The tRNA-Interacting Factor p43 Associates with Mammalian Arginyl-tRNA Synthetase but Does Not Modify Its tRNA Aminoacylation Properties[†]

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ABSTRACT: Arginyl-tRNA synthetase (ArgRS) is one of the nine synthetase components of a multienzyme complex containing three auxiliary proteins as well. We previously established that the N-terminal moiety of the auxiliary protein p43 associates with the N-terminal, eukaryotic-specific polypeptide extension of ArgRS. Because p43 is homologous to Arc1p, a yeast general RNA-binding protein that associates with MetRS and GluRS and plays the role of tRNA-binding cofactor in the aminoacylation reaction, we analyzed the functional significance of p43−ArgRS association. We had previously showed that full-length ArgRS, corresponding to the ArgRS species associated within the multisynthetase complex, and ArgRS with a deletion of 73 N-terminal amino acid residues, corresponding to a free species of ArgRS, both produced in yeast, have similar catalytic parameters (Lazard, M., Kerjan, P., Agou, F., and Mirande, M. (2000) *J. Mol. Biol. 302*, 991−1004). However, a recent study had suggested that association of p43 to ArgRS reduces the apparent K_M of ArgRS to tRNA (Park, S. G., Jung, K. H., Lee, J. S., Jo, Y. J., Motegi, H., Kim, S., and Shiba, K. (1999) *J. Biol. Chem. 274*, 16673−16676). In this study, we analyzed in detail, by gel retardation assays and enzyme kinetics, the putative role of p43 as a tRNA-binding cofactor of ArgRS. The association of p43 with ArgRS neither strengthened tRNA-binding nor changed kinetic parameters in the amino acid activation or in the tRNA aminoacylation reaction. Furthermore, selective removal of the C-terminal RNA-binding domain of p43 from the multisynthetase complex did not affect kinetic parameters for ArgRS. Therefore, p43 has a dual function. It promotes association of ArgRS to the complex via its N-terminal domain, but its C-terminal RNA-binding domain may act as a tRNA-interacting factor for an as yet unidentified component of the complex.

Aminoacyl-tRNA synthetases specifically bind their cognate tRNA isoacceptors and transfer the corresponding activated amino acid to the 3′-end of the tRNA molecule (*1*). A set of specific interactions between amino acid side chains of the synthetase and tRNA bases and of nonspecific interactions with the phosphate−sugar backbone of tRNA contribute to the accurate positioning of the 3′-end of tRNA into the active site crevice of the enzyme. Most synthetases exclusively interact with the inner, concave side of the L-shaped tRNA molecule (*2−7*). Although the mode of tRNA binding seems to be well conserved from prokaryotes to eukaryotes, one of the major differences that characterizes aminoacyl-tRNA synthetases from higher eukaryotes (from Drosophila to human), is the presence of polypeptide chain extensions that may serve as auxiliary tRNA-interacting factors (tIFs).[1]

Several types of tIFs have been described so far. They either are added to the polypeptide chain of the synthetases as N- or C-terminal extensions (association in cis) or may be appended in trans via protein−protein interaction. In mammals, MetRS, GlyRS, HisRS, TrpRS, and bifunctional GluProRS (*8*) (where XxxRS indicates the aminoacyl-tRNA synthetase and Xxx is the amino acid abbreviation) share a coiled-coil motif of ∼50 amino acid residues (*9*, *10*). It constitutes an N-terminal extension in GlyRS, HisRS, TrpRS, and ProRS and a C-terminal extension in GluRS and MetRS. In the case of human MetRS (*11*) and HisRS (*12*) or of *Bombyx mori* GlyRS (*13*), its involvement as a cis-acting tIF for aminoacylation has been established. The C-terminally appended tIF of MetRS confers on the native enzyme the ability to bind tRNA with a much higher apparent affinity ($K_d \approx 0.1\ \mu$M) as compared with a MetRS mutant lacking this domain ($K_d \approx 4\ \mu$M). Another type of cis-acting tIF has been identified in mammalian LysRS (*14*) and AspRS (*15−17*). This lysine-rich N-terminal polypeptide extension of LysRS, made of ∼70 amino acid residues, may form an α-helix-based tRNA binding motif (*18*). The cis-acting tIFs appended to eukaryotic aminoacyl-tRNA synthetases decrease dissociation constants for their cognate tRNAs. Because the concentration of non-acylated tRNA in the cytoplasm of higher eukaryotic cells is believed to be 1 order of magnitude lower than the K_m value for the cognate synthetase (discussed in ref *14*), tIFs are thought to be required for tRNA cycling during translation (*19*).

A second family of tIF concerns trans-acting general tRNA binding modules that were first described in yeast (*20−22*). In the yeast *Saccharomyces cerevisiae*, the protein Arc1p is associated in trans with MetRS and GluRS and increases

[1] Abbreviations: tIF, tRNA-interacting factor.

their aminoacylation efficiency. A human homologue of Arc1p has been described. The p43 protein, one of the nine polypeptide components of the mammalian multisynthetase complex, has the potential to bind tRNA nonspecifically (*23*) and may play the role of a trans-acting tIF for one of the synthetase components of the complex. A homologous domain is appended in cis to the C-terminus of human TyrRS, but its involvement in tRNA binding has not yet been established (*24, 25*). The crystal structure of the C-terminal moiety of p43 identified a putative OB-fold-based tRNA-binding site (*26*). This trans-acting tIF is also the precursor of the EMAPII cytokine generated during apoptosis (*27–29*). The cytokine activity has been assigned to the fragment encompassing residues 92–256 of p43 (*30*). The involvement of p43 in angiogenesis has also been proposed (*31*). Immunoelectron microscopy suggested that p43 occupies a central position within the complex and thus may serve as a donor of tRNA for several aminoacyl-tRNA synthetases (*32*). Because p43 and ArgRS are interacting proteins (*33, 34*), the involvement of p43 in modulating the activity of ArgRS has been proposed (*35*).

In this work, we investigated the role of p43 as a factor involved in the assembly of ArgRS within the multisynthetase complex and as a tIF promoting the aminoacylation activity of ArgRS. Our results stress the dual role of p43. Its N-terminal domain is responsible for association of ArgRS within the complex and its C-terminal moiety binds tRNA but does not serve as a tIF for ArgRS.

EXPERIMENTAL PROCEDURES

Protein Overexpression and Purification. Hamster arginyl-tRNA synthetase and an N-terminally truncated derivative with a deletion of the 73 terminal amino acid residues were expressed in yeast and purified as described (*36*). The auxiliary p43 subunit of the multisynthetase complex, as well as its N-terminal (p43-N) or C-terminal (p43-C) moieties, was expressed in *Escherichia coli* with a C-terminal His-tag (*23*). Plasmid for expression of *Aquifex aeolicus* Trbp111 was a gift from Paul Schimmel (The Skaggs Institute for Chemical Biology, La Jolla, CA). Trbp111 was expressed and purified as described (*37*).

Protein concentrations were determined by using calculated absorption coefficients of 0.728, 0.803, 0.257, 0.068, and 0.408 A_{280} units·mg^{-1}·cm^2, respectively, for ArgRS, ArgRS-ΔN, p43, p43-N, and p43-C.

Immunoprecipitation. His-tagged p43, p43-N, or p43-C (2 μM each) were incubated 30 min at 4 °C with ArgRS or ArgRS-ΔN (5 μM) in buffer A50 [buffer A (8 mM Na$_2$-HPO$_4$, 1.5 mM KH$_2$PO$_4$, 2.7 mM KCl, pH 7.5) including 50 mM NaCl] containing 0.1% Triton X-100 and 0.2% BSA. A preadsorption step was conducted. After a 15-min incubation with a polyclonal goat anti-rabbit-IgG antibody (1 μg), 20 μl of a 1:1 slurry of Protein-A Sepharose in buffer A50 containing 0.1% Triton X-100 was added. Incubation was continued at 4 °C for 30 min. After centrifugation, the supernatant was recovered and incubated at 4 °C for 1 h with 1 μg of Penta-His antibody (QIAGEN). After addition of 25 μl of a 1:1 slurry of Protein-A Sepharose and incubation at 4 °C for 30 min, the immunoprecipitate was washed five times with 1 mL of buffer A100 (buffer A including 100 mM NaCl) containing 0.1% Triton X-100 and

once with 100 mM Tris-HCl, pH 7.5, and the pellet was recovered in 25 μl of SDS–PAGE loading buffer, boiled for 2 min, and loaded on a 12% SDS–polyacrylamide gel. Immunoprecipitates were analyzed by Western blotting with antibodies raised in rabbit against ArgRS-ΔN.

Gel Retardation Assay. Protein–tRNA interactions were assayed using a band shift assay as previously described (*28*). Plasmid carrying the beef tRNA$_2$^{Arg} gene under control of the T7 polymerase promoter was constructed by simultaneous ligation of 10 oligonucleotides into pUC18 (5′-agcttaatac-gactcact, 5′-ctatagtgagtcgtatta, 5′-atagccccagtggcctaatg, 5′-ttatccattaggccactgggg, 5′-gataaggcattggcctccta, 5′-tggcttag-gaggccaatgcc, 5′-agccagggattgtgggttcg, 5′-ggactcgaaccca-caatccc, 5′-agtcccatctggggtcccaggatccaaacatcc, 5′-gatcggat-gtttggatcctgggaccccagatg). Plasmid ptRNA$_2$^{Arg} was linearized with *Fok*I and subjected to in vitro transcription. The amino acid acceptor minihelix (Acc$_2$^{Arg}) and anticodon microhelix (Ant$_2$^{Arg}) of beef tRNA$_2$^{Arg} were produced from *Fok*I-digested pUC18 derivatives. T7 RNA polymerase was purified from the strain BL21/pAR1219 generously provided by Prof. W. Studier (Brookhaven National Laboratory). ^{32}P-labeled tR-NAs were synthesized in a reaction mixture (50 μl) containing 1 μg of template DNA, 40 mM Tris-HCl (pH 8.0), 6 mM MgCl$_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.01% Triton X-100, 1 mM each CTP, UTP, and GTP, 10 μM [α-^{32}P]ATP (200 Ci/mmol), and 1000 U/ml T7 RNA polymerase. After incubation at 37 °C for 1 h, the transcripts were purified by electrophoresis on a denaturing 12% polyacrylamide gel (mono/bis, 19:1), recovered from the gel by soaking in H$_2$O, precipitated with ethanol, and resuspended in 5 mM MgCl$_2$. Transcripts were renatured by heating at 90 °C and slow cooling (90–30 °C in 2 h).

Homogeneous proteins were incubated at increasing concentrations with radiolabeled-tRNA (50 000 cpm per point) in an 11 μl volume containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM MgCl$_2$, 10 mM 2-mercapto-ethanol (2-ME), 10% glycerol, and BSA at 0.1 mg/mL. After incubation at 25 °C for 30 min, the mixture was placed on ice and loaded on a 6% polyacrylamide gel (mono/bis, 29:1) containing 5% glycerol in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3) at 4 °C. After electrophoresis, the gel was fixed, dried, and subjected to autoradiography. Free and bound tRNA was quantified by densitometry analysis. Because the amount of labeled transcripts added in the assays is negligible as compared with the amount of protein added, concentration of protein at which half of the tRNA forms a complex corresponds to the apparent K_d value of the complex.

Aminoacylation Assay. Initial rates of tRNA aminoacylation were measured at 25 °C in 0.1 mL of 20 mM imidazole-HCl buffer (pH 7.5), 150 mM KCl, 0.5 mM DTT, 5 mM MgCl$_2$, 3 mM ATP, 20 μM ^{14}C-labeled arginine (Perki-nElmer Life Sciences; 50 Ci/mol), and saturating amounts of tRNA, as previously described (*38*). Partially purified beef tRNA (arginine acceptance of 43 pmol/A_{260}) or homogeneous beef tRNA$_2$^{Arg} obtained by in vitro transcription (arginine acceptance of 500 pmol/A_{260}) were used as tRNA substrate. The incubation mixture contained catalytic amounts (1–10 nM) of enzymes appropriately diluted in 10 mM Tris-HCl (pH 7.5) and 10 mM 2-mercaptoethanol containing bovine serum albumin at 4 mg/mL. One unit of activity is the amount of enzyme producing 1 nmol/min of arginine–
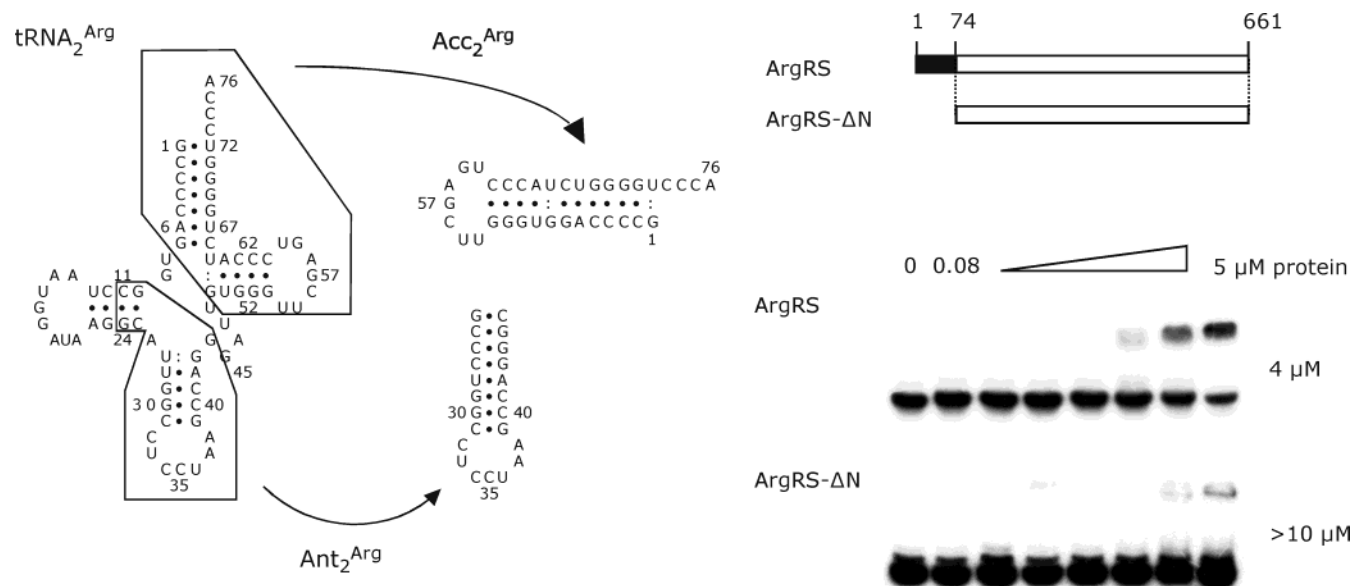
FIGURE 1: Binding of ArgRS to tRNA^{Arg}. The sequence and cloverleaf structure of tRNA$_2{}^{Arg}$ and sequence and hairpin structures of acceptor minihelix (Acc$_2{}^{Arg}$) and anticodon microhelix (Ant$_2{}^{Arg}$) used in this study are shown on the left. Full-length ArgRS (1−661) and the N-terminally truncated naturally occurring ArgRS derivative (ArgRS-ΔN; 74−661) were used. In vitro transcribed,^{32}P-labeled tRNA$_2{}^{Arg}$ was incubated with hamster ArgRS or ArgRS-ΔN at different concentrations (0.08−5 μM). After electrophoresis at 4 °C on a 6% native polyacrylamide gel, the mobility shift of tRNA was visualized by autoradiography. In each assay, the bottom band corresponds to the free tRNA species. Apparent K_d values are indicated at right.

tRNA^{Arg} at 25 °C. For the determination of K_M values for tRNA, tRNA^{Arg} concentrations of 0.02−5 μM were used. Michaelian parameters were obtained by nonlinear regression of the theoretical Michaelis−Menten equation to the experimental curve using the KaleidaGraph 3.6 software (Abelbeck Software).

For large scale synthesis of RNA substrates, in vitro transcription was conducted on 0.5 mg of linearized template DNA in a final volume of 5 mL containing 40 mM Tris-HCl (pH 8.0), 22 mM MgCl$_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.01% Triton X-100, 4 mM each CTP, UTP, GTP, and ATP, 16 mM GMP, and 500 U/ml T7 RNA polymerase. After a 1 h incubation at 37 °C, 100 units of inorganic pyrophosphatase (BioLabs) were added, and synthesis was resumed for a 4 h period. After phenol−chloroform extraction, transcripts were purified on a 1.6 mm thick 12% polyacrylamide urea-gel, electroeluted with a Bio-Trap apparatus (Schleicher & Schuell), ethanol-precipitated, and resuspended in 5 mM MgCl$_2$. Transcripts were renatured by heating at 90 °C and slow cooling (90−30 °C in 2 h).

Arginine Activation Assay. The isotopic [^{32}P]PP$_i$-ATP exchange reaction was conducted as described previously (*36*). The assay mixture contained, in a final volume of 0.1 mL, 20 mM imidazole-HCl (pH 7.5), 10 mM MgCl$_2$, 0.1 mM EDTA, and 2 mM each of ATP, [^{32}P]pyrophosphate (2.5 Ci/mol), and arginine. The reaction was started by the addition of limiting amounts of enzymes (10−20 nM) appropriately diluted in 10 mM Tris-HCl, pH 7.5, containing 10 mM 2-ME and BSA at 4 mg/mL. After 10 min at 25 °C, the reaction was stopped by the addition of 2.5 mL of a solution containing 100 mM pyrophosphate, 50 mM sodium acetate (pH 4.5), 0.35% perchloric acid, and 0.4% Norit to absorb ATP. Samples were filtered through Whatmann no. 1 filters and washed extensively with a solution of 100 mM PP$_i$. [^{32}P]-labeled ATP absorbed on Norit was quantified by liquid scintillation counting. A unit of enzyme activity is defined as the amount of enzyme required to form 1 nmol/min of [^{32}P]ATP.

For the determination of kinetic parameters in the PP$_i$−ATP exchange reaction, the concentration of tRNA in the assay was varied from 1 nM to 1 μM. Michaelian parameters were deduced as described above.

RESULTS

tRNA-Binding Capacity of Mammalian ArgRS. The native form of mammalian arginyl-tRNA synthetase (ArgRS) is a monomer of 75.6 kDa that makes protein−protein interactions with the p43 RNA-binding protein and associates with the multisynthetase complex (the ArgRS component of the complex is referred to as ArgRS-Cx) (*33, 39*). A free form of that enzyme (ArgRS-ΔN), starting at the Met74 residue (Figure 1) is also encountered in the cytoplasm of mammalian cells (*40, 41*). We previously reported that the two enzyme species ArgRS and ArgRS-ΔN, expressed in yeast and purified to homogeneity, display very similar kinetic parameters for arginine activation and tRNA arginylation (*36*). This result left open the possibility that association of ArgRS with p43 within the multisynthetase complex might influence the activity of ArgRS. However, comparison of their specific activities (*36*) and other initial investigations suggested that ArgRS and ArgRS-Cx have similar kinetic parameters. Because there is some disagreement on this point (*35*), we reanalyzed in detail the putative role of the RNA-binding protein p43 on the ability of ArgRS to bind and aminoacylate its tRNA.

We first asked whether the 73 additional residues found in ArgRS as compared with ArgRS-ΔN provide the full-length enzyme with tRNA-binding properties. Indeed, we recently reported that the eukaryotic-specific C-terminal or N-terminal polypeptide extensions of MetRS and LysRS,

Table 1: Kinetic Constants of ArgRS and ArgRS-ΔN in ATP−PP$_i$ Exchange and tRNA Aminoacylation[a]

	ATP−PP$_i$ exchange[d]	tRNA arginylation	
	tRNA$_2^{Arg}$ [b]	tRNA$_2^{Arg}$ [b]	global tRNA[c]
	ArgRS		
K_m (μM)	0.023 ± 0.007	0.16 ± 0.03	0.32[e] ± 0.04
k_{cat} (s^{-1})	3.9 ± 0.4	0.40 ± 0.08	1.8[e] ± 0.2
k_{cat}/K_m (s^{-1} μM^{-1})	170	2.5	5.6
	ArgRS-ΔN		
K_m (μM)	0.024 ± 0.006	0.085 ± 0.005	0.39[e] ± 0.04
k_{cat} (s^{-1})	4.0 ± 0.5	0.17 ± 0.03	2.6[e] ± 0.3
k_{cat}/K_m (s^{-1} μM^{-1})	167	2.0	6.7

[a] Standard errors were determined from at least two independent data sets. [b] In vitro transcribed beef tRNA$_2^{Arg}$; acceptance of 500 pmol/A_{260}. [c] Partially purified native tRNA^{Arg} from beef liver; acceptance of 43 pmol/A_{260}. [d] K_m values correspond to apparent K_{act} (activation constant) values for tRNAs. [e] Values from ref *36*.

repectively, two other components of this multienzyme complex, are tRNA-interacting factors (*11*, *14*). To assess the role of the N-terminal extension of ArgRS on tRNA binding, the two enzyme species ArgRS and ArgRS-ΔN were subjected to a band shift assay. Radiolabeled in vitro transcribed tRNA$_2^{Arg}$ was incubated with increasing amounts of ArgRS and ArgRS-ΔN (80 nM to 5 μM), and free and bound tRNA species were separated by electrophoresis on a native gel (Figure 1). The apparent K_d value of ArgRS-ΔN for tRNA$_2^{Arg}$ was higher than 10 μM, and ArgRS interacted with tRNA$_2^{Arg}$ with an apparent dissociation constant of 4 ± 1 μM. This K_d value determined for the full-length form of ArgRS is about 2 orders of magnitude higher than that obtained with MetRS (100 nM, ref *11*) and LysRS (60 nM, ref *14*) toward their cognate tRNAs but is in the range of the K_d's determined for these two enzymes after removal of their tRNA-interacting factors (4 and 6 μM, respectively, for MetRS-ΔC and LysRS-ΔN). Therefore, its eukaryotic-specific N-terminal polypeptide extension does not provide ArgRS with potent RNA binding properties.

ArgRS and ArgRS-ΔN Display Similar Catalytic Constants. We have shown previously that the two monomeric forms of ArgRS produced in yeast, ArgRS and ArgRS-ΔN, display the same kinetic parameters for tRNA in the tRNA^{Arg} aminoacylation reaction when a partially purified beef liver tRNA is used as a substrate (ref *36* and Table 1). Because we observed that the presence of a large excess of noncognate tRNAs in the aminoacylation reaction may conceal kinetic effects contributed by the eukaryotic-specific, general tRNA-binding domains of two other eukaryotic aminoacyl-tRNA synthetases, namely, MetRS and LysRS (*11*, *14*, *42*), possibly via neutralization of the nonspecific RNA binding sites, we reinvestigated kinetic parameters of the various ArgRS species using a homogeneous tRNA^{Arg} substrate. In vitro transcribed tRNA$_2^{Arg}$ proved to be an efficient substrate of ArgRS in the aminoacylation reaction, with a k_{cat}/K_m only 2 to 3-fold lower as compared with partially purified native beef tRNA^{Arg}, resulting essentially from a lower k_{cat} (Table 1).

The kinetic constants for tRNA$_2^{Arg}$ were determined in the tRNA aminoacylation reaction (Table 1). The K_m of the two recombinant free forms, ArgRS and ArgRS-ΔN, for tRNA$_2^{Arg}$ and the corresponding k_{cat} values were similar. In addition, because arginine activation by mammalian ArgRS is strictly

tRNA-dependent (*36*), K_m and k_{cat} values for tRNA$_2^{Arg}$ in the ATP−PP$_i$ exchange reaction were also determined. The N-terminal domain did not improve the catalytic efficiency (k_{cat}/K_m) of ArgRS (Table 1). The turnover number of ArgRS and ArgRS-ΔN was about 10-fold higher in the ATP−PP$_i$ exchange step as compared with the tRNA aminoacylation step. Thus, tRNA esterification or the release of aminoacylated tRNA may be the limiting step of the global reaction catalyzed by ArgRS or ArgRS-ΔN. These results, together with the tRNA binding assays described above, confirm that the eukaryotic-specific N-terminal extension of ArgRS is not a functional tRNA-interacting factor.

p43 and ArgRS Associate through Their N-Termini. The p43 component of the mammalian multisynthetase complex is a dimer of two 35.4 kDa subunits (*28*). Its C-terminal domain, from residues 147−312, is released from the complex after cleavage with caspase 7, a protease specifically activated during apoptosis, whereas its N-terminal domain remains associated with the synthetase components (*23*). A protein interaction map of the complex determined by a two-hybrid analysis revealed that p43 and ArgRS are interacting proteins (*33*). Surface plasmon resonance analysis established that ArgRS binds p43 with a dissociation constant of 93 nM whereas ArgRS-ΔN does not (*34*). The interaction of ArgRS and p43 was further addressed by in vitro pull-down experiments.

His-tagged p43 was incubated with ArgRS or ArgRS-ΔN and immunoprecipitated with anti-His antibodies. The presence of ArgRS in the immunoprecipitate was detected by Western blotting with antibodies directed to the ArgRS-ΔN moiety (Figure 2A). ArgRS but not ArgRS-ΔN was recovered in the immunoprecipitate obtained in the presence of p43. p43-N and p43-C represent the N-terminal (amino acid residues 1−146) or C-terminal (147−312) domain of p43, respectively. p43-N also co-immunoprecipitated ArgRS (Figure 2B), whereas p43-C did not (Figure 2C). These results clearly established that the N-terminal domains of p43 and ArgRS are involved in protein−protein interaction.

Association with ArgRS Does Not Impair the tRNA-Binding Capacity of p43. The 312-amino acid p43 protein has a potent general tRNA binding capacity (K_d = 0.2 μM) (*23*). In vitro transcribed tRNA$_2^{Arg}$ is also a good ligand for p43 with a K_d of 0.5 μM (Figure 3A). p43 was preincubated with ArgRS or ArgRS-ΔN at a 1:1 ratio to allow formation of a complex between ArgRS and p43; afterward, tRNA$_2^{Arg}$ was added to determine the tRNA binding capacity of the mixture by gel shift analysis. As shown in Figure 3A, addition of ArgRS, which associates with p43, or ArgRS-ΔN, which does not bind to p43, neither impaired nor significantly improved the tRNA binding capacity observed with p43 alone. This result suggested that the tRNA binding site of p43 is not sterically hindered after interaction of p43 with ArgRS. The supershift of tRNA induced by additional association of ArgRS to the tRNA−p43 complex is barely discernible (possibly because the two complexes have similar net charges) and could be best observed after longer electrophoresis in a less cross-linked gel (see below).

To ascertain that the gel shift of tRNA observed in the presence of ArgRS and p43 (Figure 3A) did correspond to a ternary complex, we performed a control experiment in which unlabeled tRNA$_2^{Arg}$ was mixed with ArgRS, p43, or both, and the gel shift of ArgRS and p43 was followed by
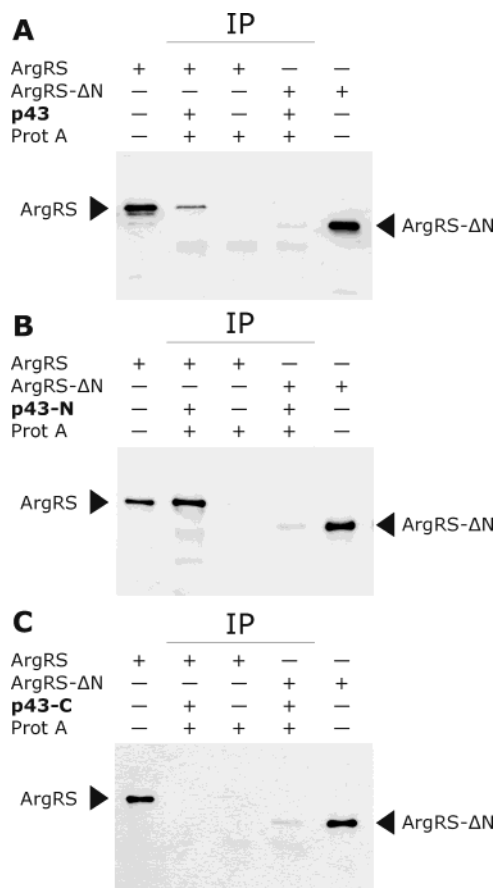
FIGURE 2: ArgRS associates with p43. His-tagged p43 (A), p43-N (B), or p43-C (C) was incubated with ArgRS (or ArgRS-ΔN) and subjected to immunoprecipitation (IP) using anti-His antibodies and Protein-A Sepharose. Immunoprecipitates were analyzed by Western blotting using antibodies directed to the conserved catalytic domain of ArgRS (ArgRS-ΔN). ArgRS (left lane) and ArgRS-ΔN (right lane) were analyzed in parallel (25 ng of homogeneous protein).

Western blotting with specific antibodies (Figure 3B). When p43 or ArgRS were added separately to tRNA$_2^{Arg}$, the electrophoretic mobility of p43, but not of ArgRS, was altered and corresponded to the single shift of tRNA. By contrast, when the two proteins were added together, ArgRS and p43 were both shifted and comigrated within the same complex, corresponding to a supershift of tRNA (Figure 3B).

We also verified that ArgRS and p43 remained associated in solution in the presence of tRNA. When His-tagged p43 was incubated with ArgRS in the presence of tRNA$_2^{Arg}$ at molar ratios of p43/tRNA of 1:1, 1:5, or 1:20, the immunoprecipitates obtained with anti-His antibodies also contained an invariable amount of ArgRS (Figure 1S, Supporting Information). Addition of tRNA did not antagonize p43−ArgRS interaction. Thus, the tRNA- and ArgRS-binding sites of p43 are not overlapping. Therefore, association of p43, a potent tRNA-interacting factor, might provide ArgRS with enhanced catalytic properties provided that p43-bound tR-NA^{Arg} is suitably presented to the catalytic center of ArgRS.

p43 Preferentially Binds the Acceptor-Stem, T-Stem−Loop Structure of tRNA. To further address the RNA binding properties of p43, the mode of binding of tRNA to p43 was investigated. To probe the interaction of tRNA with p43, the acceptor-TΨC stem−loop (Acc$_2^{Arg}$) and anticodon stem−loop (Ant$_2^{Arg}$) structures of tRNA$_2^{Arg}$ were synthesized in
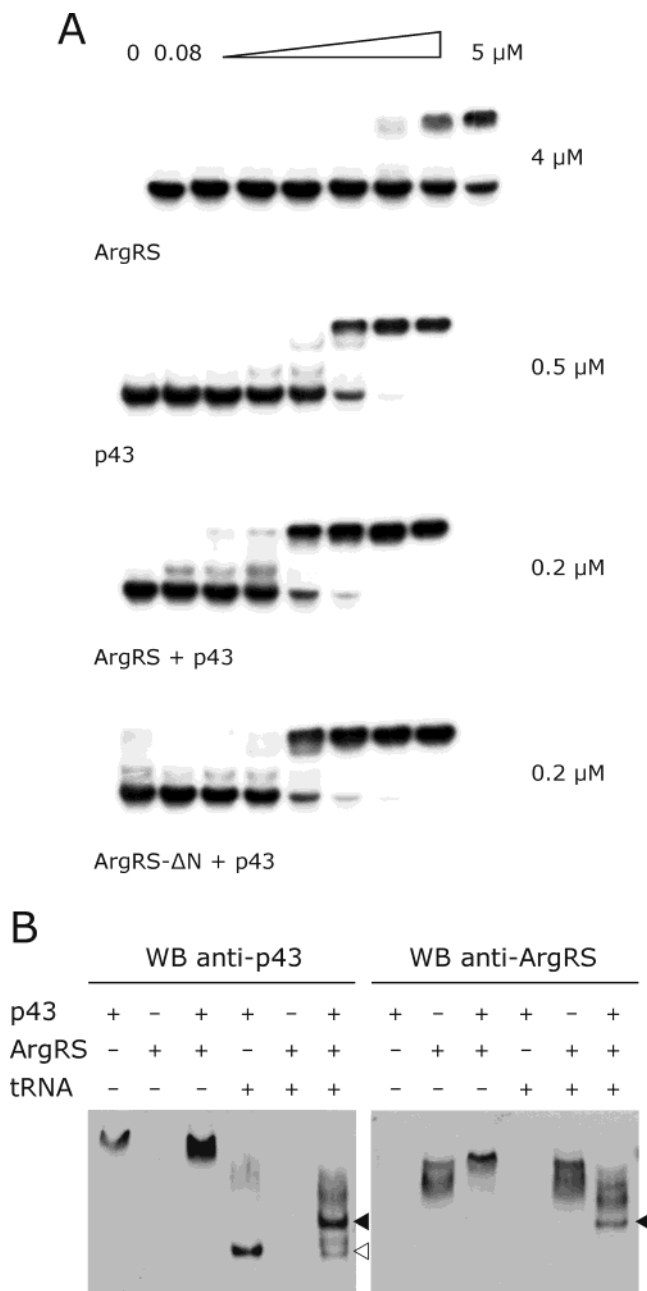

FIGURE 3: Binding of tRNA^{Arg} by the ArgRS−p43 complex. In panel A, ^{32}P-labeled tRNA$_2^{Arg}$ was incubated with ArgRS (0.08−5 μM), p43 (0.04−2.5 μM; monomer concentration), or a mixture of ArgRS and p43 or ArgRS-ΔN and p43 (0.04−2.5 μM of each protein). After electrophoresis (30 min, 100 V) at 4 °C on a 6% native polyacrylamide gel, the mobility shift of tRNA was visualized by autoradiography. Apparent K_d values are indicated at right. In panel B, ArgRS, p43, or a mixture of both (0.5 μM each, monomer concentration) was incubated with or without in vitro transcribed tRNA$_2^{Arg}$ (0.3 μM). The mixture was subjected to electrophoresis (2 h; 100 V) at 4 °C on a 4% native polyacrylamide gel. The mobilities of ArgRS and p43 were identified by Western blotting (WB) with specific antibodies. The white and black arrows indicate the single mobility shift of tRNA in the presence of p43 or the supershift of tRNA in the presence of ArgRS and p43, respectively.

vitro (Figure 1) and used in a gel mobility shift assay to analyze their association with ArgRS, p43, and ArgRS−p43 complexes (Figure 4). Neither Acc$_2^{Arg}$ nor Ant$_2^{Arg}$ showed significant binding to ArgRS. The acceptor minihelix formed a stable complex with p43 with an apparent K_d value of about 0.5 μM, similar to the binding capacity of p43 for full-length
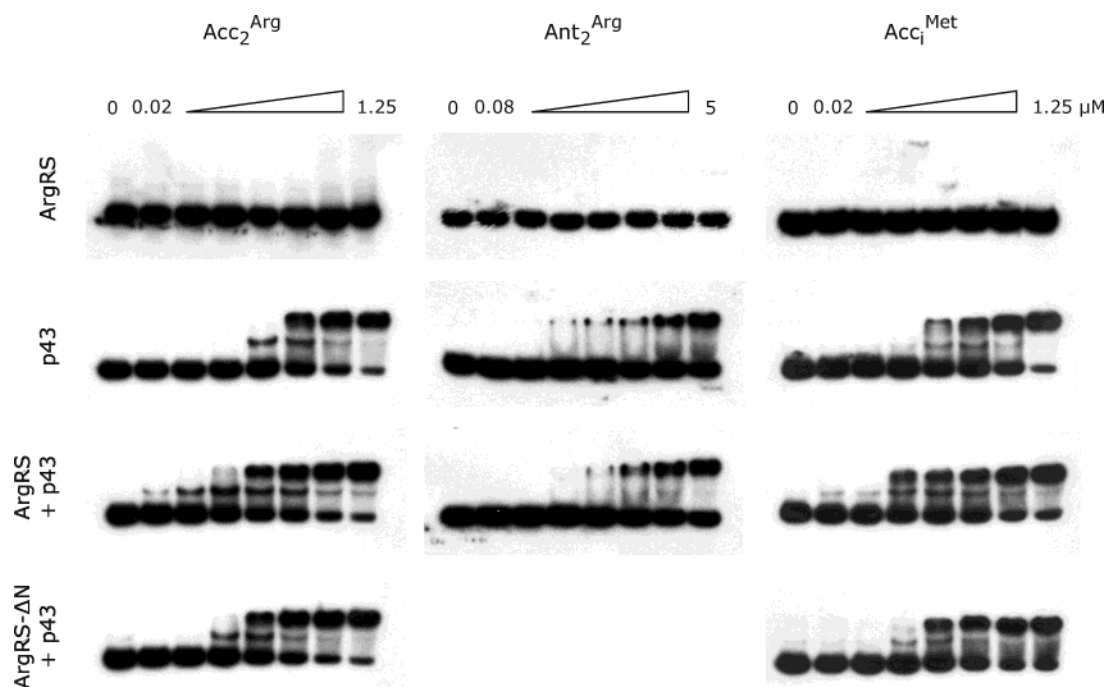
FIGURE 4: The mode of tRNA binding by p43 is not altered following its association with ArgRS. Binding of the acceptor-TΨC stem−loop (Acc_2^{Arg}) and anticodon stem−loop (Ant_2^{Arg}) minihelices of beef $tRNA_2^{Arg}$ by ArgRS, p43, and p43 in the presence of equimolar amounts of ArgRS or ArgRS-ΔN is shown. The mobility shift of the ^{32}P-labeled RNA minihelices was analyzed as described in the legend of Figure 1. A similar analysis was conducted in parallel using a ^{32}P-labeled minihelix corresponding to the acceptor-TΨC domain of yeast initiator $tRNA^{Met}$.

tRNA. By contrast, p43 displayed a much weaker affinity for the anticodon domain of $tRNA_2^{Arg}$ ($K_d \approx 5$ μM). We concluded that p43 preferentially binds the acceptor-TΨC stem−loop of $tRNA_2^{Arg}$. As observed above for the binding of full-length $tRNA_2^{Arg}$, the tRNA binding capacity of p43 for Acc_2^{Arg} or Ant_2^{Arg} was not significantly improved in the presence of ArgRS (K_d's of about 0.3 and 5.0 μM, respectively). The slight increase in binding of Acc_2^{Arg} (but not of Ant_2^{Arg}) observed after incubation of p43 with ArgRS is also detectable after incubation of p43 with ArgRS-ΔN, a derivative of ArgRS that does not associate with p43. These results suggest that association of p43 with ArgRS neither alters the tRNA-binding capacity of p43 nor modifies the mode of tRNA−p43 interaction.

p43 in solution is a nonspecific tRNA-interacting factor (*23*). We sought to check whether its association with ArgRS rendered p43 specific for $tRNA^{Arg}$. When full-length yeast $tRNA_i^{Met}$ (not shown) or Acc_i^{Met} (Figure 4) and Ant_i^{Met} (not shown) minihelices were used in the band shift assay in place of $tRNA_2^{Arg}$, the tRNA binding potential of p43 was not impaired after incubation with ArgRS or ArgRS-ΔN.

p43 Is Not a Trans-Acting tRNA-Interacting Factor for ArgRS. The possibility that p43 might act as a tRNA-presenting protein for ArgRS was investigated. ArgRS and p43 were mixed together at molar ratios ranging from 1:0.25 to 1:20 000 to allow protein−protein interaction, and the mixture was directly used in the tRNA aminoacylation assay. Because we sought to determine the putative effect of p43−ArgRS association on the efficiency of the aminoacylation reaction independent of any potential effect that might be attributed to the binding of p43 to tRNA, we used the bacterial tRNA-binding protein Trbp111 as a control. Trbp111 is a dimeric protein that binds tRNA without specificity of

sequence (*37*) and can form a ternary complex with tRNA and tRNA synthetase without impairing the aminoacylation activity of the synthetase (*43*). When increasing concentrations of p43 or Trbp111 were added to ArgRS, the $tRNA^{Arg}$ aminoacylation capacity of the enzyme was not significantly affected (Figure 2S, Supporting Information). In particular, the aminoacylation activity of ArgRS was not enhanced in the presence of a 2-fold molar excess of p43, as previously reported by others (*35*).

Activity of ArgRS from the Multienzyme Complex Is p43-Independent. ArgRS and p43 are two components of the mammalian multisynthetase complex. The results obtained with free recombinant species of ArgRS and of p43, two ectopic proteins, strongly suggested that p43 is not a trans-acting tIF of ArgRS in the $tRNA^{Arg}$ aminoacylation reaction. To investigate whether p43 may be a tIF of ArgRS under native conditions, when the two proteins are associated within the multisynthetase complex, kinetic parameters for tRNA in the $tRNA^{Arg}$ aminoacylation reaction were determined for ArgRS-Cx, the ArgRS component of the complex. To assess the role of p43 on ArgRS activity, we used the multisynthetase complex treated with caspase 7. The p43 component of the mammalian multisynthetase complex is a substrate for caspase 7, a protease activated upon onset of apoptosis on mammalian cells (*23*). After treatment with caspase 7, the complex retains native ArgRS but is completely deprived of the EMAPII C-terminal domain of p43 (*23*). Thus, the potential tIF activity of p43 is lost. The kinetic parameters for $tRNA_2^{Arg}$ determined for ArgRS-Cx in the ATP−PP_i exchange and in the tRNA aminoacylation reactions were not affected by proteolytic subtraction of the tRNA-binding domain of p43 (Table 2).

Table 2: Kinetic Constants of ArgRS-Cx in ATP−PP$_i$ Exchange and tRNA Aminoacylation[a]

	tRNA$_2^{Arg}$ [b]	
	ATP−PPi exchange[c]	tRNA arginylation
ArgRS-Cx		
K_m (μM)	0.006 ± 0.001	0.20 ± 0.02
k_{cat} (s^{-1})	1.1 ± 0.2	0.16 ± 0.03
k_{cat}/K_m (s^{-1} μM^{-1})	183	0.8
ArgRS-Cx/Casp7		
K_m (μM)	0.008 ± 0.002	0.20 ± 0.02
k_{cat} (s^{-1})	1.4 ± 0.2	0.13 ± 0.03
k_{cat}/K_m (s^{-1} μM^{-1})	175	0.65

[a] Standard errors were determined from at least two independent data sets. [b] In vitro transcribed beef tRNA$_2^{Arg}$; acceptance of 500 pmol/A_{260}. [c] K_m values correspond to apparent K_{act} values for tRNA.

DISCUSSION

The work reported here showed that p43 forms a binary complex with ArgRS (Figure 2) and that this association neither modulates the tRNA binding capacity of p43 (Figures 3 and 4) nor modifies the amino acid activation and tRNA aminoacylation activities of ArgRS (Tables 1 and 2; Figure 2S, Supporting Information). These results contrast with an earlier report showing that addition of p43 to ArgRS led to a 2.5-fold increase in ArgRS activity (*35*). They described that a 2-fold molar excess of p43 to ArgRS reduces the apparent K_m of ArgRS for tRNA, whereas the k_{cat} value is not affected. Surprisingly, when p43 was added in excess the enhancement of ArgRS activity was no longer observed. Whether these apparent discrepancies arise from differences in experimental procedure could not be assessed because detailed experimental procedures were not given in this study (*35*). Surprisingly, no quantitative data on enzyme activity (specific activity of the purified enzyme, k_{cat}, and K_m parameters) are furnished, and all the results are indicated as relative values. Nevertheless, the reported experiments provide some indications of possible sources of the discrepancies. Perhaps critically, the enzyme preparation used by Park et al. (*35*) was obtained after expression in *E. coli*. Indeed, we previously reported that expression of ArgRS in *E. coli* cells produces inclusion bodies and that the fraction of soluble enzyme has a reduced (100-fold) specific activity as compared with the enzyme used here, produced in yeast (*36*). Therefore, the enhancement of ArgRS activity observed by Park et al. (*35*) in the presence of stoichiometric amounts of p43 could be due to a chaperone effect of the binding of p43 on an ArgRS species that would not be properly folded.

ArgRS and p43 are the first example in which the association of an eukaryotic aminoacyl-tRNA synthetase with a tRNA-interacting factor does not provide kinetic advantages to the synthetase. The yeast homologue of p43, Arc1p, forms a ternary complex with MetRS and GluRS and stimulates the activity of MetRS mainly by reducing the K_m for tRNA^{Met} by about 100-fold (*21*, *22*). When Arc1p in solution forms a complex with several yeast tRNAs, only elongator and initiator tRNA^{Met} associated with the Arc1p−MetRS complex (*22*). Thus, in the yeast system, MetRS determines the specificity of the interaction and Arc1p strengthens association. By contrast, we observed that tRNA^{Arg} but also tRNA^{Met} are able to associate with p43 or with the p43−ArgRS complex with similar affinities. These data also support the

conclusion that p43 is not a functional tIF for ArgRS and therefore does not provide catalytic advantages for the synthetase. Because the N-terminal domain of p43 is essential for association of ArgRS with the complex, our results support the proposal that this auxiliary component of the complex has a dual function: its N-terminal domain is a structural determinant for the assembly of this macromolecular structure and its C-terminal moiety is a tIF for another component of the complex.

Our results also provide some clues for understanding the mode of binding of tRNA by p43 and Trbp111. The crystal structure of the complex between *S. cerevisiae* ArgRS and tRNA^{Arg} was reported at high resolution (*44*). The contact areas between the synthetase and its tRNA include the end of the acceptor stem of the tRNA with the catalytic center of the protein, the anticodon loop with the C-terminal α-helical domain, and the D-loop with the N-terminal α/β globular domain. The latter contact that involves the outer corner of the L-shaped tRNA molecule is rather unusual among aminoacyl-tRNA synthetases but should be conserved in hamster ArgRS, which displays a primary sequence and hence a structural organization similar to yeast ArgRS. In the present study, we showed that tRNA$_2^{Arg}$ or a minihelix corresponding to the acceptor-TΨC stem−loop bind to p43 with similar affinities. We also reported earlier that the C-terminal appended domain of plant MetRS, homologuous to the C-terminal tRNA-binding domain of p43, preferentially binds the acceptor stem of tRNA (*42*). This is in contrast to Trbp111, which is believed to bind to the convex side of the tRNA structure (*45*). Convincing evidence for the simultaneous binding of Trbp111 and IleRS to a single tRNA molecule was presented (*43*). However, the docking model proposed for the Trbp111−tRNA complex is not compatible with the simultaneous binding of Trbp111 and ArgRS to the same tRNA molecule. Indeed, according to this model, the N-terminal α/β globular domain of ArgRS should compete with Trbp111 for binding to the D-loop region of tRNA. The finding that saturating amounts of Trbp111 or of p43 do not inhibit aminoacylation of tRNA^{Arg} by ArgRS suggests that the two proteins bind tRNA without steric hindrance.

In view of the finding in this paper that p43 is not a functional tIF of the ArgRS component of the mammalian complex, the functional partner of p43 within the multisynthetase complex must now be searched out. The intricate structural organization of this particle could reveal that two nonphysically interacting proteins may be close in the 3D-structure and so may be functionally connected.

SUPPORTING INFORMATION AVAILABLE

Results of His-tag immunoprecipitations of ArgRS and His-tagged p43 in the presence of tRNA and of aminoacylation assays of p43−ArgRS and Trbp111−ArgRS mixtures. This material is available free of charge via the Internet at http://pubs.acs.org.

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