochondria of procyclic forms of the trypanosomatid *Trypanosoma brucei* (17). Therefore, the ASCT/SCS cycle is the only catabolic pathway present in both

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5 The abbreviations used are: PFO, pyruvate:ferredoxin oxidoreductase; ASCT, acetate:succinate CoA-transferase; *Tv*ASCT, *T. vaginalis* acetate:succinate CoA-transferase; UTR, untranslated region.
Acetate:Succinate CoA-transferase of T. vaginalis

mitochondria and hydrogenosomes. Identification of genes encoding mitochondrial or hydrogenosomal ASCT could therefore provide valuable information to unravel the evolutionary relation between these organelles.

The formation of acetate from acetyl-CoA as an end product of energy metabolism is observed in many prokaryotes and in a few eukaryotic species, all predominantly living in anaerobic or hypoxic environments (18, 19). Besides the formation of acetate by ASCT activity (Fig. 1, reaction 1), three alternative pathways for acetate production from acetyl-CoA have been identified: (i) acetate can be produced from acetyl-CoA via a two-step mechanism in which acetyl-phosphate occurs as an intermediate, involving the enzymes phosphate acetyltransferase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1), as occurs in many eubacteria (Fig. 1, reaction 2) (20); (ii) acetate can be produced from acetyl-CoA via an acetyl-CoA synthetase (EC 6.2.1.13, also known as acetate thio kinase) a process that concomitantly produces ATP from ADP in a reaction that is known to occur in several archaea and in the amitochondriate protist Giardia intestinalis and Entamoeba histolytica, (Fig. 1, reaction 3) (21, 22); (iii) furthermore acetate can be produced via hydrolysis of acetyl-CoA catalyzed by an acetyl-CoA hydrolase (EC 3.1.2.1), as is present in Saccharomyces cerevisiae (23, 24) (Fig. 1, reaction 4).

The first gene encoding a eukaryotic ASCT was identified in T. brucei (25). This gene showed high sequence similarity to mammalian succinyl-CoA:3-oxoacid CoA-transferase (SCOT, EC 2.8.3.5). However, no sequence homolog of this ASCT gene from T. brucei could be detected in the T. vaginalis genome data base at TIGR. On the other hand, we could identify in the T. vaginalis data base another candidate for acetate production by BLAST searches using the sequence of acetyl-CoA hydrolase from S. cerevisiae (ACh1 YBL015W) as a template. Based on the obtained sequence (corresponding to contig 921979, annotated in GenBank™ as XM_001330141) two specific oligonucleotides containing BamHI and PstI sites (underlined) at the initiating methionine and 90-bp downstream of the stop codon, respectively, were designed: 5′-TATAGGATCCATGTTGTCATCCTTCACTGTGCTATTC3′ and 5′-TATACTGCAGAAATTATACCCACATTCCACCCCTC3′. PCR amplification was performed and the predicted 1600-bp DNA fragment was purified from the gel and ligated into the eukaryotic expression vector pcDNA3.1+ and the bacterial expression vector pQE30. The sequences of the resulting constructs were verified by sequencing. TvASCT (in pcDNA 3.1+) was transfected into CHO K-1 cells using Lipofectamine Reagent (Invitrogen, Karlsruhe, Germany), following the standard, manufacturer’s protocol. Cells were harvested 24 h after transfection.

Antibody Preparation—Recombinant TvASCT (in pQE30) was expressed by M15 (pREP4) Escherichia coli and purified from inclusion bodies by subsequent lysis with lysosome (1 mg/ml), sonication (Soniprep 150), and centrifugation at 10,000 × g for 30 min at 4 °C. The pellet was washed two times with 1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) and 1 time with PBS only, after which the pellet was dissolved in 10 mM Tris-HCl, 50 mM MgCl2, 0.05% (v/v) Triton X-100. The resulting supernatant was used for purification of TvASCT by column chromatography.

MonQ anion exchange chromatography was performed with a monoQ HRS5/5 FPLC column (Amersham Biosciences Fine Chemicals, Uppsala, Sweden). The column was equilibrated with 15% (v/v) glycerol in 20 mM Hepes pH 8.3, at a flow rate of 0.5 ml/min, after which 1 ml of supernatant was loaded. UV absorption was recorded at 254 nm and fractions of 2 ml were collected during the entire run. The column was eluted for 10 min with 15% (v/v) glycerol in 20 mM Hepes pH 8.3, followed by a linear gradient increasing from 0 mM NaCl to 200 mM NaCl in 64 min, and from 200 mM NaCl to 1 M NaCl in 24 min.

ASCT Activity Assay—The activity of ASCT was determined by a radioassay as described before (17). In brief, the assay mix contained 1 mM [1-14C]acetyl-CoA (0.2 MBq, Amersham Biosciences), 50 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 50 mM succinate (pH 7.4), and 0.05% (v/v) Triton X-100. The assay was started by addition of the lysate, incubated at 20 °C for 30 min, and terminated by the addition of ice-cold trichloroacetic acid (6% w/v, final concentration). Reaction products were then separated by anion-exchange chromatography and quantified by liquid scintillation counting.

Gene Identification and Recombinant Expression—BLAST searches (29) for acetyl-CoA hydrolase related sequences in the available T. vaginalis TIGR genome data base were performed using acetyl-CoA hydrolase from S. cerevisiae (ACh1 YBL015W) as a template. Based on the obtained sequence (corresponding to contig 921979, annotated in GenBank™ as XM_001330141) two specific oligonucleotides containing BamHI and PstI sites (underlined) at the initiating methionine and 90-bp downstream of the stop codon, respectively, were designed: 5′-TATAGGATCCATGTTGTCATCCTTCACTGTGCTATTC3′ and 5′-TATACTGCAGAAATTATACCCACATTCCACCCCTC3′. PCR amplification was performed and the predicted 1600-bp DNA fragment was purified from the gel and ligated into the eukaryotic expression vector pcDNA3.1+ and the bacterial expression vector pQE30. The sequences of the resulting constructs were verified by sequencing. TvASCT (in pcDNA 3.1+) was transfected into CHO K-1 cells using Lipofectamine Reagent (Invitrogen, Karlsruhe, Germany), following the standard, manufacturer’s protocol. Cells were harvested 24 h after transfection.
of homologs of the acetate:succinate CoA transferase of *T. brucei*, acetate kinases and acetyl-CoA synthetases of several organisms, and acetyl-CoA hydrolase of *S. cerevisiae*. For the first three acetate producing enzymes no significant sequence homologs could be detected in the data base, only for the latter one, the acetyl-CoA hydrolase, significant hits were found. Four high-scoring segment pairs (TVAG\_395550, TVAG\_113870, TVAG\_164890, TVAG\_393850) were identified with an open reading frame of 1569 bp encoding a polypeptide of 523 amino acids. The calculated molecular mass of this protein is 57 kDa with a theoretical pI of 7.99 (ProtParam tool). Computer analysis of the predicted protein revealed 55% sequence identity to acetyl-CoA hydrolase from *S. cerevisiae*.

Data base searches with the derived amino acid sequence of this acetyl-CoA hydrolase homolog from *T. vaginalis* produced no significant matches to typical eukaryotic homodimeric CoA-transferases from eukaryotes such as succinyl-CoA:3-oxoadipate CoA-transferase from human or acetate:succinate CoA-transferase from *T. brucei*. However, significant similarity was observed with a family of proteins of both eukaryotic and prokaryotic species. These proteins are annotated as acetyl-CoA hydrolase/acetyl-CoA transferase/succinyl-CoA:CoA-transferase (Fig. 2). Here we define the *T. vaginalis* homolog of this family of proteins as *T. vaginalis* acetate:succinate CoA-transferase (*TvASCT*), as our further experiments showed that this *T. vaginalis* gene codes for an acetate:succinate CoA-transferase.

**Anti-\(TvASCT\) Antibody Generation**—For Ab generation *TvASCT* was expressed in *E. coli*, which resulted in minor amounts of soluble *TvASCT* and predominantly in insoluble *TvASCT* in inclusion bodies. Neither the complete lysate containing all soluble proteins, nor soluble *TvASCT* purified on nickel-nitritolactric acid resin (Qiagen) showed any detectable ASCT activity. This is in contrast to recombinant expression of the *Clostridium kluyveri* succinyl-CoA:CoA-transferase (*cat1*), in *E. coli*, which was active, even though measured enzyme activities were very low (27). *TvASCT* purified from inclusion bodies was subsequently used for antibody generation, which resulted in rabbit sera containing polyclonal antibodies against *TvASCT*. These antibodies specifically recognized the purified recombinant His tag *TvASCT*, the recombinant His tag *TvASCT* in a total lysate of transfected *E. coli*, and also the native *T. vaginalis* ASCT in a total lysate (Fig. 3). On the other hand, these antibodies did not recognize any other protein present in a total lysate of *E. coli* transfected with an empty vector. To further confirm the specificity of the anti-*TvASCT* antibodies, proteins of the complete hydrogenosomal fraction of *T. vaginalis* were separated on a two-dimensional gel, blotted and probed with the *TvASCT* antisemur. This resulted in specific detection of only *TvASCT* (Supplementary data) because the spots detected were confirmed to be *TvASCT* by ESI-Q-TOF-MS/MS analysis.

**Expression and Subcellular Localization of *TvASCT***—Two specific oligonucleotides were designed against the open reading frame in contig 921979. This ORF corresponds to the *T. vaginalis* gene XM\_001330141 in GenBank\(^\text{TM}\). A 1569-bp fragment could be amplified by PCR using *T. vaginalis* cDNA.
The fragment was isolated and sequence analysis confirmed the amplification of the *TvASCT* gene. This result clearly demonstrates that the gene is expressed at the mRNA level. ASCT activity in *T. vaginalis* has been localized to hydrogenosomes (12), and therefore, the *TvASCT* protein sequence is expected to contain a mitochondrial-like targeting sequence, as the import system into hydrogenosomes is comparable to that of mitochondria (37–39). The eight N-terminal amino acids of *TvASCT* (Fig. 2) and of its three paralogs in the *T. vaginalis* genome (Supplemental data), strongly fit the consensus of hydrogenosomal targeting sequences (40). The programs TargetP (41), WoLF Psort (42), and MitoPred (43) predicted *TvASCT* to contain a mitochondrial targeting sequence. Therefore *TvASCT* expression at the protein level was investigated in purified hydrogenosomes.

Hydrogenosomal proteins of *T. vaginalis* were separated by isoelectric focusing in the first dimension and by SDS-PAGE in the second dimension. Two 57-kDa protein spots were excised from the Coomassie-stained gels, digested with trypsin and analyzed by ESI-Q-TOF-MS/MS (Supplemental data). Sequenced peptides were compared with the *T. vaginalis* genome data base (TIGR) and all originated from the same ORF in contig 921979 (Table 1). A Western blot of cellular fractions probed with anti-*TvASCT* antibody showed strong accumulation of *TvASCT* in the hydrogenosomal fraction (Fig. 4). These observations clearly demonstrate that the *TvASCT* protein is expressed and located inside hydrogenosomes. The *TvASCT* gene encodes a protein with ASCT activity.

**Expression of *TvASCT* in CHO Cells**—CHO K1 cells were transfected with *TvASCT*, and the cells were harvested after 24 h. The cells transfected with *TvASCT* showed a 15-fold increase in ASCT activity, whereas cells containing the empty vector showed no increased ASCT activity (Fig. 5). The amount of ASCT protein in transfected CHO cells was, however, very low and the average ASCT activity was 7 nmol/min per mg protein, which is two orders of magnitude lower than the ASCT activity in *T. vaginalis* cells (Fig. 6). The observed conversion of a minute

![Alignment of acetyl-CoA hydrolases/transferases](image-url)

**FIGURE 2. Alignment of acetyl-CoA hydrolases/transferases.** Alignment of the gene product of contig 921979 from *TvASCT*, with the gene products from *S. cerevisiae* ACI1, P32316; *Candida glabrata* Q6FP3; Pelobacter carbinolicus 1935792; Clostridium klyuyerii 1, P38946; and Artemia franciscana AAP9833. Identical residues among 4 of 6 aligned proteins are shown in **black**, whereas conserved residues among 4 of 6 aligned proteins are **shaded**. The putative N-terminal targeting sequence of *TvASCT* is printed in **bold**.
amount of acetyl-CoA by untransfected CHO cells might be caused by side activity of the mammalian succinyl-CoA:3-oxoacid CoA-transferase (SCOT; EC 2.8.3.5) (Ref. 17).

Overexpression of TvASCT in T. vaginalis—T. vaginalis T1 cells were transfected with TvASCT in pTagvag2 to yield strain TvtagvagASCT and were selected for transient transformation with G418. Expression of TvASCT was quantified on mRNA, and protein levels in TvtagvagASCT as well as wt T. vaginalis and TvtagvagFrataxin cells (pTagvagFrataxin was kindly provided by Jan Tachezy, Prague) as controls and total cell lysates were assayed for ASC activity. TvASCT transcript and protein levels in TvtagvagASCT cells were increased to 409 ± 136% and 518 ± 171% of the wild type, respectively, while they remained largely unchanged in Frataxin-overexpressing controls (101 ± 10% and 95 ± 21%, respectively) (Fig. 6). ASC activity in TvtagvagASCT was raised to 426 ± 54% of the wild type activity and again remained unchanged in TvtagvagFrataxin (115 ± 10%) (Fig. 6).

Co-elution of ASC Activity and TvASCT Protein in Different Protein Purifications—Three principally different column chromatography methods for protein purification were performed using complete T. vaginalis lysates. Fractions were collected and tested for both ASC activity using an enzyme assay, and for the presence of the TvASCT protein by Western blot. In all experiments, using chromatography based on anion exchange, isoelectrofocusing and size exclusion, the elution pattern of ASC activity corresponded to that of the TvASCT protein (Fig. 7 and Supplemental data). In all three purification methods ASC activity was highly purified as it eluted in fractions containing only a small percentage of the total amount of protein. Combination of the anion exchange and electrofocusing columns could not be used for further purification of the ASC enzyme since this always resulted in a complete loss of ASC activity.

Peptide sequences identified from the described 57-kDa protein spots by mass spectrometry after two-dimensional-gel separation of isolated hydrogenosomal proteins from T. vaginalis.

The position of the identified peptides in the amino acid sequence of the TvASCT protein as encoded on contig 921979.
activity that could not be regained in reconstitution experiments (not shown). In diluted lysates TvASCT activity always declined rapidly, although native TvASCT was stable for days at 4 °C in total cell lysate or during incubation for 15 min at 55 °C, while precipitation with 37.5% (NH₄)₂SO₄ resulted in only 10% activity loss (not shown).

Succinate Dependence and Apparent Km for Succinate of TvASCT—Regarding the fact that TvASCT shows a high similarity to the protein family of acetyl-CoA hydrolases/transferases, the succinate dependence of TvASCT was re-examined. Using our specific and sensitive radioactive enzyme assay, in which substrates and end products were identified by anion exchange chromatography, we confirmed that TvASCT is succinate dependent, and therefore, is a true acetate:succinate CoA-transferase and not a hydrolase (Fig. 8, panel A and Ref. 12).

Furthermore, the affinity for succinate of the TvASCT enzyme as determined in a total T. vaginalis lysate, resulted in an apparent $K_m$ of 0.36 mM ($\pm$ 0.03) (Fig. 8, panel B) and in an apparent $K_m$ of 0.75 mM ($\pm$ 0.05) for TvASCT purified by anion exchange chromatography. Such partially purified enzyme preparations were also used to confirm that TvASCT activity is succinate-dependent (not shown). The apparent $K_m$ values were calculated using direct linear plots in EZ-fit (44).

**DISCUSSION**

Recently, the draft genome sequence of T. vaginalis was published (40). The Supporting Online Material (SOM) of that manuscript (Table S22) shows a list of all hydrogenosomal proteins involved in carbohydrate metabolism and ASCT is the only enzyme lacking a complete annotation. Here we demonstrate that the homolog of the acetyl-CoA hydrolase/transferase protein family, present in the T. vaginalis genome data base, functions as the missing ASCT of T. vaginalis, for the following reasons. Firstly, heterologous expression of TvASCT in CHO cells resulted in the expression of an active ASCT enzyme albeit at a very low level when compared with T. vaginalis cells (Fig. 5). Second, homologous overexpression of TvASCT in T. vaginalis resulted in a more than 4-fold increase in TvASCT mRNA and protein levels and also ASCT activity (Fig. 6) Third, upon protein fractionation, using different chromatographic methods, the elution pattern of the activity of ASCT matched with the protein pattern of the acetyl-CoA hydrolase/transferase homolog (Fig. 7 and Supplemental data). Fourth, the T. vaginalis homologs of the acetyl-CoA hydrolase/transferase family contain a hydrogenosomal targeting signal (Fig. 2 and Supplemental data) and we have demonstrated the expression and hydrogenosomal localization of the gene product of at least one of these gene copies (Fig. 3). Finally, no homologs of any of the other known types of acetate producing enzymes (see Fig. 1) are present in the T. vaginalis genome, whereas multiple copies of homologs to the acetyl-CoA hydrolase/transferase protein family have been detected (Supplemental data).

Taken together, the total of all our analyses clearly demonstrates that the gene product of this acetyl-CoA hydrolase/transferase homolog functions as the hydrogenosomal ASCT. Therefore, this gene can now be annotated as the T. vaginalis ASCT (TvASCT). TvASCT is thereby the first hydrogenosomal acetate-producing enzyme to be identified in any organism. The reason for the observed loss of activity during subsequent purification steps and the poor heterologous expression of TvASCT are unknown. In this respect it is unknown yet
The enzyme of the crustacean four of these annotations are supported by biochemical data.

whether or not TvASCT might need a cofactor or post-translational modification for maximal activity.

Even though TvASCT belongs to a protein family, which contains acetyl-CoA hydrolases as well as acetyl-CoA transferases, it is a true ASCT, because it converts acetyl-CoA to acetate in a succinate-dependent way (Fig. 8, panel A). This is consistent with the earlier reported characteristics of acetate production in T. vaginalis (12).

By measure of similarity, ASCT from T. vaginalis hydrogenosomes belongs to a family of proteins annotated as either acetyl-CoA hydrolases or acetyl- or succinyl-CoA transferases. However, it shares no similarity whatsoever with the functionally equivalent mitochondrial SCOT sequences from trypanosomatids (25). A sequence similarity network of acetyl-CoA hydrolase/transferase family sequences (Supplemental data) displays two well defined major groups. Both groups contain prokaryotic as well as eukaryotic sequences. Some proteobacteria (Rhodoferax ferrireducens, Geobacter sp. or Bordetella parapertussis) and firmicutes (Clostridium difficile) are represented in both groups, suggesting an ancient prokaryotic gene duplication. The eukaryotic sequences are divided into two major clusters. All animal sequences form a monophyletic cluster in one of the two major groups of the network, while T. vaginalis, D. discoideum, and the fungi form a separate distinct cluster that is strongly associated with a set of δ-proteobacterial homologs in the other.

Functional annotation of most sequences in the network is based on sequence similarity rather than on any biochemical characterization and both major clusters contain sequences annotated as acetyl-CoA transferases, acetyl-CoA hydrolases, and acetyl-CoA transferase/hydrolase family members. Only four of these annotations are supported by biochemical data. The enzyme of the crustacean Artemia franciscana has 4-hydroxybutyrate transferase activity (45), while three enzymes of the other major cluster are succinyl-CoA transferase (C. kluveri) (27), acetyl-CoA hydrolase (S. cerevisiae) (23), and ASCT (T. vaginalis) (this study), respectively. Thus clustering does not strictly correlate with function in this gene family.

Surprisingly, TvASCT does not share any similarity with the mitochondrial ASCT from T. brucei, the only other eukaryotic ASCT identified so far. Furthermore, trypanosomatid ASCT is kinetically different from TvASCT, as demonstrated by their different apparent $K_m$ values for succinate; 2.8 ± 0.4 mM for Leishmania mexicana ASCT (17) and 0.75 ± 0.05 mM for TvASCT. Apparently, two completely different enzymes for succinate-dependent acetate production have evolved independently in ATP-generating organelles. The identification of TvASCT does not only illuminate the identity of the last, unidentified hydrogenosomal protein involved in carbohydrate metabolism, but it also means the identification of a completely different, second class of succinate-dependent acetyl-CoA transfers in eukaryotes.

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