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Comments

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Functional Association between Three Archaeal Aminoacyl-tRNA Synthetases*

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Aminoacyl-tRNA synthetases (aaRSs) are responsible for attaching amino acids to their cognate tRNAs during protein synthesis. In eukaryotes aaRSs are commonly found in multi-enzyme complexes, although the role of these complexes is still not completely clear. Associations between aaRSs have also been reported in archaea, including a complex between prolyl-(ProRS) and leucyl-tRNA synthetases (LeuRS) in *Methanothermobacter thermautotrophicus* that enhances tRNA^{Pro} aminoacylation. Yeast two-hybrid screens suggested that lysyl-tRNA synthetase (LysRS) also associates with LeuRS in *M. thermautotrophicus*. Co-purification experiments confirmed that LeuRS, LysRS, and ProRS associate in cell-free extracts. LeuRS bound LysRS and ProRS with a comparable K_D of about 0.3–0.9 μM , further supporting the formation of a stable multi-synthetase complex. The steady-state kinetics of aminoacylation by LysRS indicated that LeuRS specifically reduced the K_m for tRNA^{Lys} over 3-fold, with no additional change seen upon the addition of ProRS. No significant changes in aminoacylation by LeuRS or ProRS were observed upon the addition of LysRS. These findings, together with earlier data, indicate the existence of a functional complex of three aminoacyl-tRNA synthetases in archaea in which LeuRS improves the catalytic efficiency of tRNA aminoacylation by both LysRS and ProRS.

To ensure that the correct amino acid is incorporated into the growing polypeptide chain during protein biosynthesis, the aminoacyl-tRNA synthetases (aaRSs)² must attach the correct amino acid to the corresponding tRNA molecule (1). This essential process, in turn, supplies the ribosome with the aminoacyl-tRNAs that are critical for the synthesis of proteins in all cells. The aaRSs can be divided into two groups, class I and II, based on the architecture of the catalytic domain (2–4). Class I aaRSs, which are normally monomeric, attach the aminoacyl group to the 2'-OH of the 3' terminal nucleotide of the tRNA,

whereas class II aaRSs catalyze the addition to the 3'-OH of the tRNA and are usually dimeric. In bacteria, the aaRSs normally act alone as free-standing proteins, although in some instances their activities are enhanced by association with other translation factors (5, 6). In eukaryotic organisms, aaRSs are commonly found in multi-enzyme complexes within the cell that are believed to stabilize the interaction between tRNAs and synthetases, thereby increasing the efficiency of aminoacylation. These multi-enzyme complexes vary depending on the specific aaRSs and accessory proteins involved. For example, a complex forms between two synthetases and a non-synthetase protein in *Saccharomyces cerevisiae* (7), and there is also evidence for functional interactions between seryl-tRNA synthetase and a peroxisomal protein (8) as well as between tyrosyl-tRNA synthetase and a protein involved in the regulation of cell wall assembly in yeast (9). A complex of valyl-tRNA synthetase and human elongation factor-1H (10) as well as a larger complex consisting of nine aaRS activities and three non-synthetase proteins (MARS) are both found in mammals (11–13). Association with non-synthetase components in the larger complex may not only promote efficient aminoacylation and substrate channeling (14) but also function in other processes beyond translation such as gene silencing, apoptosis, and inflammation (12, 15).

No bacterial multi-aaRS complexes have yet been described, and there is only sparse data concerning their existence in archaeal species. An early report indicated the presence of a multi-synthetase complex in the extreme halophile *Haloarcula marismortui*, with many if not all of the aaRSs purified in one or possibly two large complexes (16). A subsequent study in *Methanocaldococcus jannaschii* revealed the presence of a non-synthetase protein bound to purified prolyl-tRNA synthetase (ProRS) and further suggested that both aspartyl- and the class I-type lysyl-tRNA synthetase (LysRS1) might also be part of this complex (17). The cellular role of the *M. jannaschii* complex, however, remains unclear because the aaRS activities were essentially unchanged upon complex formation. Recently, a yeast two-hybrid screen also revealed a complex between ProRS and leucyl-tRNA synthetase (LeuRS) in *Methanothermobacter thermautotrophicus* (18). Steady-state kinetic analyses showed that the catalytic efficiency (k_{cat}/K_m tRNA^{Pro}) of ProRS increases 5-fold in this complex compared with the free enzyme, whereas aminoacylation by LeuRS is unchanged. Preliminary investigation of this interaction in cell-free extracts of *M. thermautotrophicus* suggested that LysRS1 might also associate with the LeuRS-ProRS complex, but these initial findings were not definitive.

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² The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; HMD, H₂-forming N⁵-N¹⁰-methylene tetrahydromethanopterin dehydrogenase; LeuRS, leucyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; MARS, multi-aminoacyl-tRNA synthetase complex; ProRS, prolyl-tRNA synthetase; AF, Alexa fluor.

To further investigate the composition of multi-aaRS complexes in archaea, we have now undertaken a search for proteins that interact with LysRS1. The choice of LysRS1, a primarily archaeal protein, was based upon its proposed presence in multi-aaRS complexes in *M. jannaschii* and *M. thermautotrophicus* and its possible role in the assembly of an aminoacylation ternary complex in *Methanosarcina barkeri* (17–19). LeuRS was found to interact with LysRS1, and the association of these two aaRSs with ProRS was demonstrated in cell-free extracts. These findings, together with *in vitro* kinetic analyses, indicate the existence of a functional complex of three aaRSs in archaea.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid, Strains, Media, and Plasmid Construction—Yeast transformation was done according to Ref. 20. Yeast media were made according to the manual for the ProQuest two-hybrid system (Invitrogen) and as described (21). All of the primers were from Integrated DNA Technologies. The bait vector pDBLeu, prey vector pDEST22 or pPC86, and the yeast host strain MaV203 (*MAT α leu2-3, 112, trp 1-901, his3 Δ 200, ade2-101, gal4 Δ , gal80 Δ , SPAL10::URA3, GAL1::lacZ, HIS3_{UAS} GAL1::HIS3@LYS2, can1^R, cyh2^R) were from the ProQuest two-hybrid system (Invitrogen). The yeast two-hybrid bait vector containing the *M. thermautotrophicus lysK* gene was constructed as follows. The LysRS encoding gene (*lysK*, MTH1541) was isolated by PCR using genomic *M. thermautotrophicus* DNA as template, the primers 5'-GGTG GTGTCGACCATGAAGTTCACACAC-3' and 5'-GGTGGTGTCTGACTCAGGCCTCCAGTCT-3' flanked by SalI sites and *Pfu* DNA polymerase (Stratagene). The *lysK* PCR product was cloned into PCR-Blunt II-TOPO vector (Invitrogen), sequenced, and subsequently subcloned into the yeast ProQuest two-hybrid bait vector pDBLeu using the SalI restriction sites. Construction, amplification, and screening of the *M. thermautotrophicus* cDNA-based yeast two-hybrid library were as previously described (18). The two-hybrid screen was carried out essentially as described in the manual of the ProQuest two-hybrid system (Invitrogen) by sequential or co-transformation of bait and prey vectors. Potential positive protein-protein interacting colonies were identified by growth on selective media (SC lacking histidine, leucine, and tryptophan) supplemented with 3-aminotriazole (10–25 mM). After rescreening on the same media, bait vectors were isolated from the respective colonies and sequenced.*

A vector producing an N-terminally tagged His₆ fusion derivative of LysRS was prepared by inserting the relevant PCR-amplified gene into the *Escherichia coli* expression plasmid pET15b (Novagen). For the His₆-LysRS construct, forward primer 5'-CATATGAAGTTCACACACTTT-3' and reverse primer 5'-GGTGGTCTGCAGTCAGGCCTCCAGTCT-3' were used. Cloning into pET15b was done by isolating the respective NdeI and PstI fragment and ligating it into NdeI- and PstI-digested pET15b.

Protein Production and Purification—His₆-LeuRS and His₆-ProRS were produced and purified as previously described (18). His₆-LysRS was produced by transforming *E. coli* BL21-RIL (Stratagene) with pET15b-Mt*lysK*. The resulting transformants were grown and induced to produce protein using the Over-

night Express Auto-induction System 1 (Novagen) following the manufacturer's protocol. Cell-free extract was produced by sonication of the cells in buffer A (50 mM Hepes, pH 7.2, 25 mM KCl, 10 mM MgCl₂, 5 mM dithiothreitol, and 10% glycerol) containing protease inhibitor mixture tablet (Complete Mini, EDTA-free; Roche Applied Science) followed by centrifugation at 75,000 × *g* for 20 min (all of the following procedures were performed at 4 °C unless otherwise stated). To reduce the amount of contaminating *E. coli* proteins, the supernatant was incubated at 55 °C for 10 min followed by a brief centrifugation to remove precipitated protein and then ultracentrifugation at 100,000 × *g* for 1 h. The supernatant from ultracentrifugation was loaded onto a HiPrep 16/10 Q Sepharose FF column (GE Healthcare) equilibrated in buffer A and washed extensively in the same buffer. The His₆-LysRS was eluted with a NaCl gradient (0–1 M) in the same buffer. Fractions containing His₆-LysRS, as determined by aminoacylation activity, were pooled and concentrated by ultrafiltration (Amicon 30; Millipore). The concentrated sample was further purified using gel filtration on a Superose 12 column (GE Healthcare) equilibrated in buffer A containing 100 mM NaCl. Fractions containing His₆-LysRS, as determined by SDS-PAGE and Coomassie Brilliant Blue staining, were pooled, concentrated by ultrafiltration, and stored at –80 °C.

tRNA Purification—*M. thermautotrophicus* cells, a gift from J. Reeve (The Ohio State University), were used to prepare total tRNA as previously described (19). The total RNA concentration was estimated spectrophotometrically (A_{260}/A_{280}), and individual tRNA acceptor levels were measured in plateau charging reactions (tRNA^{Lys} = 0.41 pmol/μg; tRNA^{Leu} = 0.24 pmol/μg; tRNA^{Pro} = 0.40 pmol/μg). Preparation of *in vitro* transcribed *M. thermautotrophicus* tRNA^{Pro} and tRNA^{Leu} was as previously described (18). *In vitro* transcribed *M. thermautotrophicus* tRNA^{Lys} was found to be inactive in aminoacylation assays (data not shown).

Co-purification of LeuRS, LysRS, and ProRS—All of the steps were performed at 4 °C. *M. thermautotrophicus* cells (5 g) were resuspended in buffer A (as above) containing protease inhibitor mixture tablet (Complete Mini, EDTA-free; Roche Applied Science), 1 mM phenylmethylsulfonyl fluoride, and 1 mM *N*-α-(*p*-toluene sulfonyl)-L-arginine methyl ester to a final volume of 10 ml. The cells were then passed twice through a French pressure cell, sonicated, and finally centrifuged at 48,000 × *g* for 30 min. The supernatant was then removed and centrifuged for 40 min at 100,000 × *g*. The resulting cell-free extract was then loaded onto a Sephacryl S300 26/60 column (GE Healthcare) previously equilibrated in buffer B (buffer A containing 150 mM KCl) and developed in the same buffer. Fractions containing LeuRS, LysRS, and ProRS activity were pooled and applied to a HiPrep 16/10 Q Sepharose FF column (GE Healthcare) equilibrated in buffer B, and proteins were eluted with a KCl gradient (0.125–1.125 M) in the same buffer.

Size Exclusion Chromatography of aaRSs—Size exclusion chromatography was performed using a Superose 12 column (GE Healthcare) pre-equilibrated in buffer A containing 200 mM KCl and calibrated using Gel Filtration Standards (Bio-Rad). The samples were prepared in the same buffer as a mixture of 5 μM His₆-ProRS, 30 μM His₆-LeuRS, and 14 μM His₆-

TABLE 1
Proteins identified as interacting with *M. thermautotrophicus* LysRS

Function	ORF ^a	Description ^b
Methanogenesis	MTH1130	Methyl CoM RDase II, γ -sub
	MTH1134	Methyl viologen-reducing hydrogenase
	MTH1161	N_5 -methyl-tetrahydromethanopterin: CoM methyltransferase, C-sub
	MTH1165	Methyl CoM RDase I, γ -sub
	MTH1166	Methyl CoM RDase c-sub
	MTH1168	Methyl CoM RDase I, β -sub
	MTH1300	Co-F420-reducing hydrogenase
	MTH1752	F420-dependent N^5 , N^{10} -methylene tetrahydromethanopterin reductase
	MTH1878	CoB-CoM heterodisulfide reductase Fe-S, C-sub
Protein binding and modification	MTH32	Centromere/microtubule-binding protein
	MTH357	Transglutaminase
	MTH412	Transglutaminase
	MTH678	Prefoldin β -sub
	MTH794	Chaperonin (thermosome β -sub)
Other	MTH810	Putative ski2-type helicase
	MTH1588	Ferripyochelin-binding protein
	MTH1913	Putative membrane protein
Translation	MTH1508	Leucyl-tRNA synthetase
	Unknown function	
Unknown function	MTH247	
	MTH479	
	MTH545	
	MTH609	
	MTH674	
	MTH1191	

^a Open reading frames (ORFs) are numbered according to the annotated genome sequence of *M. thermautotrophicus* (26).

^b Predicted functions of the corresponding proteins are taken from the TIGR Comprehensive Microbial Resource (cmr.tigr.org/tigr-scripts/CMR/shared/AnnotationSearch.cgi).

LysRS (40 μ l of sample volume) and preincubated for 20 min at room temperature prior to injection. The samples were applied at a flow rate of 0.25 ml min⁻¹, and 0.5-ml fractions were then collected. 0.3 ml was then removed from each fraction, and proteins were precipitated with 5 volumes acetone at -80 °C overnight. Precipitated proteins were recovered by centrifugation at 16,000 \times g for 30 min at 4 °C; the resulting pellet was dried and finally resuspended in protein gel loading buffer.

Aminoacylation Assays—L-[U-¹⁴C]Leucine (306 mCi/mmol), L-[U-¹⁴C]lysine (312 mCi/mmol), and L-[U-¹⁴C]proline (241 mCi/mmol) were all from Amersham Biosciences. A pre-reaction mixture containing 250 mM KCl, 100 mM Hepes, pH 7.5, 10 mM dithiothreitol, 10 mM MgCl₂, 50 μ g/ml bovine serum albumin, 6 mg/ml *M. thermautotrophicus* total tRNA, or *in vitro* transcribed tRNA at the concentrations indicated, and aaRSs at concentrations indicated for specific experiments were preincubated for 20 min at room temperature. The appropriate radiolabeled amino acid was then added to the mixture, and the temperature was increased to 50 °C. After 1 min, 5 mM ATP was added to start the reaction. The aliquots were spotted onto 3MM paper presoaked in 5% trichloroacetic acid (w/v) and washed in trichloroacetic acid, and the radioactivity was counted. For Lys K_m determination, L-[¹⁴C]-Lys was added at concentrations varying between 0.2 and 3 times K_m . For tRNA^{Lys} K_m determination, total tRNA was added to provide final concentrations of tRNA^{Lys} varying between 0.2 and 3 times the K_m .

Labeling of *M. thermautotrophicus* LeuRS—Fluorescent labeling and anisotropy measurements (see below) were based upon previously published procedures (5, 22). A solution of Alexa fluor (AF) 488 tetrafluorophenyl ester (Molecular Probes,

Eugene, OR) was prepared in dimethyl sulfoxide, according to the manufacturer's instructions. Prior to labeling LeuRS with AF, the protein was desalted using a 1-ml Sephadex G25 spin column (Amersham Biosciences). LeuRS (40 μ M) was then combined with AF at a molar ratio of 1:25 in 40 mM Hepes, pH 7.5, 50 mM NaCl, and 50 mM KCl and incubated for 45 min at room temperature. Excess unreacted dye was immediately removed by passage through a 1-ml Sephadex G25 spin column pre-equilibrated in the same buffer. Traces of dye were then removed from the labeled protein, LeuRS-AF, by dialysis overnight against a buffer containing 50 mM Hepes, pH 7.5, 25 mM KCl, 10 mM MgCl₂, 10% glycerol, 50 mM NaCl, and 5 mM dithiothreitol. LeuRS-AF was then applied to a Microcon YM-50 concentrator (Amicon) to remove residual free dye and concentrate the protein. LeuRS-AF was visualized on a 10% SDS-polyacrylamide gel and subjected to ultraviolet illumination, which confirmed that the final labeled product contained little or no free fluorophore. The final labeling stoichiometry was determined to be 1:1 (LeuRS to fluorophore) based on Equation 1 (as per the manufacturer's protocol).

$$R = [A_{494} \times (\text{dilution factor})]/(\epsilon_{494} \times Y) \quad (\text{Eq. 1})$$

R represents the molar ratio of LeuRS to fluorophore, A_{494} is the absorbance of labeled protein at 494 nm, ϵ_{494} is the extinction coefficient of the AF, and Y is the final concentration of LeuRS-AF determined by the Bradford assay (Bio-Rad). Prior to use in fluorescence anisotropy measurements, the activity of LeuRS-AF was verified by aminoacylation assays, and protein concentrations were determined via active site titration (23).

Fluorescence Anisotropy Measurements—The fluorescence anisotropy of LeuRS-AF was measured as a function of increasing concentrations of unlabeled protein to determine equilibrium dissociation constants (K_D). Prior to fluorescence anisotropy measurements, 100 nM LeuRS-AF was incubated with varying amounts of unlabeled protein (25–2000 nM LysRS or 25–5500 nM ProRS) for 20 min at room temperature in a buffer containing 50 mM Hepes, pH 7.5, 250 mM KCl, 10 mM MgCl₂, 125 mM dipotassium glutarate, and 5 mM dithiothreitol. Fluorescence anisotropy was measured using a Fluorolog-3 spectrofluorimeter (Horiba Jobin Yvon), with excitation and emission wavelengths of 495 and 519 nm, respectively, slit widths of 5 nm, and the time-based function for 30 s (integration time = 1 s; resolution = 8 s), and the data were then averaged. All of the measurements were carried out at least three times. The titration curves were fitted to Equation 2, which assumes a 1:1 binding stoichiometry.

$$A = A_{\min} + [(Y + S + K_D) - \{(Y + S + K_D)^2 - (4YS)\}^{1/2}]/(2Y) \quad (\text{Eq. 2})$$

A is the measured anisotropy at a particular total concentration of unlabeled protein (S) and LeuRS-AF (Y), A_{\min} is the minimum anisotropy, A_{\max} is the maximum anisotropy, and K_D is the dissociation constant.

TABLE 2

Yeast two-hybrid interaction between LeuRS and LysRS

Growth phenotypes of MaV203 transformed with control bait and prey vectors on selective media for monitoring interactions between reference proteins (A–D controls; ProQuest two-hybrid system; Invitrogen), and LysRS and LeuRS using MaV203 transformed with the bait vector pDBleu-*lysK* (encoding LysRS) and the prey vector pPC86-B55 (containing a LeuRS encoding library insert). Growth of transformants was judged after 48 h at 30 °C on SC medium lacking leucine, tryptophan, and uracil or SC medium lacking leucine, tryptophan, and histidine and supplemented as indicated. – indicates no growth, + indicates weak growth, ++ indicates moderate growth, and +++ indicates strong growth.

Plasmids	–Ura	–His +25 mM 3-AT	+0.2% 5-FOA	Description
A controls	–	–	+++	No interaction standard
B controls	–	+	++	Weak interaction standard
C controls	+/-	+	+	Moderate interaction standard
D controls	+	++	+/-	Strong interaction standard
pDBleu- <i>lysK</i> /+ pPC86-B55	–	+	+/-	LeuRS insert in prey vector
pDBleu- <i>proS</i> /+ pPC86	–	+/-	+++	Empty prey vector control

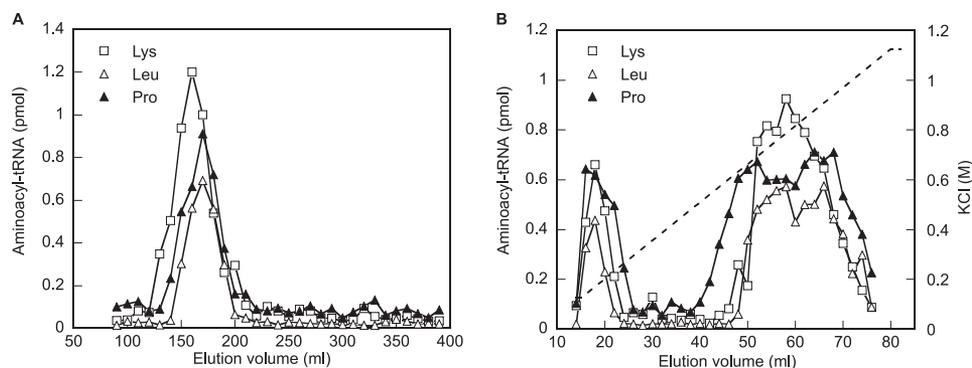


FIGURE 1. Co-purification of LysRS, LeuRS, and ProRS. *A*, *M. thermautotrophicus* cell-free extracts were applied to a Sephacryl S300 column, and the aminoacylation activity was monitored in the eluted fractions. *B*, active fractions from *A* were pooled and applied to a Q-Sepharose column and then extensively washed prior to development with a KCl gradient (see text for details). Aminoacylation activities were monitored in the eluted fractions. ◆, Lys-tRNA; △, Leu-tRNA; ▲, Pro-tRNA.

RESULTS

Identification of LysRS Interacting Proteins—Approximately 4.1×10^5 transformants were screened for protein-protein interacting phenotypes using the bait vector pDBleu harboring the *M. thermautotrophicus lysK* gene and library inserts encoded by the prey vectors pDEST or pPC86. From these transformants, 560 potential positive protein-protein interacting clones were selected and rescreened according to the growth phenotype on selective media supplemented with 3-aminotriazole (Table 1). A total of 133 clones showing growth phenotype similar to the weak or moderate interacting standard (Table 1) were picked, and bait vectors were isolated and subsequently sequenced. The LysRS interacting proteins were then sorted into four classes based upon the predicted function of the corresponding open reading frames (Table 1). An additional group of proteins with no significant similarity to proteins of known function was also identified but was not further pursued in this study.

The largest group of proteins identified as interacting with LysRS in the two-hybrid screen were all involved in different steps of the methanogenesis pathway. Of these, MTH1130, MTH1161, MTH1165, MTH1168, MTH1300, MTH1752, and MTH1878 were all previously identified as false positive clones in two-hybrid screens of the same library using translation factors or DNA-binding proteins as bait (18).^{3,4} The remaining two clones, MTH1134 and MTH1166, are both very closely

related to the other methanogenic proteins identified here and elsewhere as false positives and were not further investigated. The second major group of potential LysRS-associating proteins identified from the two-hybrid screen all have known roles in protein binding and modification. MTH357, MTH412, and MTH794 have all been previously identified as false positive clones (18).^{3,4} The other two clones, MTH32 and MTH678, both encode proteins expected to bind to different factors in the cell and thus are more likely to act as false positive clones rather than as proteins that

specifically bind LysRS. Of the other proteins found to interact with LysRS, MTH1588 and MTH1913 are both normally expected to be membrane-associated, suggesting that their association here with LysRS may be nonspecific. MTH810 is usually found as part of a multimeric complex (24), suggesting that the interaction observed here with LysRS might be nonspecific.

The only known component of protein synthesis identified from the two-hybrid screen was LeuRS (Tables 1 and 2). LeuRS has previously been shown to form functional interactions with ProRS both in the human multi-aaRS complex (reviewed in Refs. 11 and 25) and in *M. thermautotrophicus*. LysRS, albeit of the class II rather than archaeal class I type, is also a component of the human multi-aaRS complex, leading us to investigate whether LysRS is associated with ProRS and LeuRS in archaea.

Association of LeuRS with LysRS and ProRS—To investigate the possible interactions between the LeuRS, LysRS, and ProRS proteins in *M. thermautotrophicus*, the co-purification of the corresponding activities from cell-free extracts was monitored. An S100 fraction was prepared and applied to a gel filtration column, and the collected fractions were tested for LeuRS, LysRS, and ProRS aminoacylation activities in the presence of the corresponding radiolabeled amino acid and *M. thermautotrophicus* total tRNA. The LeuRS, LysRS, and ProRS activities all eluted together (Fig. 1A), whereas a control aminoacylation activity showed a different elution profile (alanyl-tRNA synthetase; data not shown). Calibration of the column with molecular mass standards indicated that the peak in the three activities corresponded to a molecular mass of ~200–255 kDa, broadly

³ Z. Kelman, personal communication.

⁴ C. D. Hausmann and M. Ibba, unpublished results.

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consistent with a 1:1:1 complex stoichiometry (predicted molecular mass, ~280 kDa). The fractions eluting between 145 and 200 ml were pooled and applied to an anion exchange column, which was then extensively washed prior to development with a KCl gradient. LeuRS, LysRS, and ProRS again eluted together, this time showing co-activity peaks both early and late in the applied KCl gradient (Fig. 1B), suggesting partial dissociation of the complex under these conditions. Attempts to further co-purify the LysRS·LeuRS·ProRS complex by separately applying the active pools to a cation exchange column were not successful (data not shown).

Reconstitution of the putative LysRS·LeuRS·ProRS complex was investigated *in vitro* using the corresponding recombinant proteins. Following co-incubation of the three proteins at room temperature, the possible formation of a ternary com-

plex was investigated by size exclusion chromatography (Fig. 2). A peak was observed with an approximate molecular mass of 600 kDa, consistent with a complex stoichiometry of 2LysRS·2LeuRS·2ProRS and in agreement with the approximately equal amount of each protein seen in the corresponding fraction. This is also consistent with previous reconstitution of the LeuRS·ProRS interaction, which also suggested a 2:2 stoichiometry (18). No evidence was seen for reconstitution of a possible interaction between LysRS and ProRS (data not shown), consistent with two-hybrid data (this work and Ref. 18).

To further characterize the multi-synthetase interactions, fluorescence anisotropy experiments were employed to measure the K_D values for LeuRS binding to LysRS and ProRS. In these experiments, LeuRS was labeled with an amine-reactive fluorophore, Alexa Fluor® 488, because of the significantly greater stability of LeuRS over LysRS and ProRS in the labeling procedure.⁴ Prior to fluorescence anisotropy measurements, the activity of the labeled LeuRS-AF was verified by aminoacylation assays, and protein concentrations were determined via active site titration (data not shown). The binding affinity between LeuRS-AF and ProRS was investigated (Fig. 3A). LeuRS-AF in the presence of increasing concentrations of unlabeled ProRS resulted in a significant increase in anisotropy and a K_D of 970 ± 10 nM. Similarly, titration of LeuRS-AF with LysRS resulted in an increased anisotropy and a binding constant of 270 ± 50 nM (Fig. 3B). These results are in accordance with the biochemical studies confirming an association between aaRSs in *M. thermautotrophicus*. Although it would also be of interest to measure the binding affinity in the presence of tRNA, the use of *in vitro* transcribed tRNA^{Lys} was not a viable option because the tRNA is inactive in aminoacylation.

Effects of Association of LeuRS with LysRS and ProRS on Aminoacylation—Our previous studies showed that the association between LeuRS and ProRS specifically enhances the steady-state kinetics of tRNA^{Pro} aminoacylation, leading to a 5-fold increase in the catalytic efficiency of the reaction (k_{cat}/K_m) (18). The potential impact on aminoacylation of the association between LeuRS, LysRS, and ProRS was investigated by monitoring aminoacyl-tRNA synthesis by each enzyme in the presence or absence of the other components of the complex.

The addition of LeuRS or ProRS increased the aminoacylation activity of LysRS (Fig. 4A), whereas the presence of both together did not lead to any further enhancement in activity over that seen with the individual proteins (data not shown). In contrast, the addition of LysRS did not significantly enhance aminoacylation by either LeuRS (Fig. 4B) or ProRS (Fig. 4C). Steady-state aminoacylation kinetics for LysRS in the presence or absence of LeuRS indicated that complex formation specifically decreased the K_m for tRNA^{Lys} 3-fold but had no effect on the K_m for Lys or the k_{cat} (Table 3). The addition of ProRS had a less

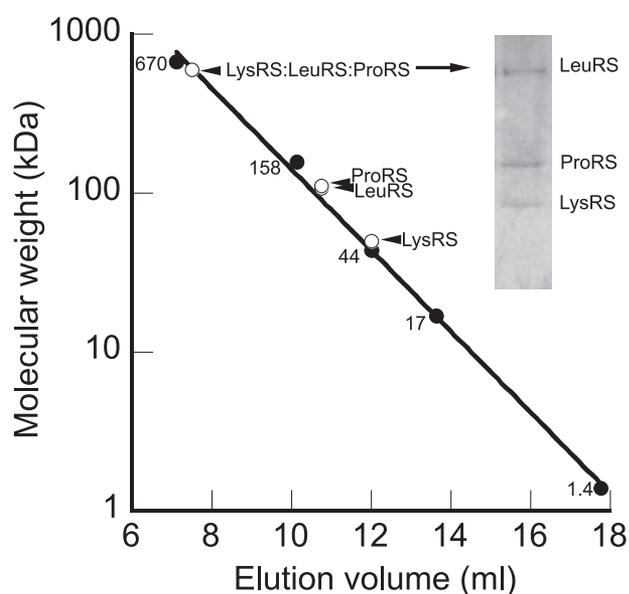


FIGURE 2. Gel filtration chromatography of LysRS, LeuRS, and ProRS. LysRS, LeuRS, and ProRS were applied either alone or combined after preincubation to a Superose 12 column. Calibration curves show the elution positions of both individual and combined proteins. ●, molecular mass standards; ○, aaRS samples. SDS-PAGE (10%) analysis of the ~600 kDa fraction is shown in the inset.

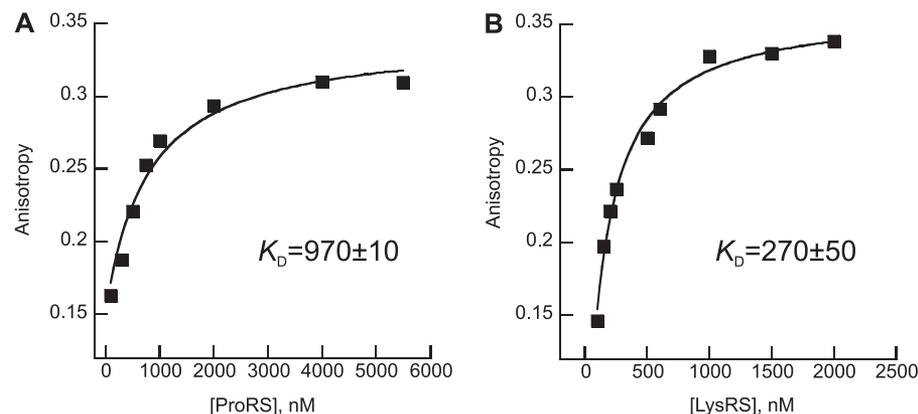


FIGURE 3. Fluorescence anisotropy experiments. The binding affinities of Alexa Fluor-labeled LeuRS to ProRS (A) and LysRS (B) were measured using 100 nM of LeuRS-AF as a function of increasing concentrations of unlabeled protein. A representative data set is shown, with values shown representing the means and corresponding standard deviations from three independent determinations. The data were fitted to Equation 2 ("Experimental Procedures").

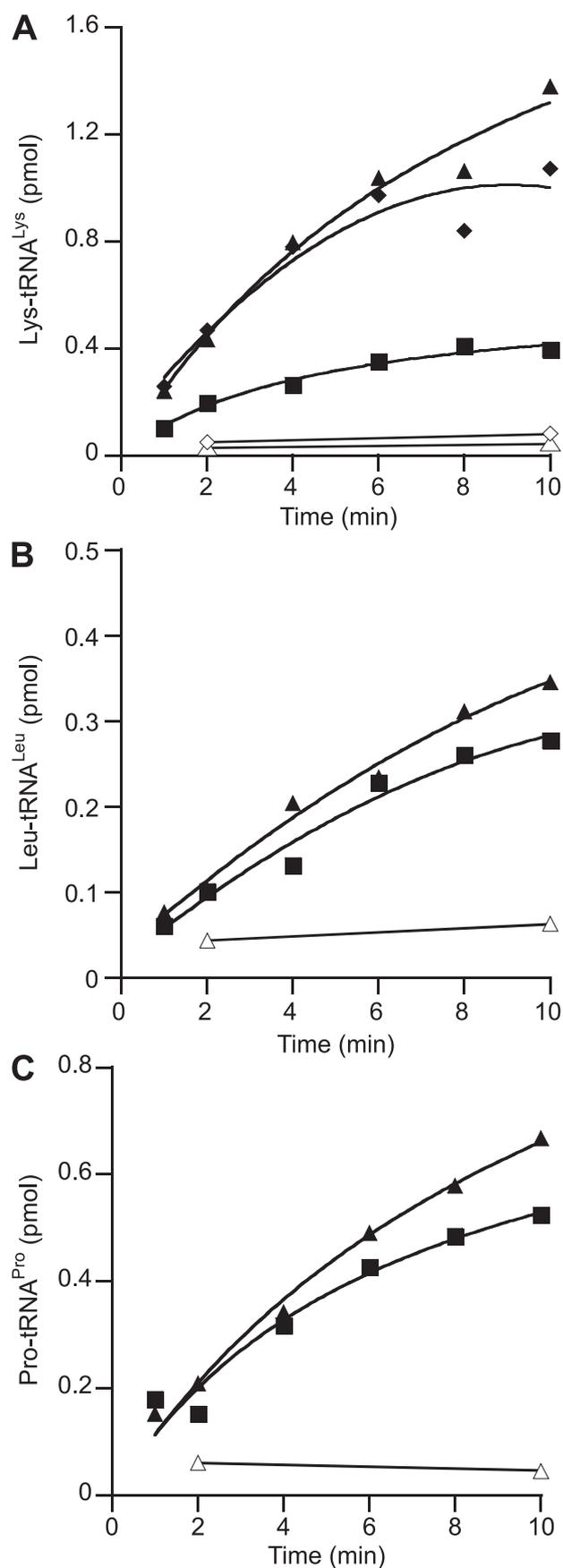


FIGURE 4. Effects of aaRS addition on aminoacylation. A, aminoacylation of tRNA^{Lys}. *M. thermautotrophicus* total tRNA (6 mg/ml) was aminoacylated under standard conditions (8- μ l samples) in the presence of LysRS (80 nM)

pronounced effect on steady-state aminoacylation by LysRS, decreasing the K_m for tRNA^{Lys} by about 30%. The addition of LeuRS or ProRS did not lead to any significant change in the K_m for Lys of LysRS, indicating that the interactions between the three synthetases specifically improve tRNA binding.

DISCUSSION

Utilization of a yeast two-hybrid assay to screen for *M. thermautotrophicus* proteins interacting with LysRS provided similar results to those previously obtained when screening for interactions with ProRS (18). In both cases the largest group of interacting proteins consisted of components of the methanogenesis pathway, nearly all of which could be accounted for as false positives based on comparison to other *M. thermautotrophicus* two-hybrid assays. As with ProRS, the LysRS-based screen did not reveal any interaction with H₂-forming N⁵-N¹⁰-methylene tetrahydromethanopterin dehydrogenase (HMD), a protein previously shown to enhance tRNA binding by ProRS in *M. jannaschii* (17). Although HMD itself was not found, the metabolically related protein F420-dependent N⁵-N¹⁰-methylene tetrahydromethanopterin reductase, which acts immediately downstream of HMD (26), was detected in the two-hybrid assay with LysRS as bait. This echoes our previous finding of another HMD-related protein interacting with ProRS (18) and provides further evidence of a possible association between protein synthesis and metabolism in archaea as originally proposed in *M. jannaschii* (17). The significance of this interaction remains unclear; recent proteomics studies in other methanogens do not indicate a regulatory role in methanogenesis, suggesting that the interaction may impact other aspects of metabolism (27).

The most significant interaction identified in the current two-hybrid assay was that between LysRS and LeuRS, suggesting that a ternary complex exists between these two aaRSs and ProRS. The existence of a ternary complex was also supported by both native and reconstituted size exclusion chromatography. Although both approaches indicate that the proteins are present in equimolar amounts, the exact stoichiometry is unclear and now requires more detailed analysis by alternative biophysical approaches.

The LysRS, LeuRS, and ProRS synthetase activities are found in the mammalian MARS (11), and their presence in a single complex is also consistent with predictions based on the physical chemistry of the corresponding substrate amino acids (28). Sequencing of the LeuRS-encoding clone identified an N-terminal fragment as the interacting partner with LysRS. This is complementary to our previous finding of a C-terminal fragment of LeuRS that interacted with ProRS and suggests how the three aaRSs could form a ternary complex mediated by LeuRS.

alone (◻) or with the addition of LeuRS (800 nM) (▲) or ProRS (800 nM) (◆). Control reactions were also performed with LeuRS alone (800 nM) (△) or ProRS alone (800 nM) (◇). B, aminoacylation of tRNA^{Leu}. *M. thermautotrophicus* total tRNA (6 mg/ml) was aminoacylated under standard conditions (8- μ l samples) in the presence of LeuRS (80 nM) alone (◻) or with LysRS (800 nM) (▲). Control reactions were also performed with LysRS alone (800 nM) (△). C, *in vitro* transcribed tRNA^{Pro} (2.2 μ M) was aminoacylated under standard conditions (8- μ l samples) in the presence of ProRS (80 nM) alone (◻) or with LysRS (800 nM) (▲). Control reactions were also performed with LysRS alone (800 nM) (△).

TABLE 3
Steady-state aminoacylation kinetics of *M. thermautotrophicus* LysRS

Enzyme ^a	Additions ^b	K_m Lys μM	K_m tRNA ^{Lys} μM	k_{cat} min^{-1}	k_{cat}/K_m tRNA (relative)
LysRS	None	6.6 ± 1.6	0.74 ± 0.15	0.86 ± 0.1	1
LysRS	LeuRS	6.0 ± 2	0.24 ± 0.02	0.91 ± 0.03	3.3
LysRS	ProRS	7.9 ± 0.9	0.51 ± 0.13	0.63 ± 0.08	1.1

^a Each enzyme was added at a final concentration of 100 nM.^b Other components were added at 1 μM each.

The idiosyncratic C-terminal extensions of human and yeast LeuRS have previously been shown to mediate protein-protein interactions (29, 30). This suggests that comparable regions of the structurally distinct C terminus of archaeal LeuRS (31) might also mediate interactions with ProRS, as observed in the human MARS (12, 13, 32). Although no comparable precedent exists for interactions between the N terminus of LeuRS and other proteins, this region of the protein is phylogenetically distinctive. For example, the first 60 amino acids of archaeal LeuRSs more closely resemble bacterial-type sequences, as opposed to the rest of the protein, which closely resembles eukaryotic examples (33). Mapping of domain-domain interactions between LeuRS and LysRS and ProRS is now required to clarify the specific roles of the idiosyncratic N- and C-terminal regions of LeuRS in complex formation.

The association of LysRS, LeuRS, and ProRS in a ternary complex, suggested by two-hybrid assays and co-purification, was supported by the observed parameters for physical association. The K_D values determined for the interaction of ProRS and LysRS with LeuRS were ~ 0.3 – $0.9 \mu\text{M}$, 5–10-fold lower than predicted cellular concentrations for the aaRS proteins in bacteria (34). If similar cellular concentrations prevail for LysRS, LeuRS, and ProRS in *M. thermautotrophicus*, the majority of these proteins would be expected to be found in the ternary complex based on the experimental K_D values. Although dissociation constants for components of MARS are not available, bacterial ProRS was recently found to associate with a *trans*-editing factor with a comparable affinity to that found here (5). Taken together with previous data from other related systems, our present findings indicate the existence of an aaRS ternary complex in archaea with the composition: LysRS_N·LeuRS_C·ProRS.

The functional consequences of the formation of this aaRS ternary complex in archaea are the specific enhancement of both tRNA^{Lys} and tRNA^{Pro} aminoacylation, with no other effects observed. This catalytic enhancement most closely resembles the MetRS·Arc1p·GluRS complex of yeast, where the functional consequence of association is improved aminoacylation. Enhancement is specifically mediated by Arc1p rather than by direct interaction between the synthetases, in much the same way that LeuRS acts here. A possible explanation as to why eukaryotic complexes utilize accessory proteins such as Arc1p, which are absent in archaea, may be related to their role in subcellular localization. Recent studies have shown that Arc1p is important for the exclusion of certain synthetases from the nucleus (35, 36), a redundant role in archaea that lack nuclei. In the absence of this specifically eukaryotic requirement for aaRS association, the role of the archaeal complex is likely solely to improve aminoacylation. Whether this, in turn,

improves the efficiency of protein synthesis by promoting substrate channeling, as proposed in eukaryotes, remains unclear. Recent studies in bacteria suggest that elongation factor Tu (1a in archaea) may facilitate channeling by promoting product release from class I aaRSs (6), which would include LeuRS and LysRS in archaea. Further studies are now warranted to probe whether the archaeal aaRS ternary complex does in fact promote substrate channeling, either directly or via EF-1a.

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