Characterization of the Gene Encoding Serine Acetyltransferase, a Regulated Enzyme of Cysteine Biosynthesis from the Prostiotic Parasites Entamoeba histolytica and Entamoeba dispar

REGULATION AND POSSIBLE FUNCTION OF THE CYSTEINE BIOSYNTHETIC PATHWAY IN ENTAMOEBAP

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The enteric prostiotic parasites Entamoeba histolytica and Entamoeba dispar possess a cysteine biosynthetic pathway, unlike their mammalian host, and are capable of de novo production of L-cysteine. We cloned and characterized cDNAs that encode the regulated enzyme serine acetyltransferase (SAT) in this pathway from these amoebae by genetic complementation of a cysteine-autotrophic Escherichia coli strain with the amoebic cDNA libraries. The deduced amino acid sequences of the amoebic SATs exhibited, within the most conserved region, 36–52% identities with the bacterial and plant SATs. The amoebic SATs contain a unique insertion of eight amino acids, also found in the corresponding region of a plasmid-encoded SAT from Synechococcus sp., which showed the highest overall identities to the amoebic SATs. Phylogenetic reconstruction also revealed a close kinship of the amoebic SATs with cyanobacterial SATs. Biochemical characterization of the recombinant Entamoeba histolytica SAT revealed several enzymatic features that distinguished the amoebic enzyme from the bacterial and plant enzymes: 1) inhibition by L-cysteine in a competitive manner with L-serine; 2) inhibition by L-cystine, and 3) no association with cysteine synthase. Genetically engineered amoeba strains that overproduced cysteine synthase and SAT were created. The cysteine synthase-overproducing amoebae had a higher level of cysteine synthase activity and total thiol content and revealed increased resistance to hydrogen peroxide. These results indicate that the cysteine biosynthetic pathway plays an important role in antioxidative defense of these enteric parasites.

The cysteine biosynthetic pathway plays an important role in incorporation of inorganic sulfur into organic compounds. In bacteria and plants, L-cysteine is the precursor of most sulfur-containing metabolites including methionine and glutathione. Extracellular sulfate is first imported by specific transporters. Intracellular sulfate is then activated by ATP sulfurylase and adenosine-5'-phosphosulfate kinase to form adenosine-5'-phosphosulfate and 3'-phosphoadenosine 5'-phosphosulfate, respectively. These activated sulfates are further reduced to sulfide. Sulfide then reacts with O-acetylserine, which is produced from serine and acetyl-CoA by serine acetyltransferase (SAT), EC 2.3.1.30. This final reaction forming L-cysteine, by transfer of the alanil moiety of O-acetylserine to sulfide, is catalyzed by L-cysteine synthase (CS; O-acetyl-L-serine (thiol)-lyase, EC 4.2.99.9). In contrast to bacteria and plants, animals are presumed to lack the sulfur assimilation pathway and thus require exogenous methionine as a sulfur source. Biochemical studies using purified (1–4) and recombinant enzymes (5), as well as a genetic approach using a yeast two-hybrid system, revealed that CS and SAT form a heteromeric complex. SAT activity and O-acetylserine availability are the major regulatory factors in the control of the L-cysteine production in plants (6, 7). Cytosolic isoforms of the SAT from Citrullus vulgaris and Arabidopsis thaliana are regulated by feedback inhibition by L-cysteine, but not by L-cystine, glutathione, D-cysteine, or other structurally similar amino acids (5, 8). Thus, the plant cytosolic SATs appear to monitor the amount of L-cysteine and its redox balance. However, mitochondrial and chloroplast isoforms of the A. thaliana SATs are feedback-insensitive (8), which indicates that the mode of regulation of cysteine biosynthesis is organelle-dependent.

Entamoeba histolytica is an enteric prostist parasite that causes amebic colitis and extraintestinal abscesses (i.e., hepatic, pulmonary, and cerebral) (9). This organism is amitochondriate, and its core metabolism involves several proteins that contain low midpoint redox potential iron-sulfur centers coordinated by cysteines, i.e. pyruvate:ferrodoxin oxidoreductase and a [4Fe-4S] ferredoxin. It requires a reduced medium for in vitro growth.

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† To whom correspondence should be addressed: Dept. of Parasitology, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel.: 81-3-5285-1111 (ext. 2733); Fax: 81-3-5285-1173; E-mail: nozaki@nih.go.jp.

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The abbreviations used are: SAT, serine acetyltransferase; EhSAT, SAT from E. histolytica; EhCS, a gene encoding EhSAT; CS, cysteine synthase; EhCS, CS from E. histolytica; EhCS, a gene encoding EhCS; EdSAT, SAT from E. dispar; EdCS, a gene encoding EdCS; ML, maximum likelihood; GST, glutathione S-transferase; ORF, open reading frame; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; DB, GAL4 DNA-binding domain; AD, GAL4 activation domain; LUC, firefly luciferase gene; LUC, firefly luciferase; pBS, pBluescript II SK+; NEO, neomycin phosphotransferase gene; BP, bootstrap proportion.
Entamoeba Serine Acetyltransferase

vitro growth, although, in the host, it invades aerobic tissues. The mechanisms of its antioxidative defenses are poorly understood. Superoxide dismutase is present, but catalase and the glutathione system involved in antioxidative defenses of other organisms, including of its host, are absent (12). The amino acid, l-cysteine, is the major thiol (12) that plays an important role in the biology of this organism, including its antioxidative defenses and in its attachment to matrix, elongation, motility, and growth in vitro (14, 15).

The source of l-cysteine for E. histolytica remains a puzzling question. The media used for in vitro cultivation contain high levels of this amino acid, and the organism is assumed to be dependent on exogenous cysteine. We have recently shown, however, that E. histolytica possesses the cysteine biosynthetic pathway (10, 11). We isolated and characterized the genes encoding two important enzymes in the pathway: ATP sulfurylase and CS. The significance of this pathway for E. histolytica is indicated by the observation that the steady state CS mRNA comprises a large proportion (up to 2%) of total mRNA (10). However, regulatory mechanisms and functional significance of this pathway have not been studied at the molecular level in E. histolytica.

In this study, we describe the isolation and characterization of cDNA and genomic DNA encoding SAT from a clonal strain of E. histolytica and Entamoeba dispar by functional complementation. We show that the amoebic SAT is a regulated enzyme in the pathway and probably plays an important role in cysteine biosynthesis. In addition, unlike the bacterial and plant SATs, the amoebic SAT revealed several unique biochemical features including a feedback inhibition by both l-cysteine and l-cystine and a lack of association with CS. We also show that overproduction of CS, but not of SAT, confers partial resistance to hydrogen peroxide, implying the importance of cysteine biosynthesis in antioxidative defense.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Restriction endonucleases and DNA-modifying enzymes were purchased from Takara Bichemical (Tokyo, Japan), New England Biolabs (Beverly, MA), and CLONTECH (Palo Alto, CA). [α-32P]dCTP (6000 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Tokyo, Japan). All oligonucleotides were synthesized with Oligo 1000M (Beckman, Tokyo, Japan). All other chemicals were commercial products of the highest purity available.

**Microorganisms**—Trophozoites of the pathogenic E. histolytica clonal strain HMI: IMSS 6 (16) were axenically cultivated in TYI-S-33 medium at 35 °C as described previously (17). P. aeruginosa PA: RE10 strain (19) was a gift from the Laboratory of Clinical Microbiology, University of La Jolla, CA. The cycling parameters were: 1) denaturation at 94 °C for 1 min; 2) annealing at 60 °C for 1 min; 3) elongation at 72 °C for 1 min; 4) 30 cycles. The 0.9-kilobase PCR fragment was digested with BamHI, electrophoresed, DNA transfer, and hybridization with 32P-dCTP-labeled EhSAT cDNA probe were carried out as described (11, 25).

**Enzyme Assays**—The enzymatic activity of SAT was measured by two methods, either by monitoring the decrease of 1 mol of the thioester bond of acetyl-CoA cleaved or 1 mol of l-cysteine synthesized at 25 °C for 1 min.

Isolation of E. histolytica and E. dispar SAT cDNA Clones—E. histolytica and E. dispar SAT cDNA clones were obtained by complementation of the cysteine auxotrophic E. coli strain JM39/5 (F′, cysE51, recA1) (24) with the E. histolytica or E. dispar phagemid cDNA library (10). The JM39/5 cells were transformed with 0.1 µg of the E. histolytica or E. dispar phagemid cDNA library by electroporation. One-half of the transformed JM39/5/E. coli was cultured on a M9 agar plate (25) containing 100 µg/ml carbenicillin, 0.5 mm isoprropyl β-D-thiogalactoside, (M9 cys−); the other half of the transformed JM39/5 cells was cultured on the M9 agar plate supplemented with 200 µg/ml l-cysteine (M9 cys+). The plates were incubated at 37 °C for 4–5 days. Eleven and nine colonies grew on the M9 cys− plate, on which the E. histolytica and E. dispar cDNA library-transfected cells were plated, respectively, whereas approximately 10 colonies grew on the M9 cys+ plates. After screening twice, these putative E. histolytica or E. dispar SAT cDNA clones were confirmed to complement cysteine auxotrophy of JM39/5 cells.

**Amplification of Amino-terminal and Upstream Flanking Regions by Polymerase Chain Reaction (PCR)**—Total DNA was isolated and purified from trophozoites as described previously (26). The 5′–3′ end of the E. histolytica SAT gene (EhSAT) coding region and the upstream flanking region were obtained by nested PCR of the genomic fragment using nested oligonucleotide primers: 5′-ttgtagtttttggcttcataaa-gaataagg-3′ and 5′-tgaggtatggctgtagctgggaagactcttc-3′ and an in vitro cloning kit (Takara Biochemical).

**Sequence Analysis**—Twenty-eight SAT sequences were retrieved from the National Center for Biotechnology Information by using the BLAST network service (27). The amino acid sequences were aligned with E. histolytica and two E. dispar sequences using the Clustal W program (28). The alignment was manually edited with the ED program of the MUST package (29). A maximum likelihood (ML) method (30) of protein phylogeny was used to infer phylogenetic relationships among the sequences. All ML analyses were performed with the PROTML program, version 2.3 (31).

**Southern Blot Analysis**—Restriction digest of genomic DNA, agarose electrophoresis, DNA transfer, and hybridization with the 32P-dCTP-labeled EhSAT cDNA probe were carried out as described (11, 25).

**Chromatographic Separation of CS and SAT from E. histolytica**—For the coupled assay, the reactions were performed in 50 mM Tris-HCl, pH 8.0, 0.2 mM acetyl-CoA, 1 mm l-serine, 1 mm NaS₂O₄, 5 mm dithiothreitol, 0.02 unit of the recombinant EhCS1, and enzyme solution in a final volume of 100 µl. Protein concentration was determined by the Bradford method (22) with bovine serum albumin as a standard. l-Cysteine and CS were assayed as described (10, 23). One unit of the enzymatic activity was defined as 1 µmol of thioester bond of acetyl-CoA cleaved or 1 µmol of l-cysteine.
Sepharose 4B Column—Approximately 10 μg of the purified recombinant GST-EhSAT was mixed with a 10-fold excess amount of purified recombinant EhCS1 (10) either in PBS or buffer A at room temperature for 1 h with gentle shaking. The mixture was then passed through an equilibrated glutathione-Sepharose 4B column. After extensive washing with PBS or buffer A, 1 ml each of 1 mM glutathione was sequentially applied to the column, and eluents were collected and concentrated with Centricon 10 (Amicon Inc., Beverly, CA). Each fraction was subjected to CS and SAT assays as well as SDS-PAGE analysis.

Assay of CS-SAT Interaction Using the Yeast Two-hybrid System—A possible interaction between CS and SAT was assayed using the yeast two-hybrid system (for reviews, see e.g. Refs. 32 and 33). An ORF of EhCS1 and EhSAT was amplified by PCR using a set of oligonucleotide primers containing appropriate restriction sites and cloned into pAS2–1 (CLONTECH Laboratories) that contained GAL4 DNA-binding domain (DB), and pACT2 that contained GAL4 activation domain (AD) to make plasmid constructs (i.e. pDB-CS, pDB-SAT, pAD-CS, and pAD-SAT) to produce fusion proteins (DB-CS, DB-SAT, AD-CS, and AD-SAT, respectively). The entire ORF of each fusion construct was sequenced to verify the absence of mutations or frameshifts. pDB-CS, pDB-SAT, or pTD1 containing ORF that had been PCR-amplified using oligonucleotide primers -ctagtcgacttaaatcgatg- and -gtagtcgacttaaatcgatg- was cloned into the pCSC I site of pCLC to produce pNEOSAT. To construct pNEOCs, the NEO cassette was cloned into the SmaI site of pCSC to produce pNEOSAT. Distances between the putative regulatory elements in the EhCS1 upstream region (38) and the initiation codon of LUC, EhCS1, or EhSAT in pNEOCS, pNEOLUC, and pNEOSAT were identical to those in the chromosomal EhCS1 locus.

Production of CS- and SAT-overproducing E. histolytica Cell Lines—To create the E. histolytica cell lines that overproduced firefly luciferase (LUC), EhCS1, and EhSAT, the wild-type trophozoites were transformed with pNEOLUC, pNEOSAT, or pNEOCS by liposome-mediated transfection (39). Approximately 10^6 trophozoites were seeded onto 35-mm diameter wells of a six-well culture plate and cultivated in 12 ml of TYI-S-33 medium with a sticky attachment tape covering the top of the wells at 35 °C for 15 h. The LipofectAMINE-plasmid DNA complexes were prepared in OPTI-MEM I medium (Life Technologies) supplemented with 5 mg/ml l-cysteine and 1 mg/ml ascorbic acid (transfection medium). Thirty μl of the transfection medium containing 3 μg of one of the plasmids was mixed with 15 μl of LipofectAMINE PLUS (Life Technologies) and kept at room temperature for 15 min. This mixture was combined with 20 μg (10 μl) of LipofectAMINE, kept at room temperature for 15 min, diluted with 945 μl of transfection medium, and added to the seeded trophozoites after removing TYI-S-33 medium. The plate was then incubated at 35 °C for 3 h. After incubation with the LipofectAMINE-DNA complex, 70–90% of the trophozoites were viable. The trophozoites were transferred to fresh medium and further cultivated at 35 °C for 18 h. G418 was then added to the cultures at 6 μg/ml.

Determination of the Total Thiol Content—The amoeba lysates were electrothermally reduced at a current of 6 mA for 1 h (40, 41) before deproteinization with perchloric acid. The thiol content was determined by measuring A_412 of the reduced product of 5,5'-dithiobis(2-nitrozoic acid), 5-mercaptop-2-nitrobenzoate (42). An extinction coefficient of 14150 sr cm^-1 at 412 nm was used for 5-mercaptop-2-nitrobenzoate.

Assay for Hydrogen Peroxide Sensitivity—To assess the short term sensitivity of the transformants to hydrogen peroxide, the trophozoites were washed with ice-cold PBS containing 1% glucose (PBSG) three times and resuspended in PBSG at 2.5 × 10^6/ml. One hundred μl of the cell suspension was mixed with an equal amount of PBSG containing various concentrations of hydrogen peroxide and incubated at 25 °C. A 5-μl aliquot was taken at various times and mixed with 0.4% trypan
blue, and viable cells were counted. To evaluate long term effect of hydrogen peroxide on growth, 3 x 10^5 trophozoites of the transformants were inoculated to 6 ml of TYI-S-33 medium in the presence or absence of 4, 8, or 12 mM hydrogen peroxide and cultivated at 35 °C for 48 h.

We have designated this gene as EdSAT1. The fact that EdSAT1 and EdSAT2 cDNA clones were obtained with comparable frequencies indicated that both of the EdSAT1 and EdSAT2 cDNAs encoded the functional SAT and that they were expressed in comparable amounts at the steady-state mRNA level. The composite sequence of EhSAT revealed a 918-bp ORF that encoded a protein with a calculated molecular mass of 34,404 Da and a pl of 6.63. EdSAT1 contained a 918-bp ORF that encoded a protein with a calculated molecular mass of 34,264 Da and a pl of 6.51.

Southern blot analysis using the 32P-dCTP-labeled EhSAT cDNA probe showed that the restriction endonucleases that did not cut the EhSAT protein coding region gave a single band (data not shown). This indicates that the EhSAT gene exists as a single copy in the trophozoites of E. histolytica.

Deduced Amino Acid Sequences of EhSAT and EdSATs—

The deduced amino acid sequences of EhSAT, EdSAT1, and EdSAT2 were homologous to those of the SATs of bacterial and plant origins (Fig. 2) and revealed 36–52% identities within the most conserved region (corresponding to amino acids 106–261 of EhSAT). Among the homologues from other species, the amoebic SATs appeared to be devoid of amino-terminal transit peptides found in the cytosolic isoforms.
conserved in the amoebic SATs at the primary sequence level. This structure has been found in various acetyl- and acyltransferases, including UDP-N-acetylglucosamine 3-O-acetyltransferase (47), chloramphenicol acetyltransferase (48), thigaloctoside acetyltransferase (49), and Rhizobium nodulation protein NodL (50). Second, the amoebic SATs contained a unique insertion between the coil regions 2 and 3. A similar insertion was found only in the plasmid-encoded Synechococcus SrpH protein among the 28 available SAT sequences. Third, 0.2 mM L-serine and 0.1 mM acetyl-CoA were used. However, only weak or no inhibition was observed at 30 mM with L-cysteine (<5%), βL-homocysteine (10%), N-acetyl-L-cysteine (<7%), and DL-homoserine (5%) under the same condition.

Chromatographic Separation of EhSAT and EhCS—To test whether CS and SAT form a complex in amoebae, the lysate of E. histolytica trophozoites was subjected to gel filtration chromatography. As shown in Fig. 5, both CS and SAT activities were detected as a single peak. This elution profile was nearly identical in the presence or absence of 0.4 mM NaCl in elution buffer. The peaks of CS and SAT activities were eluted at 59.1 ± 4.8 and 71.0 ± 3.0 kDa, respectively, which suggests that both enzymes exist as a homodimeric form. However, unlike the bacterial and plant CS and SAT, which form a multimeric “cysteine synthase” complex (2, 53), neither the homo- nor heteromultimeric form was demonstrated in the E. histolytica lysate.

An Attempt to Co-purify CS with GST-EhSAT Using a Glutathione-Sepharose 4B Column—The results obtained by conventional chromatographic separation supported the assumption that EhCS and EhSAT did not interact in vivo. To verify this assumption, we tested whether the two enzymes co-purified by affinity chromatography. The GST-EhSAT recombinant protein was mixed with either the recombinant EhCS1 or the E. coli lysate, which contained an endogenous CS activity. The mixture was incubated to let any protein interaction occur and then applied to the glutathione-Sepharose 4B column. If EhCS1 and/or the E. coli CS interacts with the GST-EhSAT fusion protein, they should be retained on the column, and eluted with either O-acetylseryne or glutathione as shown for plant enzymes (54). However, SDS-PAGE analysis and CS assay of the eluents revealed that neither EhCS1 nor the E. coli CS was retained in the column (data not shown; also see Fig. 4A, lane 1, for a lack of the co-purified E. coli CS). These results further indicate that neither homologous (E. histolytica) nor heterologous (E. coli) CS associates with GST-EhSAT. However, we cannot exclude the possibility that the recombinant enzymes failed to co-purify because of non-native folding of one or both expressed proteins.

Assay of CS-SAT Interaction Using Yeast Two-hybrid System—Since the expression of DB-SAT showed autonomous activation of GAL4 transcription (data not shown), possible interaction between EhCS1 and EhSAT was investigated by cotransformation of Y187 strain of S. cerevisiae with pDB-CS and pAD-SAT. No interaction was detected between DB-CS and AD-SAT or between DB-CS and AD-CS. These results were not due to a failure of expression of DB-CS, AD-SAT, or AD-CS, since CS activity was detected in the lysate of pDB-CS-transformed yeasts, and the AD-CS and AD-SAT fusion proteins...
were detected in the lysate of pAD-CS- and pAD-SAT-transformed yeasts by immunoblot analysis using a monoclonal antibody against the GAL4-AD (data not shown). These data supported the premise that there is no detectable interaction of EhCS and EhSAT in vitro and in vivo. The two-hybrid system also failed to demonstrate self-association of a plant CS (55). Since the apparent molecular size of the native amoebic CS, determined by gel filtration chromatography, suggests that CS protein probably exists as a homodimer (see above), the failure to detect self-interaction of EhCS1 in the yeast two-hybrid system indicates that fusion of GAL4-AD or GAL4-DB at the amino terminus of the CS may interrupt self-interaction of the CS. This result also suggests that the amino terminus of the CS may be involved in dimerization of the CS.

**Enzyme Activities and Thiol Content in the CS- and SAT-overproducing Amoebae**—The lysate of the pNEOLUC transformant contained CS activity of 6.25 ± 1.12 units/mg of protein and SAT activity of 4.76 ± 0.75 × 10^{-3} units/mg of protein (Fig. 6), activities comparable with those of the wild-type amoebae (Ref. 10; data not shown). The pNEOCS transformant revealed 2.7-fold higher CS activity and 2.1-fold higher SAT activity than the control LUC-producing amoebae. The reason for the concomitant increase in SAT activity in the CS-overproducing amoebae is unknown. The pNEOSAT transformant showed a comparable level (1.15-fold) of CS activity and a 13.3-fold higher SAT activity compared with the control LUC-producing amoebae. Although an overproduction of plant SAT in *E. coli* resulted in the induction of CS expression (54) by accumulation of putative inducers, O-acetylserynine and N-acetylseryline (56), the CS activity in the SAT-overproducing amoebae was unchanged. The amounts of total thiol in the perchloric acid extracts of the CS- and SAT-overproducing transformants were 2.3- and 1.5-fold higher than the control LUC-producing transformants, respectively. Thus, it appears that thiol content increases in proportion with CS activity but not with SAT activity. The amount of CS protein expressed in these transformants was also quantified with immunoblot analysis using serial dilutions of the lysates and a polyclonal antiserum raised against purified EhCS proteins (10). The lysate of the pNEOCS transformants contained a 2.5- to 5-fold greater amount of CS protein than the pNEOSAT- and the control LUC-producing transformants (data not shown). These data agreed well with the results of CS activity.

**Hydrogen Peroxide Sensitivity of the CS- and SAT-overproducing Amoebae**—To evaluate whether CS and SAT overproduction affects short term sensitivity to hydrogen peroxide, the LUC-, CS-, or SAT-overproducing cell lines were incubated in PBSG containing hydrogen peroxide (Fig. 7). The CS-overproducing amoebae were more resistant to hydrogen peroxide (10 and 100 mM) than the control LUC-overproducing amoebae.
amoeba cell lines to hydrogen peroxide. The suspension of 2.5 × 10³ trophozoites of the cell lines that had been transformed with pNEOCS (circles, unbroken lines), pNEOLUC (triangles, dotted lines), or pNEOSAT (squares, broken lines) was incubated at 35 °C in the presence or absence of 10, 100, or 1000 nM hydrogen peroxide, and the percentage of viable cells was estimated. Differences considered statistically significant by Student’s t test (p < 0.05) between the pNEOLUC and pNEOCS transformants or between the pNEOLUC and pNEOSAT transformants are marked with asterisks.

For example, at 10 and 20 min after the hydrogen peroxide addition, the CS-overproducing amoebae were 1.8- and 3.1-fold, respectively, more resistant to 10 mM hydrogen peroxide than the control LUC-producing amoebae as expressed in the percentage of viable cells. On the contrary, the SAT-overproducing amoebae were as sensitive to hydrogen peroxide (10 mM) as the control LUC-producing amoebae. The SAT-overproducing amoebae were slightly more sensitive to a high concentration of hydrogen peroxide (100 mM) than the control LUC-producing amoebae. The CS-overproducing amoebae grew as fast in TYI-S-33 medium containing 4 mM hydrogen peroxide as in the absence of hydrogen peroxide, whereas neither the SAT- nor LUC-overproducing amoebae were viable after 48 h of cultivation. When the CS-, SAT-, and LUC-overproducing amoebae were cultivated in the presence of 8 or 12 mM hydrogen peroxide, all trophozoites of these transformants were killed after 48 h (data not shown).

**DISCUSSION**

In this study, we have shown that SAT is a regulated key enzyme in the biosynthetic pathway of L-cysteine, which is assumed to play an important role in the microaerophilic protist parasites Entamoeba, Trichomonas, and Giardia. These protists lack antioxidant systems normally present in aerobic or aerotolerant eukaryotic cells (13) and, instead, possess alternative mechanisms for detoxification similar to those known to exist in certain prokaryotes (13, 57, 58). The amoebic SAT was found to differ in its biochemical properties and evolutionary relationships from the bacterial and plant enzymes. First, the amoebic SAT was feedback-inhibited by physiological concentrations of L-cysteine and L-cysteine with comparable efficiency. This implies that EhSAT plays a role as “fuel gauge” of the redox state of L-cysteine. Second, the mechanisms and specificities of EhSAT inhibition by L-cysteine differed from the SATs from other organisms and organelles (1, 5, 8), suggesting organism- and organelle isoform-specific structures. Third, unlike the bacterial and plant SATs, an association between the amoebic SAT and CS was absent. This has been supported by three independent methods: gel filtration chromatography, affinity chromatography, and a yeast two-hybrid system. It is worth noting that an interaction between amoebic SAT and E. coli CS was also undetectable. Thus, the fact that we cloned the amoebic SAT cDNA by functional rescue using the E. coli SAT-deficient mutant implies that CS-SAT interaction is not essential either in the amoeba or in E. coli. Fourth, the overproduction of EhSAT did not result in the induction of CS, unlike in γ-proteobacterial and plant counterparts (54, 56). This implies that coordinated induction of CS expression by the accumulation of O-acetylserine or N-acetylserine, which is the isomeric product converted from O-acetylserine, is probably absent in amoebae. Alternatively, the overproduced O- and N-acetylserine may be decomposed without being accumulated in amoebae. These results, together with other biochemical peculiarities of EhSAT, also indicate that the mode of regulation of the cysteine biosynthetic pathway in amoebae highly diverged from other organisms.

Phylogenetic analysis also revealed unusual characteristics of EhSAT. Specific relationship of the amoebic SATs with the plasmid-encoded Synechococcus SrpH was supported by 1) a high BP value (100%) of this monophyletic relationship, 2) high primary structure similarities, 3) specific insertion of the 7- or 8-amino acid region that differentiates Entamoeba SATs and Synechococcus SrpH from all of the others, and 4) amino-terminal extensions that were 20–120 amino acids longer than the cytosolic SATs from bacteria (e.g. E. coli and B. subtilis). Although we cannot rule out the possibility that the amoebic sequences were misplaced due to the long branch attraction effect, Synechococcus SrpH and the Entamoeba SATs represent highly divergent genes within this group. It will be interesting to determine whether the similarity observed at the primary structure level is reflected in similarities of the biochemical properties of the amoebic SAT and Synechococcus SrpH. It is worth noting that the amoebic CS was distant from the chlorosomal copies of cyanobacterial CS in phylogenetic analysis (data not shown; Ref. 10). Together with the absence of the interaction between the two amoebic enzymes, this leads us to speculate that the two amoebic enzymes may have evolved in different ancestral organisms and have been delivered to amoebae by horizontal transfer as suggested for many other proteins in amoebae (e.g. Ref. 60). However, we cannot exclude the possibility that the ancestral organism of Entamoeba possessed the divergent CS and SAT genes (e.g. CS1, CS2, SAT1, and SAT2) and subsequently retained one set of genes (e.g. CS1 and SAT2) and lost the other pair (e.g. CS2 and SAT1) during evolution.

In addition to the importance of the feedback-mediated regulation of cysteine biosynthesis by EhSAT, we have also demonstrated that the level of CS protein and activity also plays an important role in the control of L-cysteine production in vivo. We have shown using the genetically engineered amoeba strains that overproduction of CS, but not that of SAT, affected the total thiol content and sensitivity to hydrogen peroxide. The reason for the lack of increment of thiol content in the SAT-overproducing amoebae is unknown. Overproduction of EhSAT may interfere with coordination of CS and SAT, although no association of EhCS and EhSAT has been demonstrated. The ratios of CS activity to SAT activity in the lysate of the control LUC-producing and wild-type amoebae were 924 and 1370 (data not shown), whereas, in the SAT-overproducing amoebae, the ratio dramatically decreased to 114. By using recombinant enzymes, an 860–1100-fold excess amount of the recombinant EhCS1 over the recombinant EhSAT was required for the maximal in vitro L-cysteine production (data not shown). Thus, a large excess of CS (approximately 10³-fold) over SAT was necessary for efficient production of L-cysteine both in vitro and in vivo. Therefore, an inadequate proportion of the two enzymes in the SAT-overproducing amoeba may contribute to a lack of increase in thiol content of these amoebae. Alternatively, the overproduced O-acetylserine may be converted or degraded to molecules that do not serve as substrates of CS. The second explanation also agrees with a lack of the concomitant induction of CS activity in the SAT-overpro-
duc ing amoebae, which is dissimilar to the cases in bacteria and plants (54, 56).

Although amoeba trophozoites are exposed to various oxidatives and reactive oxygen species including hydrogen peroxide, superoxide, and hydroxyl radical during infection of mammalian hosts, amoebicidal effect has been attributed solely to hydrogen peroxide (61). Thus, defense against hydrogen peroxide seems to be important for an amoeba’s survival in the mammalian hosts. In this study, we showed that overproduction of CS resulted in increase of the total cellular thiol content and resistance to hydrogen peroxide. Bruchhaus et al. (62) showed that the 29-kDa cysteine-rich protein removes hydrogen peroxide only in the presence of thiols. The bacterial homologue of the 29-kDa amoebic protein, thiol-specific antioxidant protein, is also active in the removal of hydrogen peroxide only in the presence of thiols such as diithiothreitol or dihydroxylic acid (63). Thus, it is conceivable that the overproduced l-cysteine or unidentified thiol compounds, in the CS-overproducing amoeba, may modulate the thiol-dependent peroxidase activity of the 29-kDa protein. The fact that the increase in hydrogen peroxide resistance of the CS-overproducing amoeba was observed in both short and long term incubations indicates that the increase in resistance may be attributable to the changes in membrane sensitivity against hydrogen peroxide. Our finding supports the presence and importance of cell surface thiol groups, which were implicated for defense against extracellular stimuli (64).

One big question related to the biological importance of the cysteine biosynthetic pathway in Entamoeba is why Entamoeba apparently requires a high concentration of l-cysteine in its media for growth and survival in vitro (14, 15) if it is capable of synthesizing this amino acid. It has been shown that E. coli is able to produce l-cysteine from sulfite and incorporate extracellular l-cysteine (65). These mechanisms are reciprocally regulated by extracellular amino acid concentrations in E. coli (65). One possible explanation for the observed requirement of extracellular l-cysteine in the culture system (14, 15) is that de novo synthesized l-cysteine may not be efficiently utilized for the maintenance of the redox state of the surface thiol molecules (64). L-Cysteine biosynthesis may be biologically important in Entamoeba due to its reliance on iron-sulfur proteins for its electron transport chain (66, 67). It will be interesting to determine whether extracellular l-cysteine is incorporated in the amoeba and used for the synthesis of iron-sulfur proteins or solely serves to maintain surface thiol molecules in the reduced state.

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