A dual-specificity aminoacyl-tRNA synthetase in the deep-rooted eukaryote *Giardia lamblia*

Shipra Bunjun*, Constantinos Statopoulos*, David Graham†, Bokke Min†, Makoto Kitabatake†, Alice L. Wang‡, Ching C. Wang三位, Christian P. Vivares†, Louis M. Weiss†, and Dieter Söll***

*Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06520-8114; **Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06511; †Department of Microbiology, University of Illinois at Urbana-Champaign, Urbana, IL 61801; ‡Department of Pharmaceutical Chemistry, University of California, San Francisco, CA 94143-0446; ¶Laboratoire de Parasitologie Moleculaire et Cellulaire, Université Blaise Pascal, 63177 Aubière Cedex, France; and †Department of Pathology, Albert Einstein College of Medicine, Bronx, NY 10461

*Contributed by Dieter Söll, September 15, 2000*  

Cysteinyl-tRNA (Cys-tRNA) is essential for protein synthesis. In most organisms the enzyme responsible for the formation of Cys-tRNA is cysteinyl-tRNA synthetase (CysRS). The only known exceptions are the euryarchaea *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*, which do not encode a CysRS. Deviating from the accepted concept of one aminoacyl-tRNA synthetase per amino acid, these organisms employ prolyl-tRNA synthetase as the enzyme that carries out Cys-tRNA formation. To date this dual-specificity prolyl-cysteinyl-tRNA synthetase (ProCysRS) is only known to exist in archaea. Analysis of the preliminary genomic sequence of the primitive eukaryote *Giardia lamblia* indicated the presence of an archaeal prolyl-tRNA synthetase (ProRS). Its proS gene was cloned and the gene product overexpressed in *Escherichia coli*. By using *G. lamblia*, *M. jannaschii*, or *E. coli* tRNA as substrate, this ProRS was able to form Cys-tRNA and Pro-tRNA *in vitro*. Cys-AMP formation, but not Pro-AMP synthetase, was tRNA-dependent. The *in vitro* data were confirmed in *vivo*, as the cloned *G. lamblia proS* gene was able to complement a temperature-sensitive *E. coli* cysS strain. Inhibition studies of CysRS activity with proline analogs (thiaproline and 5-aminopropionyl-l-threonine) in a *Giardia* S-100 extract predicted that the organism also contains a canonical CysRS. This prediction was confirmed by cloning and analysis of the corresponding cysS gene. Like a number of archaea, *Giardia* contains two enzymes, ProCysRS and CysRS, for Cys-tRNA formation. In contrast, the purified *Saccharomyces cerevisiae* and *E. coli* ProRS enzymes were unable to form Cys-tRNA under these conditions. Thus, the dual specificity is restricted to the archaeal genre of ProRS. *G. lamblia*’s archaeal-type prolyl- and alanyl-tRNA synthetases refine our understanding of the evolution and interaction of archaeal and eukaryal translation systems.

Aminoacyl-tRNA synthetases (AARSs) are essential for the faithful translation of the genetic code. They ensure the fidelity of protein synthesis by correctly acylating a tRNA species with its cognate amino acid (1). This crucial family of enzymes is divided into two distinct classes (I and II) based on characteristic signature motifs. These and other conserved structural features allow facile recognition of orthologous enzymes in many organisms by sequence similarity searches of the available databases. They also allow comparison between the AARSs and thus classification into subtypes with respect to their phylogenetic origin (2). These enzymes have exquisite specificity for their substrates (amino acid and tRNA), a process mediated in some cases by intricate editing mechanisms (3–5). It is commonly accepted that each cell contains 20 such enzymes, one for each canonical amino acid. This assumption was supported by the description of 20 AARSs found in some bacteria (e.g., *E. coli*) and in the eukaryotic cytoplasm. However, recent discoveries arising from functional genomics studies in bacteria and archaea have overturned this concept and revealed that most organisms do not use a full complement of 20 canonical AARSs (6–8). The major exception is the route to Gln-tRNA or Asn-tRNA formation, which in many organisms involves an amidation of a mischarged Glu-tRNA or Asp-tRNA (8–10). Most surprisingly, it was demonstrated *in vitro* and *in vivo* that the ProRS of *M. jannaschii*, *M. thermoautotrophicum*, and *Methanococcus maripaludis* is able to catalyze the formation of cysteinyl-tRNA (Cys-tRNA; ref. 7). The fact that the two former organisms lack a canonical cysteinyl-tRNA synthetase (CysRS) implies that these organisms use the dual-specificity prolyl-cysteinyl-tRNA synthetase (ProCysRS) for the synthesis of both Cys-tRNA and Pro-tRNA required for protein synthesis. The fact that a single AARS is capable of and required for supplying two different aminoacyl-tRNAs for protein synthesis challenged the accepted view of these enzymes (1).

As we showed earlier, the genome of the lower eukaryote *Giardia lamblia* genome contains genes encoding the archaeal genre of ProRS and alanyl-tRNA synthetase (AlaRS; ref. 2). *G. lamblia* is a parasitic, amitochondrial protist that diverged early in the eukaryotic lineage (11, 12). By comparing genes from this organism with homologous eukaryotic and archaeal genes it may be possible to infer the events that led to the formation of the modern eukaryal cell type. The parasite *G. lamblia* is found in every region of the United States and throughout the world and infects about 200 million people annually worldwide (13). It has become recognized as one of the most common causes of waterborne disease in humans in the United States, causing diarrhea, abdominal cramps, and nausea. Further knowledge of the essential enzymes of protein synthesis may reveal new targets for development of antiparasitic agents. Here we report the existence of the dual-specificity ProCysRS and its activity in *G. lamblia*.

**Materials and Methods**

**Cloning of the *G. lamblia* proS, alaS, and cysS Genes.** Partial sequence data of the *G. lamblia* genes proS, alaS, and cysS, obtained from the *Giardia lamblia* genome project (supported by the National Institute of Allergy and Infectious Diseases) at the Josephine Bay Paul Center web site at the Marine Biological Laboratory in Woods Hole, MA (http://hermes.mbl.edu/baypaul/Giardia-HTML/index2.html), were used to design primers that enabled cloning of...
complete gene sequences from a genomic library of G. lamblia WB (14). The PCR products were cloned into the pCR-TOPO vector (Invitrogen) and sequenced to confirm the identity of the gene. Upon digestion with the restriction enzymes NdeI and BamHI, the gene was ligated into pET15b (Invitrogen) for subsequent expression of an N-terminal His-tagged protein and into pCBS1 (15) for subsequent complementation tests. The sequences were deposited in GenBank (accession numbers: alaS, AF245445; cyoS, AF299082; proS, AF245446). A plasmid for tRNA expression, pTECH, constructed by the introduction of a multiple cloning site, was cloned into the HincII/EcoRV site of a mutant pACYC184 (New England Biolabs), which lacks the EcoRI site in the chloramphenicol resistance gene. A tRNA gene cloned in the multiple cloning site is expressed constitutively from the lpp promoter, and the transcription is terminated by an rrm terminator.

Overexpression and Purification of G. lamblia, S. cerevisiae, and E. coli ProS. For overexpression of G. lamblia proS, clone pET15b-proS was used to transform the E. coli strain BL21-Codon Plus-RIL (Stratagene). Transformants were grown in 5 ml LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol at 30°C for 15 h. After inoculation of 500 ml of LB medium with the addition of ampicillin (100 µg/ml), the expression of the His6-tagged proS gene was induced for 5 h with the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Harvesting of cells, lysis, and purification of the cell extract by Ni-nitrilotriacetic acid-agarose chromatography (Qiagen, Chatsworth, CA) were performed as previously described (16). The G. lamblia His6-ProCysRS was >95% pure, as judged by Coomassie brilliant blue staining after SDS-PAGE. Active fractions were pooled, concentrated with solid polyethylene glycol 20,000, dialyzed against ammonium chloride buffer containing 20% (vol/vol) glycerol, and stored at −20°C.

Both S. cerevisiae cytoplasmic and mitochondrial proS genes, YHR020w (sp | P38708) and YER087w (sp | P39965), respectively, were cloned by PCR, using a genomic library as DNA template. The pBAD-TOPO/TA system (Invitrogen), which allows the expression of the gene of interest with a His6 tag at the C terminus, was used for expression in E. coli. Expression of both genes was induced in the presence of 0.02% l-arabinose in the growth medium. The E. coli proS gene was a gift from T. Li (Yale University). All three enzymes were purified as described above for G. lamblia His6-ProCysRS.

G. lamblia tRNA-Free Cell Extract Preparation. G. lamblia WB cells were cultured as described (17) and harvested as a confluent sheet formed on the surface of the culture flask. Cells were washed once in 100 vol of 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5) and centrifuged at 3,000 × g for 15 min. The pellet was resuspended in 4.5 ml of 50 mM Hepes (pH 7.0), 50 mM KCl, 15 mM MgCl2, 5 mM DTT and a mixture of protease inhibitors (10 µg/ml each of PMSF, pepstatin, leupeptin, aprotinin, and 16 µg/ml benzamidine). After sonication (Fischer Sonic Dismembrator 550) at 4°C, the lysate was centrifuged at 4°C in a Beckman TL-100 centrifuge at 100,000 × g for 1 h. The supernatant was diluted with an equal volume of glycerol and stored at −70°C. For the aminoaacetylation assay this solution was dialyzed overnight at 4°C against the reaction buffer of 50 mM Hepes (pH 7.0), 50 mM KCl, 15 mM MgCl2, and 5 mM DTT.

Aminoaacetylation and ATP-PP Exchange Assays. Cys-tRNA and Pro-tRNA synthesis was assayed by measuring the acid-precipitable aminoaacetyl-tRNA as described (7), after incubation at 37°C. The reaction mixture contained 50 mM Hepes (pH 7.0), 50 mM KCl, 15 mM MgCl2, and 5 mM DTT in the presence of 10 mM ATP, 20–50 µM [35S]cysteine (1075 Ci/mmole; NEN DuPont), or [3H]proline (103 Ci/mmole; Amersham). The substrate used was unfraccionated M. jannaschii tRNA (1 mg/ml final concentra-
tion) unless indicated otherwise. Also used was total tRNA from E. coli (Roche Molecular Biochemicals), S. cerevisiae (Roche Molecular Biochemicals), and G. lamblia (prepared by standard methods). The enzyme concentration ranged from 0.05 to 0.5 µM. Assays for inhibition of enzyme activity were performed in the presence of 2 mM thiopropine (Sigma) or 1 mM 5’-O-[N-(L-
prolyl)-sulfamoyl]-adenosine (gift of S. Cusack, European Molecular Biology Laboratory, Grenoble Outstation). ATP-PP, exchange assays were performed at 37°C in the presence or absence of total M. jannaschii tRNA with [32P]PP, (NEN DuPont; 4.6 Ci/mmol). The reaction mixture also contained 2 mM proline or cysteine and 1 mM ATP in a total volume of 200 µl. Samples (40 µl) were taken at various time points, and the reaction was stopped by adding 200 µl of 1% activated carbon in 0.4 M sodium pyrophosphate and 15% (vol/vol) perchloric acid. The mixture was filtered through glass microfilter filter disks (GF/C, 24 mm diam.; Whatman), and radioactivity was measured by liquid scintillation counting.

Complementation of E. coli Strain UQ818 (cys54). The G. lamblia proS gene was cloned into the plasmid pCBS1 to yield pCBS- proS. The E. coli cys54 strain UQ818 (18), with an additional pTet plasmid for expression of the M. jannaschii tRNA54 gene (pTet-Mj-tRNA54), was transformed with the compatible plasmid pCBS-Gl-proS. For use as positive and negative controls, UQ818 was transformed with the plasmid pCBS-Ec-cysS and the empty vector pCBS1, respectively; both of these, as well as the pTet-Mj-tRNA54, were gifts from T. Li (Yale University). The resulting transformants were tested for growth on LB agar supplemented with ampicillin (100 µg/ml), chloramphenicol (34 µg/ml), and cysteine (5 mM) at 30°C and 42°C, as has been previously described (7). Transformants were also tested for growth in the absence of pTet-Mj-tRNA54, without chloramphenicol in the growth medium.

Protein Sequence Alignments. The following amino acid sequences for previously described AlaRS proteins were obtained from the nonredundant protein database at the National Center for Biotechnology Information: Aeropyrum pernix (sp | Q9YY9 × 3), Aquifex aeolicus (sp | O67323), Arabidopsis thaliana (sp | P36428), Archaeoglobus fulgidus (sp | O28029), Bacillus subtilis (sp | O34526), Borrelia burgdorferi (sp | O51238), Caenorhabditis elegans (gb | AAB52339.1), Chlamydia trachomatis (sp | O84754), Dictyostelium discoideum (gb | AAFO5592.1), Drosophila melanogaster (gb | AAFO5593.1), Escherichia coli (sp | P00957), Homo sapiens (sp | P49588), Methanobacterium thermoautotrophicum (sp | O27718), Methanococcus jannaschii (sp | Q57984), Mycobacterium tuberculosis (sp | O07438), Pyrococcus horikoshii OT3 (sp | O58035), Rickettsia prowazekii (sp | Q9ZCA4.1), Saccharomyces cerevisiae (sp | P40825), Sulfobolus solfataricus (sp | P96041), Synechocystis sp. (sp | P74423), Thermotoga maritima (sp | Q9 X1B6), Thermus aquaticus (sp | P74941), and Treponema pallidum (sp | O83980). The following amino acid sequences for previously described ProRS proteins were also obtained from the nonredundant protein database at the National Institute for Biotechnology Information: Aeropyrum pernix (dbj | BAA81340.1), Aquifex aeolicus (gb | AAC06648.1), Archaeoglobus fulgidus (sp | O28664), Bacillus subtilis (emb | CAB13530.1), Borrelia burgdorferi (gb | AAC66767.1), Caenorhabditis elegans (cytoplasmic: gb | AA50660.1, mitochondrial: emb | CAB04884.1), Chlamydia pneumoniae (gb | AAD18640.1), Chlamydia trachomatis (sp | P36431), Deinococcus radiothermus (gb | AAFO10387.1), Dictyostelium discoideum (dbj | C24346.1), Escherichia coli (sp | P16659), Homo sapiens (mitochondrial: emb | CAB55948.1), Methanobacterium thermoautotrophicum (sp | O26708), Methanococcus jannaschii (sp | Q58635), Mycobacterium leprae (emb | CAB36573.1), Mycobacterium tuberculosis (sp | O05814), Mycoplasma genitalium (sp | P47525), Mycoplasma
deduced AlaRS and ProRS sequences are of the archaeal genre (GenBank accession nos. AF245445, AF299082; Rickettsia prowazekii (sp | Q92DE7), Saccharomyces cerevisiae (cytoplasmic: sp | P38708; mitochondrial: sp | P39965), Schizosaccharomyces pombe (cytoplasmic: emb | CAA19574.1; mitochondrial: emb | CA2A1147.1), Sulfolobus solfataricus (gi | 6015904), Synechocystis sp. (sp | P73942), Thermotoga maritima (gb | AAD35599.1), Treponema pallidum (sp | O83195), and Ureaplasma urealyticum (gb | AAF30864.1). Sequence data from partial genome sequences for Clostridium acetobutylicum were from http://www.genomecorp.com, Porphyromonas gingivalis and Trypanosoma brucei were obtained from http://www.tigr.org. Pyrococcus furiosus was from http://www.genome. utah.edu, and Streptomyces coelicolor and Plasmodium falciparum were from http://www.sanger.ac.uk. Sequences not yet publicly available were from Thermoplasma acidophilum (courtesy of A. Ruepp, Max-Planck-Institut für Biochemie, Martinsried, Germany), Halobacterium salinarum (courtesy of S. Schuster and D. Oesterhelt, Max-Planck-Institut für Biochemie, Martinsried, Germany), Methanosarcina mazei G01 (courtesy of T. Hartsch, Götttingen Genomics Laboratory, Germany), and Pyrococcus aerophilum (courtesy of S. Fitz-Gibbon, Univ. of California, Los Angeles). Thirty-three amino acid sequences from AlaRS were aligned using the CLUSTALW (v.1.7.4) program (19). Forty-three amino acid sequences from ProRSs were separated into groups of archaeal- and bacterial-type enzymes and then aligned using CLUSTALW. These alignments were combined manually using the AE2 alignment editor (T. Macke, Ribosomal Database Project). From the human and Drosophila bifunctional Gluprol-tRNA synthetase only regions homologous to other ProRS proteins were used in this analysis.

Phylogenetic Inference. From the alignment of 33 AlaRS proteins, 717 positions were deemed to be confidently aligned. These were analyzed as previously described (2), by protein maximum parsimony methods using a heuristic search algorithm (PAUP* 4.0 beta 2; D. Swofford, Sinauer). The 1,000 shortest trees were evaluated by maximum likelihood criteria, using the PROTML program (v 2.2; D. Swofford, Sinauer). The 1,000 shortest trees were evaluated using the RELL weightings. Phylogenetic trees were viewed and edited with the TREEVIEW program (v. 1.5.2; ref. 22). In the alignment of 43 ProRSs, 579 positions were analyzed by the same techniques.

Results

Genomic Giardia lamblia Sequence Data Suggest the Presence of Archaeal ProRS and AlaRS Enzymes and of a Canonical CysRS. Our recent analysis of aminoacyl-tRNA synthetase genes in a large number of organisms revealed the presence of an archaeal ProRS and AlaRS in Giardia lamblia (2). This finding piqued our interest for two reasons: analysis of these genes may provide additional data on the evolutionary position of Giardia. More intriguingly, it suggested that even eukaryotes may possess the novel dual-specificity ProCysRS, which to date was biochemically demonstrated to be present in at least three archaeal genomes, even though sequence data suggested that the same enzyme activity will be found in other archaea (ref. 7; C.S., unpublished work). Thus, based on the partial sequence information (see Materials and Methods), we cloned the G. lamblia proS, alaS, and cysS genes. Their sequences were determined (GenBank accession nos. AF245446, AF245445, AF299082); the deduced AlaRS and ProRS sequences are of the archaeal genre (see below), whereas the CysRS showed much similarity to other known CysRS proteins (15).

G. lamblia ProRS Exhibits Dual Specificity in Vitro and Requires tRNA for Cysteine Activation. To determine whether G. lamblia ProRS possesses dual aminoaacylation specificity, the G. lamblia proS gene was recloned with a 5’-terminal His epitope tag. Overexpression in E. coli and purification over a Ni-nitrilotriacetic acid column led to a His-tagged protein of the expected molecular mass (61 kDa) and purity (>95%). This enzyme charged unfractionated M. jannaschii tRNA almost as well as Giardia tRNA; we continued experiments with the archaeal tRNA, as it was easier to obtain. The gene product of the G. lamblia proS gene did aminoaacylate fractionated M. jannaschii tRNA almost as well as Giardia tRNA; we continued experiments with the archaeal tRNA, as it was easier to obtain. The gene product of the G. lamblia proS gene did aminoaacylate fractionated M. jannaschii tRNA almost as well as Giardia tRNA; we continued experiments with the archaeal tRNA, as it was easier to obtain. The gene product of the G. lamblia proS gene did aminoaacylate fractionated M. jannaschii tRNA almost as well as Giardia tRNA; we continued experiments with the archaeal tRNA, as it was easier to obtain.

Bunjun et al.

PNAS | November 21, 2000 | vol. 97 | no. 24 | 12999
G. lamblia ProRS Synthesizes Cys-tRNA in Vivo. To examine the ability of G. lamblia ProRS to synthesize Cys-tRNA in vivo, we attempted to rescue growth at a restrictive temperature of a temperature-sensitive E. coli cysS strain with the G. lamblia proS gene. As the M. jannaschii tRNA was a better substrate for the Giardia ProRS than E. coli tRNA (see above), coexpression of the genes encoding M. jannaschii tRNA and Giardia ProRS restored growth of E. coli strain UQ818 (18) at 42°C, indicating that ProRS can synthesize Cys-tRNA in vivo (Fig. 2 A and B). The slower growth of the rescued transformants is attributed to the enzyme’s preference for homologous tRNA. This conclusion can be tested when the Giardia tRNA\textsuperscript{C\textsubscript{59}} gene becomes known. In vivo complementation proceeds even in the presence of the E. coli tRNA\textsuperscript{C\textsubscript{59}} gene (Fig. 2 C and D); however, the poor growth indicates that G. lamblia ProRS cannot charge E. coli tRNA efficiently. The ability of G. lamblia ProRS to synthesize Cys-tRNA both in vitro and in vivo indicates that this enzyme can specify two amino acids during protein synthesis.

ProCysRS Is Active in G. lamblia. In an effort to determine whether both the ProCysRS and the CysRS activities contribute to Cys-tRNA formation in this organism, a G. lamblia S-100 cell-free extract was tested for CysRS activity in the presence of G. lamblia total tRNA (Fig. 3). In the absence of antibodies against the two enzymes, we attempted to inhibit cysteinyllation with the proline analogs, which are not expected to inhibit the canonical CysRS enzyme that may also be expressed in G. lamblia. As can be seen, under the conditions tested, the major CysRS activity derives from ProCysRS, as it can be inhibited by thiaproline (24) and 5’-O-[N-(L-prolyl)-sulfamoyl]adenosine (25). However, the inhibition is not as good as with the pure ProCysRS; thus, there may be a small amount of canonical CysRS activity in G. lamblia.

G. lamblia proS and alaS Show Archaeal Origin. ProRS enzymes can be phylogenetically resolved into two main groups: a bacterial group and an archaeal/eukaryal group, which has several bacterial members as well (Fig. 4). Both bacterial and archaeal/eukaryal groups are homologous class IIa aminoacyl-tRNA synthetases, sharing three class-defining motifs in a universally conserved region (26). Surrounding those motifs, however, are significantly diverging primary sequences (2). The G. lamblia ProRS protein described here is specifically related to its archaeal homologs rather than to the eukaryal cytoplasmic ProRS enzymes (Fig. 4; ref. 2). Despite the Giardia enzyme’s similarity to archaeal ProRS, unmodified Giardia
Fig. 4. Phylogenetic tree of ProRS sequences inferred by protein ML analysis. This tree is rooted using threonyl-tRNA synthetase sequences as an outgroup (not shown). Some eukaryotic sequences are distinguished as mitochondrial (mito) or cytoplasmic (cyt). Bootstrap probabilities for each node are estimated by the resampling estimated log-likelihood method. The scale bar represents 10 substitutions per 100 amino acid positions.

Discussion

Among Giardia’s complement of aminoacyl-tRNA synthetases, only AlaRS and ProRS are of archaeal origin, whereas the remaining 18 enzymes bear close resemblance to their eukaryotic relatives. Although there is currently no obvious explanation for the presence of these archaeal synthetases in G. lamblia, it is clear that the ProRS, being of the archaeal genre, can provide Cys-tRNA for the organism in addition to that generated by the canonical CysRS. This improved capacity to form Cys-tRNA may be needed in certain circumstances during the life cycle of G. lamblia. Cysteine is known to play an important role in this organism; G. lamblia trophozoites are coated with cysteine-rich proteins, which, among other roles, function to protect against the hostile gut environment to which G. lamblia is subjected (28, 29). Unlike most eukaryotes, G. lamblia produces energy by fermentation, in keeping with its lack of mitochondria and mitochondrial enzymes. The low-oxygen environment of G. lamblia, where alanine is the major metabolic product (30), is partially maintained by cysteine and proteins containing cysteine. The pathway of cysteine biosynthesis and its efficiency in G. lamblia are unknown; the currently available genomic sequence data contain only an incomplete fragment of one of the cysteine genes (15). One cysteine gene (proS1 in Fig. 4) from that organism is phylogenetically related to the A. fulgidus gene, congruent with the small subunit ribosomal RNA phylogeny. The second cysteine gene (proS2) is related to bacterial members of the archaeal/eukaryal group. It is possible that the two enzymes discriminate among different tRNA species, although their specificities are currently uncharacterized.

In contrast to the archaeal-type ProCysRS found in G. lamblia, ProRS from the highly diverged microsporidian Encephalitozoon cuniculi is similar to the eukaryotic version. Most aminoacyl-tRNA synthetases from that parasite are related to fungal homologs, consistent with recent phylogenetic studies of other microsporidian genes (27).

The family of alanyl-tRNA synthetases consists of homologous class II enzymes divided into archaeal and bacterial/eukaryal subgroups. The G. lamblia AlaRS is an archaeal-type enzyme, which forms an outgroup to the archaeal enzymes in a protein phylogeny (Fig. 5; ref. 2). Two histories would explain these observations. Either the G. lamblia AlaRS represents an ancestral eukaryal enzyme, and other eukarya have acquired a bacterial-type AlaRS, or an archaeal-type AlaRS has displaced the eukaryal enzyme, and other eukarya have acquired a bacterial-type AlaRS. Additional alaS sequence data from diverse eukarya may help in the elucidation of these two hypotheses.

Fig. 5. Phylogenetic tree of AlaRS sequences inferred by protein ML analysis. This tree is rooted using paralogous AlaS2 sequences as an outgroup (not shown). The scale bar represents 10 substitutions per 100 amino acid positions.
Why do some organisms contain two CysRS activities (CysRS and ProCysRS)? The presence of two enzymes for the same activity endows the organism with certain advantages for optimal growth. For instance, it is known for some well-regulated enzymes that their $K_M$ value is within an order of magnitude of the physiological substrate concentration (31). If there were changes in cysteine concentration during the life cycle of Giardia, and if ProCysRS and CysRS had different $K_M$ values for cysteine, then Cys-tRNA could be formed by one or the other enzyme, based on the prevailing cellular cysteine concentration, an optimal situation. Whereas the $K_M$ values of M. jannaschii ProCysRS for cysteine and proline are comparable to those from ProRS or CysRS enzymes (C.S., unpublished work), the M. maripaludis ProCysRS appears to have a lower affinity for cysteine than does the organism’s CysRS (C.S., unpublished observations). RNA modifications are sometimes essential for tRNA recognition. Should the two CysRS activities require different RNA modifications for substrate recognition, then Giardia could deal better with changes in modification levels caused by different growth rates or gene loss (for modification enzymes) during genome reduction. Future studies of the properties of the enzymes will shed light on some of these questions.

In any case, the dual-specificity ProCysRS is an efficient enzyme, as M. jannaschii and M. thermoautotrophicum are vigorous organisms in which this enzyme is the only means of Cys-tRNA synthesis (32, 33). On the other hand, ProRS activity is usually not duplicated; only one organism (M. mazei) is known where a canonical ProRS coexists with the dual-specificity ProCysRS. ProCysRS is currently the only known dual-specificity AARS.

Did this enzyme precede the generation of the canonical CysRS or was the CysRS activity woven into the proS context after loss of the cysS gene? It is uncertain whether the contemporary ProRSs was the CysRS activity woven into the canonical ProRS coexists with the dual-specificity ProCysRS. The presence of two enzymes for the same substrates in addition to the anticodon bases. Broadly defined identity elements suggest that the eukaryal relevant systems are modular and peripheral to the cell’s other systems. The specific charging of tRNAs with their cognate amino acids is imperative for a cell, even though the cell may be indifferent to the mechanism (1, 2).

We thank M. Ibb and C. R. Woese for constructive discussions. We are indebted to S. Cusack for a sample of 5’-O-[N-(prolyl)-sulfamoyl]adenosine, and to H. D. Becker and T. Li for experimental advice. This work was supported by grants from the National Institute of General Medical Sciences and from National Aeronautics and Space Administration. M.K. was a postdoctoral fellow of the Japanese Ministry for Education.