Role of Arc1p in the Modulation of Yeast Glutamyl-tRNA Synthetase Activity†

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ABSTRACT: Yeast methionyl-tRNA synthetase (MetRS) and glutamyl-tRNA synthetase (GluRS) possess N-terminal extensions that bind the cofactor Arc1p in trans. The strength of GluRS−Arc1p interaction is high enough to allow copurification of the two macromolecules in a 1:1 ratio, in contrast to MetRS. Deletion analysis from the C-terminal end of the GluRS appendix combined with previous N-terminal deletions of GluRS allows restriction of the Arc1p binding site to the 110−170 amino acid region of GluRS. This region has been shown to correspond to a novel protein−protein interaction domain present in both GluRS and Arc1p but not in MetRS [Galani, K., Grosshans, H., Deinert, K., Hurt, E. C., and Simos, G. (2001) *EMBO J. 20*, 6889−6898]. The GluRS apoenzyme fails to show significant kinetics of tRNA aminoacylation and charges unfractionated yeast tRNA at a level 10-fold reduced compared to Arc1p-bound GluRS. The K_m values for $tRNA^{Glu}$ measured in the ATP−PP_i exchange were similar for the two forms of GluRS, whereas k_{cat} is increased 2-fold in the presence of Arc1p. Band-shift analysis revealed a 100-fold increase in tRNA binding affinity when Arc1p is bound to GluRS. This increase requires the RNA binding properties of the full-length Arc1p since Arc1p N domain leaves the K_d of GluRS for tRNA unchanged. Transcripts of yeast $tRNA^{Glu}$ were poor substrates for measuring tRNA aminoacylation and could not be used to clarify whether Arc1p has a specific effect on the tRNA charging reaction.

The primary and universal function of aminoacyl-tRNA synthetases (AARSs)[1] is to attach a specific amino acid to a cognate tRNA according to the rules of the genetic code. The "core catalytic domains" of the 20 different aminoacyl-tRNA synthetases are conserved, interact with the two branches of the L-shaped tRNA, and can be divided into two classes. Class I and class II are distinguished by the structures of their nucleotide binding fold, which interacts with the tRNA acceptor arm, whereas a more idiosyncratic RNA binding domain is responsible for the interaction with the anticodon arm (*1*, *2*). In mammalian AARSs, peptide appendices are added to the catalytic core and are involved in protein−protein interactions within a unique high molecular weight complex comprising eight AARSs and three noncatalytic proteins, p43, p38, and p18 (*3*). As an example, the N-terminal appendix of GlnRS is required to keep ArgRS and p43 within the complex (*4*). More generally, these eukaryotic-specific appendices are of variable nature (i.e., lysine-rich and leucine-rich domains, GST-like domains) and mediate interactions with the components of the complex (*5*) including p38, which is essential for the stability of the multisynthetase complex (MSC) (*6*). It has also been shown that the N-terminal appended domains of yeast GlnRS (*7*, *8*) and AspRS (*9*) appeared also to have general RNA binding properties and can represent cis-acting factors.

Yeast AARSs also possess N-terminal extensions, yet they are not organized in a MSC. Because GlnRS, MetRS, and GluRS have appendices of about 200 amino acids dispensable for activity, their role has long been obscure. A search for components that genetically interact with the yeast tRNA export factor Los1p has identified Arc1p (the yeast homologue of p43) that associates with MetRS and GluRS via their N-terminal sequences (*10*, *11*). Arc1p has a dual function: the 100 N-terminal amino acids make protein−protein interactions with GluRS and MetRS (*11*, *12*), whereas the C-terminal part of the molecule contains a novel RNA binding domain that recognizes specifically tRNA with limited discrimination between different tRNA species (*10*, *11*). Kinetic and structural studies in the MetRS system allowed the conclusion that Arc1p facilitates the binding of $tRNA^{Met}$ to the synthetase (*10*, *11*), whereas the specificity of $tRNA^{Met}$ aminoacylation resides uniquely on interactions between tRNA and MetRS (*13*−*16*). A current model for the ternary Arc1p−MetRS−$tRNA^{Met}$ complex suggests that Arc1p interacts with the top corner of the tRNA molecule (*10*, *11*) whereas the synthetase makes a precise molecular fit with the inner side of the L-shaped $tRNA^{Met}$ (*15*).

GluRS is one of the three class I AARSs, which require the cognate tRNA to activate the amino acid in the presence of ATP and $MgCl_2$. The resulting enzyme-bound aminoacyl-adenylate is believed to react in a second step with the

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[1] Abbreviations: MetRS, methionyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; AARS, aminoacyl-tRNA synthetase; GST, gluthatione-S-transferase; PCR, polymerase chain reaction.

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2-hydroxyl group of the tRNA terminal adenosine to yield the energy-rich aminoacyl-tRNA. The two other enzymes are glutaminyl- and arginyl-tRNA synthetases. It was long recognized that the tRNA activating function requires the integrity of the 3′ terminus of the tRNA (*17*, *18*). More recently, crystal studies of various substrate-bound GluRSs indicated that the interaction of three $tRNA^{Glu}$ regions with GluRS triggers a conformational change around the nucleotide-binding site allowing ATP to be in the reactive configuration (*19*).

Although Arc1p was shown to stimulate the aminoacylation activity of yeast GluRS (*12*), the exact influence of Arc1p on the kinetic properties of GluRS has so far not been investigated. In contrast to MetRS, GluRS by itself has the potential to form a stable complex with its cognate tRNA that can be isolated by gel filtration (*12*). This suggests a more complex picture for the Arc1p-mediated stimulation of the yeast tRNA glutamylation than just a role in increasing the GluRS binding affinity for $tRNA^{Glu}$.

EXPERIMENTAL PROCEDURES

Yeast Strains, Media, and Plasmids. The yeast strain RS453, auxotrophic for Ade2, Ura3, Leu2, and Trp1, has been previously described (*10*). The following recombinant plasmids were used: P_{NOP1}−ProtA−GluRS, P_{NOP1}−ProtA−GluRSΔN1(Δ9−85), P_{NOP1}−ProtA−GluRSΔN2(Δ9−131), and P_{NOP1}−ProtA−GluRSΔN3(Δ9−191) *(10)*. Yeast cells were grown in rich yeast extract peptone dextrose (YPD) medium or synthetic semisynthetic dextrose complete (SDC) medium containing the necessary amino acids and nutrients. For counter selection of cells containing *URA3* plasmids, 5-fluoorotic acid (5-FOA, Toronto Research Chem.) was used at 1 μg/mL.

Construction of Plasmids Expressing His_6-GluRS and Arc1p. The GluRS ORF (*GUS1*) was subcloned from plasmid pPP1−ProtA−GluRS (*10*) into plasmid p423GALL (*20*) using a *Pst*I site at the 5′ end that was ligated into the vector *Eco*RI site after being made compatible by fill-in and *Xho*I at the 3′ end. The GluRS was tagged N-terminally with the $MRGSH_6$ sequence using oligonucleotide AAAA ACTAGT ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC followed by 9 codons of the GluRS ORF including the methionine codon. This primer created a *Spe*I site (underlined) at the 5′-end after amplification of the 600 first nucleotides of the GluRS ORF encompassing the internal *Bam*HI site. The polymerase chain reaction (PCR) product bearing the His-tag was digested with *Spe*I and *Bam*HI to replace a corresponding restriction fragment in p423GALL−GluRS. Sequencing of the full-length *GUS1* gene revealed the presence of two point mutations downstream of the *Bam*HI site. The undesired mutations were removed by replacing the internal *Bam*HI−*Sal*I fragment with the corresponding genomic fragment isolated from a recombinant cos-yeast DNA clone (SCC7LORFS, a gift from Dr. Vandenbol, Belgium).

Plasmid pUN100−*ARC1 (10)* was used as a template for PCR reaction to produce *ARC1* flanked by *Nco*I and *Xho*I restriction sites allowing its cloning into the yeast expression vector pVTU2 (2μ, *URA3*, P_{ADH}) and pACTII (2μ, *LEU2*, P_{ADH}, GAL4-AD, Dr. S. J. Elledge, Houston). In both cases, untagged Arc1p was produced.

Protein Purification. Yeast cells (RS453) were transformed either with p423GALL−*GUS1* alone or with both p423GALL−*GUS1* and pVTU2−*ARC1* plasmids to ensure copurification of the His_6-GluRS−Arc1p complex. Cultures (5 L) of the transformed yeast cells were grown in semisynthetic galactose complete (SGC) minus His and SGC− His minus Ura medium to an A_{600} between 2 and 2.5. The cells were collected by centrifugation (28 g) and resuspended in 24 mL of breaking buffer (50 mM Tris-HCl (pH 8), 100 mM NaCl, 10 mM β-mercaptoethanol, 10% glycerol, 1 mM PMSF, and 1 μg/mL of a mixture of serine protease inhibitors, Pefablock (Pephafarm AG Basel)). Cells were broken in the presence of 140 g of glass beads for 5 × 2 min with cooling in between. The whole mixture was cleared by centrifugation at 4000 rpm for 5 min to remove cell debris and glass beads. Ribosomes were removed by ultracentrifugation at 100 000*g* for 1 h (45 000 rpm in a Ti 50 Beckman rotor); then the pH of the supernatant was adjusted to 8, and the supernatant was loaded onto a nickel nitrilotriacetic acid resin (Qiagen, Hilden, Germany; bed volume = 2.5 mL). After extensive washing with breaking buffer supplemented with 5 mM imidazole (20−25 mL), elution was done by raising the concentration of imidazole to 250 mM. Fractions with $A_{280\ nm}$ values above 0.5 were pooled, dialyzed against DEAE buffer (50 mM Tris-HCl (pH 8), 10% glycerol, 1 mM dithiothreitol) and loaded onto a TSK DEAE-5PW column (0.5 cm × 7.5 cm) equilibrated in the same buffer. The proteins were eluted with a KCl gradient from 0 to 250 mM, and the purity of the His_6-GluRS and His_6-GluRS−Arc1p complex was checked by SDS−polyacrylamide gel electrophoresis. The pooled material was concentrated by dialysis against 50 mM Tris-HCl (pH 8), 50% glycerol, 1 mM dithiothreitol buffer and stored at −20 °C.

ATP−PPi Exchange and tRNA Aminoacylation Activities of His_6-GluRS and His_6-GluRS−Arc1p. The ATP−pyrophosphate exchange was carried out at 37 °C in 144 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM [^{32} P]PPi (175 cpm/nmol), 2 mM glutamic acid, and 12 μM $tRNA^{Glu}$ with 100 nM GluRS or 75 nM GluRS−Arc1p complex. Labeled ATP was adsorbed onto charcoal and washed with water, and the radioactivity was monitored by scintillation counting. Aminoacylation was carried out as described in ref *10* using 75 nM GluRS or GluRS−Arc1p complex. The $tRNA^{Glu}$ used in both reactions was a counter-current fraction containing 5−10% of aminoacylatable $tRNA^{Glu}$.

Construction and Expression of GluRS Deletion Mutants. A 210-bp amino-terminal fragment of GluRS with *Nco*I and *Sal*I restriction sites was generated by PCR and ligated into plasmid pGG75 (*21*) to create a glutathione-S-transferase−N-ter-GluRS (GST−GluRS) fusion protein. The corresponding DNA fragment was subsequently cloned into plasmid pAS2 (Dr. S. J. Elledge, Houston, TX) containing the GAL4-binding domain. Digestion with *Bam*HI and *Sal*I followed by religation of the pAS2 recombinant plasmid yielded an N-ter-GluRS 1−171 fragment. An N-ter-GluRS 1−110 fragment was further generated by using appropriate primers and the polymerase chain reaction. *ARC1* fused to the activator domain of GAL-4 was done in plasmid pACTII. The two types of plasmids thus generated, together with a number of control constructs, were transformed into the *Saccharomyces cerevisiae* host strain Y190 (*22*) carrying the *GAL1−lacZ* gene fusion to assay Gal4p activation of *GAL1*

transcription. The activity of the β-galactosidase reporter was assayed initially by colony color, using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) and further confirmed using the β-galactosidase assay.

Expression and purification of the glutathione-S-transferase−GluRS(1−210) fragment was done as described for GST−MetRS (*21*).

Synthesis of tRNA^{Glu} Transcripts. Plasmids carrying the yeast minor and major tRNA^{Glu} genes under the control of T7 polymerase promoter were constructed by ligating 10 overlapping oligonucleotides into pUC18. To match the rules of T7 RNA polymerase activity, we replaced U1 of the minor tRNA^{Glu} by G to yield G1−A72. We also created the variant with a G1−C72 pair in the minor and major species. Runoff transcripts obtained from 100 μg of plasmid DNA digested with *Bst*NI were purified by polyacrylamide gel electrophoresis under denaturating conditions, visualized by UV shadowing, and electroeluted using standard procedures.

Labeling and Purification of 5′-[^{32}P]tRNA^{Glu} Transcript. One microgram of purified tRNA^{Glu} transcript with the G1−A72 pair was dephosphorylated at the 5′ end with shrimp alkaline phosphatase (MBI Fermentas), precipitated with ethanol after phenol extraction, and subsequently incubated with T4 polynucleotide kinase (10 units, NEB Biolabs) for 30 min at 37 °C in the presence of 50 μCi of [γ-^{32}P]ATP. The labeled transcript was purified by electrophoresis on a 10% denaturing polyacrylamide gel, identified by autoradiography, excised, and eluted by diffusion at 4 °C in a buffer containing 50 mM sodium acetate, 200 mM NaCl, 20 mM EDTA, and 0.1% SDS until 90% of the radioactivity had been extracted. Prior to gel-shift assays, the transcripts were renaturated by incubation at 70 °C for 4 min in 5 mM $MgCl_2$ followed by cooling at room temperature for 15 min.

tRNA Binding Assays. The renatured labeled tRNA^{Glu} transcript (less than 10 nM, 5000 cpm per lane) was incubated in a 10 μL reaction volume in the presence of His-GluRS, Arc1p, or His-GluRS−Arc1p complex at various concentrations for 15 min at 4 °C in the following buffer: 100 mM Tris-HCl (pH 6.8), 30 mM KCl, 12 mM $MgCL_2$, 0.1 mM EDTA. Upon RNA/protein association, 6 μL of 20% sucrose solution containing tracer dyes was added prior loading on a 6% native polyacrylamide gel prepared and run at 3 W in 50 mM Tris-base, 50 mM glycine buffer for 1 h in the cold room. After electrophoresis, the gel was dried and exposed for autoradiography, quantified using a phosphoimager (Molecular Dynamics, Sunnyvale, CA), or both.

Miscellaneous Procedures. Protein concentrations were determined using the protein assay from Bio-Rad. Western blotting using affinity-purified anti-Arc1p polyclonal antibodies (*10*) was done according to standard protocols.

RESULTS

Arc1p Copurifies with GluRS When Coexpressed in Yeast. In a previous work, the GluRS−Arc1p complex was reconstituted in vitro from its individual components that were purified independently as tagged proteins (*12*). Separation of the complex was done by gel filtration using fast protein liquid chromatography. To obtain the GluRS−Arc1p complex, we took advantage of the high binding affinity between Arc1p and GluRS. Indeed, MetRS dissociates from Arc1p at 1 M $MgCl_2$, whereas GluRS remains bound to Arc1p till 4 M $MgCl_2$ concentration is reached (*10*). This may be attributed to a conserved protein−protein interaction domain present in both GluRS and Arc1p but not in MetRS (*23*). Figure 1 (lane 3) shows that Arc1p copurifies with tagged His_6-GluRS with an apparent stoichiometry of 1:1; the differences in the intensity of the bands between the two proteins may result from the difference in their M_r values (42 kDa for Arc1p and 89 kDa for GluRS). Copurification of Arc1p with GST-tagged-MetRS clearly results in a substoichiometric complex with Arc1p (Figure 1, lane 2), in accordance with our expectations.

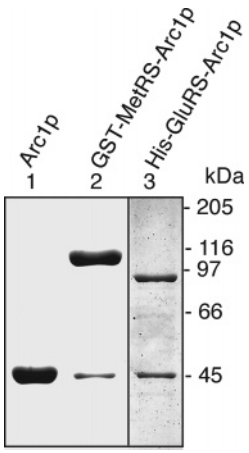

FIGURE 1: Copurification of N-terminally tagged GluRS and MetRS with untagged Arc1p from overexpressing yeast strains. The recipient yeast strain RS453 was cotransformed with plasmid-borne recombinant MetRS or GluRS and untagged Arc1p. Crude extract from 2 L of cell cultures was applied on 1 mL of the affinity column, and the recombinant MetRS or GluRS was eluted either with glutathione or with imidazole. The panels show SDS−PAGE analysis of fractions eluted from glutathione−agarose columns (GST−MetRS) and from nickel nitrilotriacetic acid columns (His−GluRS). Copurification of Arc1p was assessed by comigration with purified recombinant His-tagged Arc1p.

The C-Terminal Half of the GluRS N-Terminal Domain Is Required for Arc1p Binding. The ability of protein A−GluRS deletion mutants to bind Arc1p has been tested previously, thus leading to a map of the Arc1p binding site in the GluRS N-terminal domain (*23*). To establish an independent proof of this protein−protein interaction and to map it more precisely, we used the *GAL4*-based two-hybrid system (*24*, *25*).

The results show that neither activation domain (AD)−Arc1p nor binding domain (BD)−GluRS interact with the complementary GAL4 domain. There was also no nonspecific interaction of BD−GluRS with AD−Snf4p. Only the double transformants expressing AD−Arc1p and BD−GluRS(1−210) or the proteins Snf4p and Snf1p, which were taken as positive control, gave deep blue colonies with X-gal, suggesting strong interactions. The interaction of the truncated GluRS(1−170) with Arc1p was significantly reduced compared to the full-length N-terminal domain of GluRS, while no interaction could be detected for the GluRS(1−110) deletion since in the latter case the β-galactosidase activity is indistinguishable from background levels (Figure 2A).

To evaluate further the strength of the interaction between GluRS N-terminal domain and Arc1p, the ability of immobilized GluRS N-terminal domain to retain Arc1p was tested. Immobilization of the GluRS N-terminal domain onto

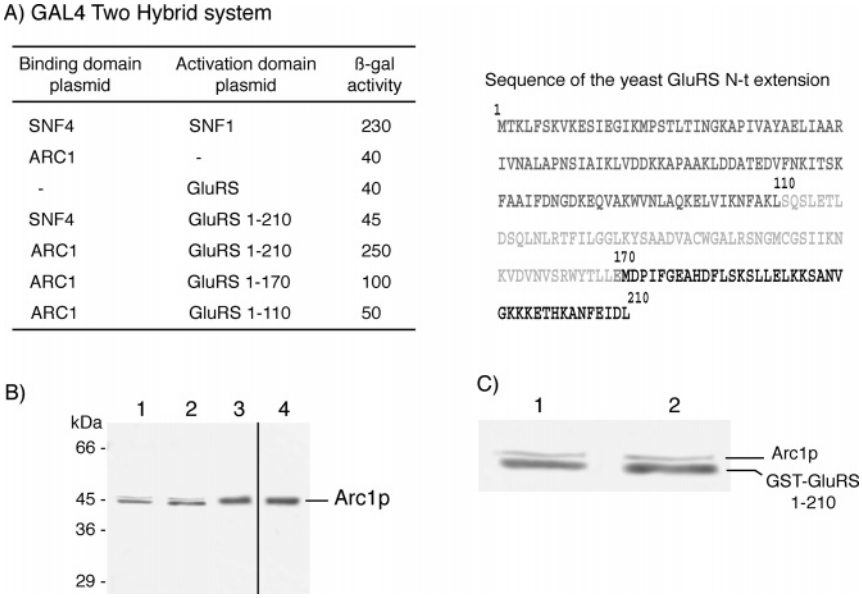

FIGURE 2: Interaction of the GluRS N-terminal domain with Arc1p. Panel A presents two-hybrid analysis of GluRS N-terminal domain with Arc1p. β-galactosidase activities of cell lysates of strain Y190 transformed with pairwise combinations of the two-hybrid vectors were determined. The *SNF* genes were taken as positives controls, whereas *ARC1* fused to the activator domain or the pair *SNF4*−*GUS1* (GluRS) were taken as negative controls. Optimal β-galactosidase activity was obtained with the entire N-terminal extension of yeast GluRS and reaches background levels when 100 amino acid residues are deleted from the C-terminal end. Panel B shows copurification of overexpressed GluRS N-terminal domain with Arc1p. Yeast strain RS453 was transformed with plasmid-borne GluRS N-terminal domain (1−210) and Arc1p. Lanes 2 and 3 show SDS−PAGE analysis of fractions eluted from the glutathione−agarose columns. Position of the Arc1p band was assessed by Western blotting (lane 4). Panel C shows zooming of the Coomassie gel staining indicating the position of Arc1p and GluRS N-terminal domain proteins.

glutathione−agarose was chosen because of the high affinity of the tag for its ligand leading to coelution of only a few nonspecific proteins. The yeast strain RS453 was cotransformed with multicopy plasmids encoding the GST−GluRS-(1−210) construct and untagged Arc1p. Under conditions where copurification of Arc1p with full-length GluRS results in a 1:1 ratio (Figure 1), the amount of Arc1p bound to the GluRS N-terminal appendix is clearly substoichiometric (Figure 2B). This result suggests that GluRS N-terminal domain has reduced affinity for Arc1p or that the two proteins are overproduced at different levels, making the amounts of Arc1p available for the interaction with GluRS N-terminal domain limiting. Indeed, using pull-down experiments with GluRS N-terminal domain expressed from a centromeric plasmid revealed the presence of quasi-stoichiometric amounts of Arc1p (*11*).

tRNA^{Glu} Aminoacylation with Free and Arc1p-Bound GluRS. Purified yeast tRNA^{Glu} is not available, and commercial unfractionated yeast tRNA behaved as a poor substrate for yeast GluRS, exhibiting a plateau charging level of less than 0.1% (data not shown). The plateau level could be significantly increased using freshly prepared total yeast tRNA. Plateau levels of 1.4% were measured in the presence 100 nM of GluRS−Arc1p complex (Figure 3A), knowing that levels of 5% are usually obtained with a given species of tRNA. Therefore, we suspect that the low activity of tRNA aminoacylation measured with the commercial tRNA is attributable to the tRNA and particularly to a modified base that may be partially degraded (see below). *Escherichia coli* tRNA^{Glu} is not a substrate for yeast GluRS−Arc1p complex (Figure 3A), indicating the absence of heterologous aminoacylation.

When the measurement of the aminoacylation plateau was repeated with 200 nM free GluRS, the value was only 0.15% (Figure 3A). One possibility for this drop in plateau is that k_{cat} for tRNA glutamylation is reduced so that the equilibrium between rates of enzymatic tRNA acylation and chemical hydrolysis of aminoacylated tRNA is affected (*26*).

Influence of Arc1p on the Kinetic Parameters of tRNA^{Glu} in the Aminoacylation and ATP−PPi Exchange Reactions. Initial rates of tRNA^{Glu} aminoacylation were measured using a yeast tRNA counter-current fraction, which was further purified on a Delta Pak C4 column (Waters, WAT011794) to yield a charging level of 15%. In the absence of Arc1p, initial rates were obtained by adding pyrophosphate to the reaction mixture, but they were too low to accurately extrapolate kinetic constants from the experimental data (Figure 3B). With a counter-current fraction containing 5% of tRNA^{Glu}, the concentration of tRNA^{Glu} could be raised to 6 μM, but the rates of tRNa aminoacylation remained too low (data not shown). In the presence of the GluRS−Arc1p complex, the initial rates were significantly increased (Figure 3C), and a plot of velocity versus tRNA^{Glu} concentration (Figure 3D) yielded a K_m value for tRNA^{Glu} of 0.45 μM and a k_{cat} of 0.016 s^{-1}. In terms of turn-over numbers, this k_{cat} value is very low and does not match protein synthesis rates, which are about 1 s^{-1} or more. However, when the activity of GluRS is measured in the ATP−PPi exchange reaction (Figure 4A,B), k_{cat} values above 1 s^{-1} are obtained indicating that the intrinsic activity of GluRS is similar to other aminoacyl-tRNA synthetases (i.e., arginyl-tRNA synthetase, *27*). The tRNA-dependent activation of glutamic acid offers a means to measure independently the K_m for GluRS in the presence and absence of Arc1p (Figure 4A,B). Lineweaver−Burk plots derived from the initial rates versus tRNA concentration yielded a K_m value for tRNA^{Glu} that is unchanged in the presence of Arc1p, whereas k_{cat} is increased 2-fold (Figure 4C).

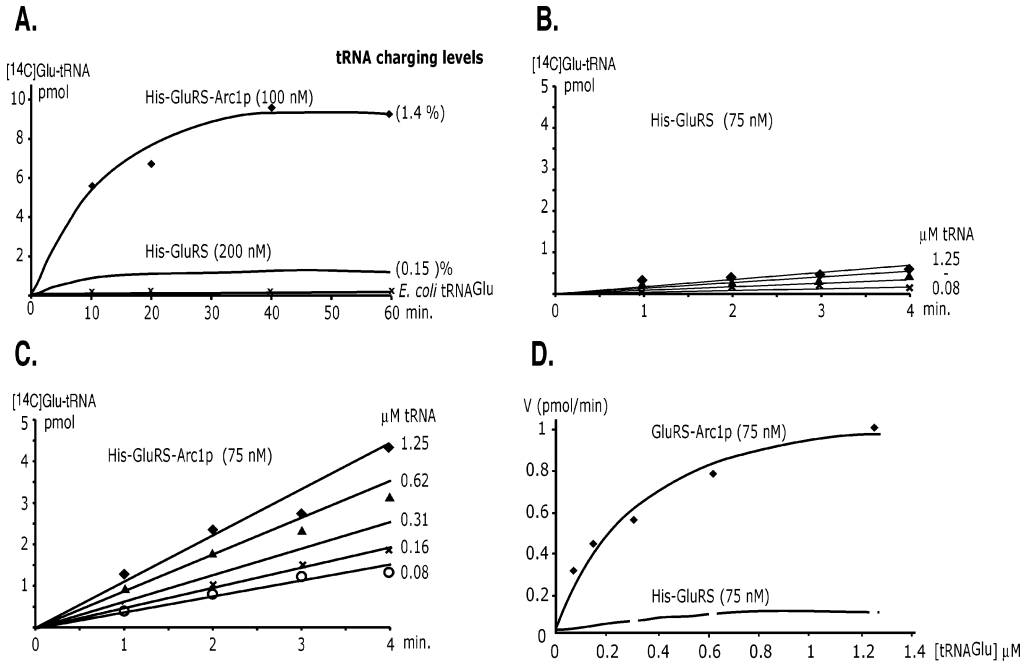

FIGURE 3: $tRNA^{Glu}$ aminoacylation by GluRS and GluRS−Arc1p complex. Panel A shows the plateau level of glutamate−tRNA aminoacylation using unfractionated yeast tRNA. Picomoles of [^{14}C]glutamate incorporated into total tRNA were measured in the presence of 200 nM His-GluRS or 100 nM His-GluRS−Arc1p complex. The unfractionated tRNA was prepared as described in Experimental Procedures. Kinetics of tRNA aminoacylation were also followed in the presence of 100 nM His-GluRS−Arc1p and commercial total tRNA or pure *E. coli* $tRNA^{Glu}$ (2 μM). The concentration of [^{14}C]glutamate and tRNA in the assay were, respectively, 35 μM (300 cpm/pmol) and 6.4 μM. The calculated plateau of tRNA aminoacylation was 1.4% in the presence of His-GluRS−Arc1p complex and 0.15% for His-GluRS. Panels B and C show the kinetics of $tRNA^{Glu}$ aminoacylation with His-GluRS and His-GluRS−Arc1p, respectively. The $tRNA^{Glu}$ concentrations were calculated from the known maximum level of glutamate incorporated into tRNA (the tRNA used in these experiments was a partially purified counter-current fraction containing 15% $tRNA^{Glu}$). [^{14}C]Glutamate was used at a concentration of 35.7 μM and had a specific activity of 190 cpm/pmol. Panel D shows plots of velocity versus $tRNA^{Glu}$ concentration.

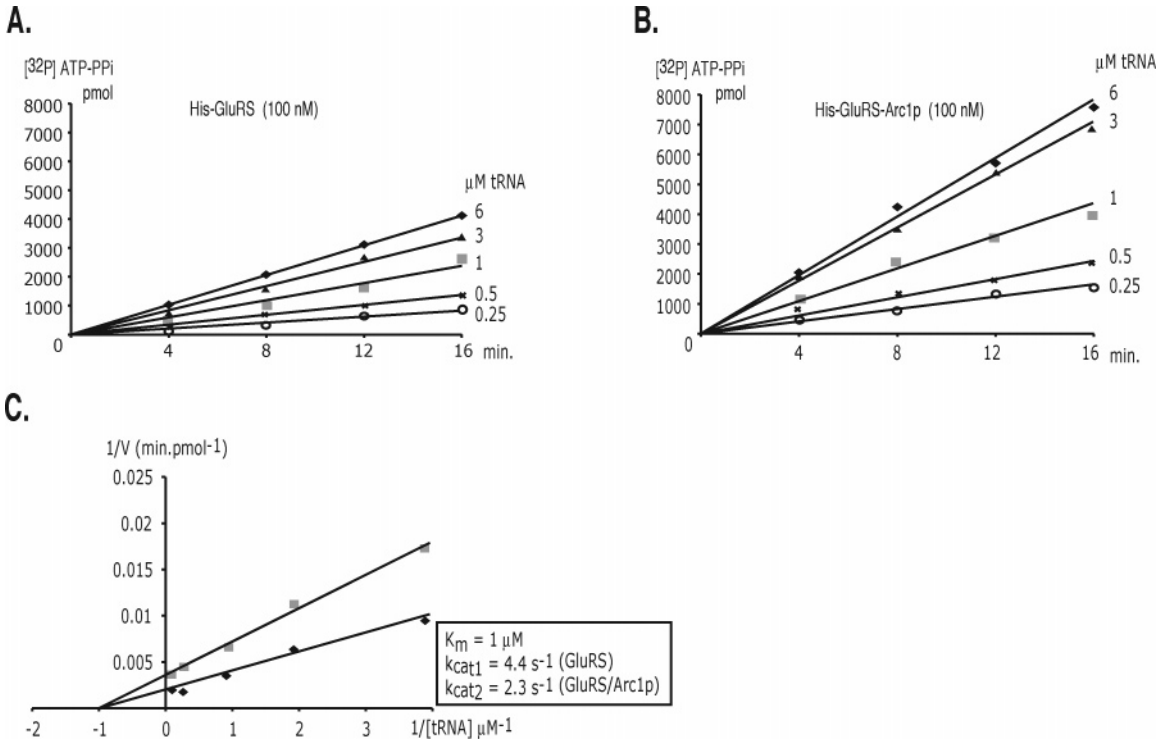

FIGURE 4: Influence of Arc1p on yeast GluRS ATP−PPi exchange activity. The ATP−PP_i exchange was followed in the presence of 100 nM free (A) and Arc1p-complexed GluRS (B). The tRNA used here is a counter-current fraction containing 5% $tRNA^{Glu}$. Specific concentrations of $tRNA^{Glu}$ were calculated as indicated in Figure 3. Glutamic acid was 5 mM, and the specific activity of the [^{32}P]pyrophosphate was 1.2 cpm/pmol. The concentration of ATP and PP_i were 2 mM. The enzyme activity was expressed as pmoles of $[^{32}P]_i$ incorporated into ATP. Panel C presents double-reciprocal plots of velocity versus $tRNA^{Glu}$ concentration calculated for His-GluRS and His-GluRS−Arc1p.

Aminoacylation of Yeast $tRNA^{Glu}$ Transcripts. Modified bases are not usually implicated in tRNA aminoacylation, but in a few cases, they behave as positive determinants for entry into the transition state of tRNA aminoacylation (*28*).

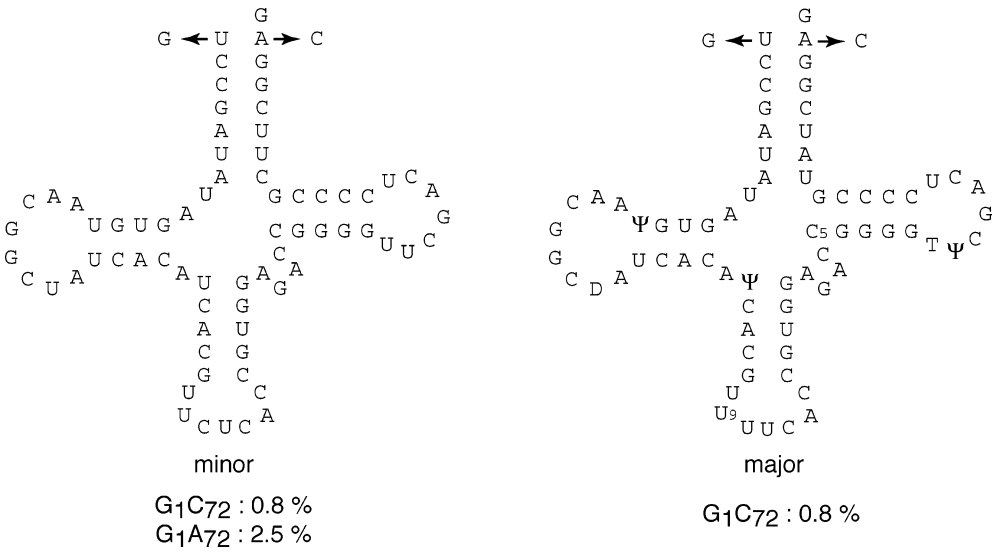

FIGURE 5: Cloverleaf structures of yeast tRNA^{Glu}. Cloverleaf structures of minor and major tRNA^{Glu} species are shown on the left and right, respectively. In the case of major tRNA^{Glu}, the native tRNA sequence is indicated whereas for the minor species the nuleotide composition is deduced from the gene sequence. The tRNA^{Glu} transcripts used in this study bear a G1 to facilitate their in vitro transcription. The compensatory base changes have been performed for both tRNAs yielding G1−C72 Watson−Crick base pairs. Minor tRNA^{Glu} was also produced as a G1−A72 variant because this variant is closer to the native U1−A72 base pair in terms of spatial organization of chemical groups. The plateau level of aminoacylation for each tRNA^{Glu} transcript is indicated. U_9 = mcm^{5}s^{2}U (5-methoxycarbonylmethyl-2-thiouridine); Ψ = pseudouridine; C_5 = 5-methylcytidine.

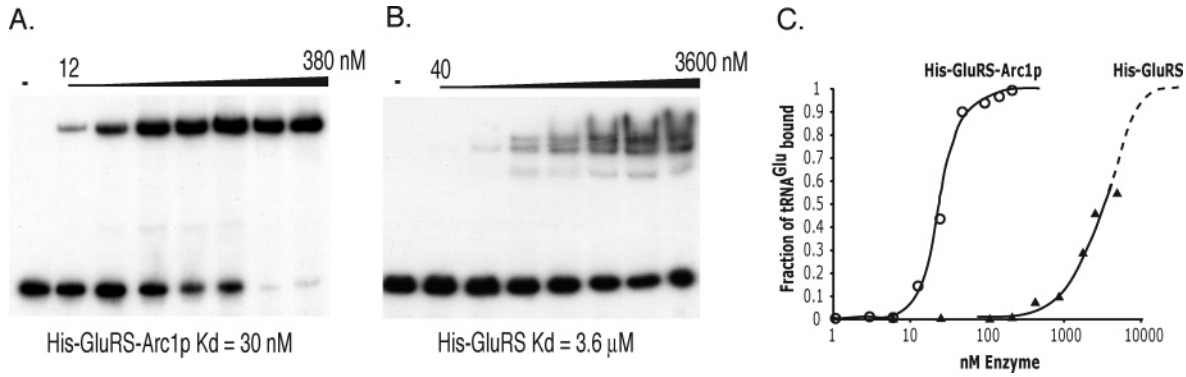

FIGURE 6: Binding of tRNA^{Glu} transcript to GluRS and GluRS−Arc1p complex. Labeled in vitro transcribed minor tRNA^{Glu} (G1−A72) was incubated with increasing concentrations of His-GluRS−Arc1p complex (A) or His-GluRS (B), and the resulting tRNA−protein complex was assayed by gel shift. The protein concentration range used in each case is indicated. The gels were submitted to phosphorimaging to quantify the free and complexed fraction of tRNA^{Glu}. The data obtained were plotted as the fraction of bound tRNA^{Glu} versus the logarithm of protein concentration (*C*). In the case of free GluRS, this value was deduced from the decrease of tRNA used in the assays. The dashed line depicts the theoretical curve of tRNA binding for His-GluRS.

In the particular case of *E. coli* tRNA^{Glu}, 5-methylaminomethyl-2-thiouridine (mnm^{5}s^{2}U) at the wobble position (position 34) interacts positively with *E. coli* glutamyl-tRNA synthetase (*29*). Yeast major tRNA^{Glu} with anticodon UUC has also an analogous modified uridine: 5-methoxycarbonylmethyl-2-thiouridine (mcm^{5}s^{2}U) at position 34 (Figure 5). Thiouridine is known to be sensitive to oxidation (*30*) and its degradation may account for the low k_{cat} of tRNA glutamylation in yeast. Major identity elements of *E. coli* tRNA^{Glu} are clustered in the augmented D helix, whereas nucleotides in the acceptor stem (G1−C72) and in the anticodon loop (positions 34 and 35) have a lesser but still important contribution in tRNA^{Glu} recogntion (*31*). Both yeast tRNA^{Glu} isoacceptors have U1−A72 in the acceptor stem. Since we failed to generate a yeast tRNA^{Glu} transcript with U1−A72 based on a ribozyme strategy, the yeast tRNA^{Glu} transcripts synthesized by T7 RNA transcription may be affected by the nucleotide substitution at position 1 in addition to the lack of modified bases. The set of modified bases was only established for the major species; that of the minor species is still unknown. We synthesized a G1−C72 variant for major and minor tRNA^{Glu}species, as well as a G1−A72 variant for the minor species. The two yeast transcripts were poor substrates for yeast GluRS as the maximum level of tRNA charging was only 2.5% (Figure 5). This value was obtained for the minor tRNA^{Glu} G1−A72 variant in the presence of GluRS−Arc1p complex. The same transcript was further used for binding assays by gel-shift mobility.

tRNA Binding to Yeast GluRS and GluRS−Arc1p Complex. Radiolabeled tRNA^{Glu} transcript was incubated with increasing amounts of either GluRS (Figure 6B) or GluRS−Arc1p complex (Figure 6A), and the fraction of tRNA that was shifted in the presence of the protein was quantified using phosphorimaging. In these experiments, the protein is in large excess over tRNA allowing assimilation of the protein added to free protein and derivation an apparent dissociation constant (K_d) from the value at which half of the tRNA is

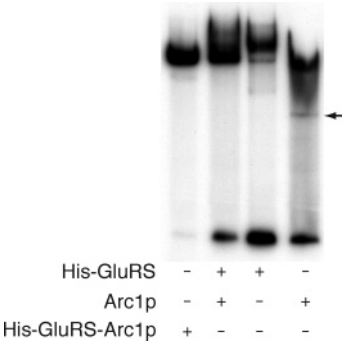

FIGURE 7: Gel-shift assays showing the increase of GluRS tRNA binding in the presence of Arc1p. His-GluRS (2.5 μM) and Arc1p (2.5 μM) were incubated in the presence of 5′-end-labeled $tRNA^{Glu}$ either alone or after GluRS−Arc1p complex formation. In the latter case, His-GluRS and Arc1p were preincubated for 15 min. prior $tRNA^{Glu}$ addition. Complexed $tRNA^{Glu}$ transcript with preformed yeast His-GluRS−Arc1p (0.7 μM) is shown as a control.

complexed to GluRS or GluRS−Arc1p complex. Figure 6A shows that complex formation is resolved in a single signal when Arc1p-bound GluRS is used, whereas two or more shifts of labeled tRNA were obtained in the presence of free GluRS (Figure 6B). These shifts probably correspond to several conformations of free GluRS as deduced from a Coomassie gel staining of the protein (data not shown). The respective binding profiles were plotted on a logarithmic scale and yielded apparent dissociation constants of 30 nM for the GluRS−Arc1p complex and 3.6 μM for free GluRS (Figure 6C). To prove that Arc1p is responsible for the increase in tRNA binding affinity, we added recombinant His-tagged Arc1p to GluRS (Figure 7). Recombinant Arc1p purified from *E. coli* shows a heterogeneous pattern of migration in the presence of tRNA, which we interpret as the result of tRNA complexes with monomeric Arc1p (indicated by an arrow) and with higher order structures of Arc1p (dimeric or aggregated forms) corresponding to the smeary band. These forms disappear in the presence of GluRS. Instead, we see an increase of the band corresponding to tRNA complexed with GluRS−Arc1p and a concomitant decrease of free tRNA. The tendency of the recombinant Arc1p to aggregate may not occur for the yeast Arc1p, which was shown to contain a biotinylated lysine residue in the N-terminal domain (*32*). Stimulation of the GluRS binding affinity for tRNA requires full-length Arc1p. The Arc1p N-terminal domain that was shown to form a heterocomplex with GluRS in vivo and in vitro (*11*, *12*) does not modify the shift and level of radiolabeled $tRNA^{Glu}$ transcript complexed to free GluRS (Figure 8A). That the Arc1p N-terminal domain effectively binds GluRS can be deduced from the shift of the Arc1p N-terminal appendix in the presence of GluRS, and this shift is not altered by adding $tRNA^{Glu}$ (Figure 8B).

DISCUSSION

Arc1p is an accessory protein of yeast MetRS and GluRS that modulates their activity. In the case of MetRS, Arc1p helps binding of $tRNA^{Met}$ to its cognate tRNA synthetase by decreasing significantly (100-fold) the K_m of $tRNA^{Met}$ (*10*). This result is in line with the property of Arc1p being a general tRNA binding factor and offers a way to regulate the activity of MetRS in vivo. Indeed, in the absence of

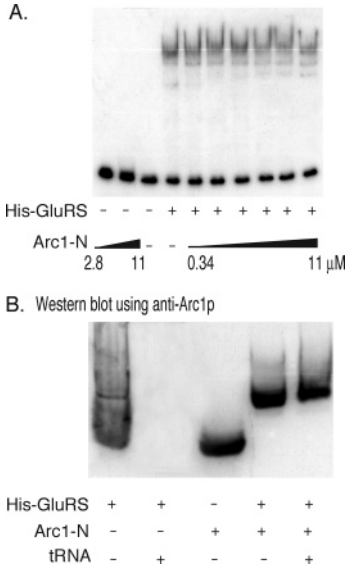

FIGURE 8: Arc N-terminal appendix does not influence $tRNA^{Glu}$ binding to GluRS. Panel A shows a gel-shift assay using 5′-end-labeled $tRNA^{Glu}$ transcript and Arc1p N-terminal appendix alone (first two lanes) or a mixture of increasing concentrations of Arc1p N-terminal appendix and His-GluRS (2.5 μM). The range of Arc1p N-terminal appendix concentration is indicated. Panel B shows a Western blot using anti-Arc1p antibodies. The gel was performed in the same conditions as the gel-shift assays (see Experimental Procedures) with the exception that the concentration of unlabeled $tRNA^{Glu}$ was increased to 0.5 μM. His-GluRS and Arc1p N-terminal appendix concentrations were 2.5 μM each.

Arc1p, MetRS has a K_m for tRNA that seems "unphysiologically" high (about 10 μM), which is 10−100-fold higher than that of a standard aminoacyl-tRNA synthetase. By decreasing the K_m for tRNA, Arc1p allows MetRS to function efficiently at low intracellular concentrations of $tRNA^{Met}$. Compared to MetRS, GluRS binds more strongly to Arc1p than MetRS and has the potential to bind tRNA in the absence of Arc1p (*12*). The strength of the interaction between GluRS and Arc1p is such that untagged Arc1p copurifies with His-tagged GluRS in a 1:1 complex. Deletion analysis of the GluRS N-terminal appendix made from the N-side (*12*) or from the carboxy-end (the present study) gives complementary results allowing restriction of the Arc1p binding site in the GluRS amino acid region 110−170. It is exactly in this region (amino acid residues 86−170) where a novel a protein−protein interaction domain has been identified in the GluRS (*23*). This domain also occurs in Arc1p (*23*), thus explaining the high affinity between the two macromolecules.

Measuring the strength of the interaction between GluRS and a transcript of yeast $tRNA^{Glu}$ by gel shift-asssay allows us to estimate an apparent K_d of tRNA for GluRS in the micromolar range (3.6 μM). In the presence of Arc1p, the value of K_d drops to 30 nM. This difference in the dissociation constants may explain why free GluRS aminoacylates poorly a partially purified yeast $tRNA^{Glu}$ fraction, because at 1.2−6 μM, the maximal $tRNA^{Glu}$ concetrations that we could use, the enzyme may not be saturated. Unfortunately, the $tRNA^{Glu}$ transcript, which is a potent substrate for complex formation with GluRS−Arc1p, is still a poor substrate for tRNA aminoacylation and cannot be used to determine K_m of tRNA for free GluRS and to check whether Arc1p influences k_{ca} for tRNA aminoacylation.

Despite this lack of clarification, the observation that the plateau of aminoacylation is reduced 10-fold in the absence of Arc is intriguing. Indeed, we know that for the establishment of maximal plateaus of aminoacylation, maximal initial rates of aminoacylation are required to overcome the chemical deacylation rate of charged tRNA (*26*).

The present study clearly shows that a major role of Arc1p is to stimulate binding for GluRS as it does for MetRS. This stimulation requires the full-length protein where the middle and C-terminal regions are responsible for tRNA binding and constitute a trans-acting RNA binding protein (TRBP) (*11*). The heterodimer formed by the Arc1p N-terminal domain and GluRS (*11* and this study) is unable to stimulate tRNA binding for the synthetase indicating that the increase in affinity is not just the result of a GluRS conformational change induced by Arc1p binding. GluRS is a class I enzyme that like MetRS interacts with the entire inner side of the L-shaped tRNA structure (*33*). The association of Arc1p with either synthetase can therefore accommodate tRNA binding on both sides of the tRNA molecule as the TRBP domain is believed to interact with the top corner of the tRNA. This was as deduced from previous studies (*10*) and also from a structural model of tRNA binding to *Aquifex aeolicus* Trbp111 3 structure, a thermophile homologue of Arc1p TRBP domain (*34*).

It is surprising that yeast has maintained Arc1p as a transactivator of GluRS and MetRS and that this role was not taken over by an appended domain of these tRNA synthetases during their evolutionary courses. This may be indicative of an additional function of Arc1p as already suggested by its probable involvement in the nuclear transport of tRNA and the tRNA-aminoacylation machinery (*10*, *35*). It is also plausible that the specific role played by Arc1p for the yeast MetRS and GluRS is closely linked to the evolutionary history of these two aminoacyl-tRNA synthetases. For Trbp111, which may be regarded as a precursor of Arc1p, Schimmel and co-workers (*36*) proposed that this protein could have participated in the establishment of the cornerstone tRNA molecule (*36*).

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