HEMIN BINDS TO HUMAN CYTOPLASMIC ARGINYL-TRNA SYNTHETASE AND INHIBITS ITS CATALYTIC ACTIVITY

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The free form of human cytoplasmic arginyl-tRNA synthetase (hcArgRS) is hypothesized to participate in ubiquitin-dependent protein degradation by offering arginyl-tRNA Arg to arginyl-tRNA transferase (ATE1). We investigated the effect of hemin on hcArgRS based on the fact that hemin regulates several critical proteins in “N-end rule” protein degradation pathway. Extensive biochemical evidence has established that hemin could bind to both forms of hcArgRS in vitro. Based on the spectral changes of the Soret band on site-directed protein mutants, we identified Cys-115 as a specific axial ligand of hemin binding that is located in the Add1 domain. Hemin inhibited the catalytic activity of full-length and N-terminal 72-amino acid truncated hcArgRSs by blocking amino acid activation. Kinetic analysis demonstrated that the $K_m$ values for tRNA$^{Arg}$, arginine and ATP in the presence of hemin were not altered, but $k_{cat}$ values dramatically decreased compared with those in the absence of hemin. By comparison, the activity of prokaryotic ArgRS wasn’t affected obviously by hemin. Gel filtration chromatography suggested that hemin induced oligomerization of both the isolated Add1 domain and the wild type enzyme, which could account for the inhibition of catalytic activity. However, the catalytic activity of a hcArgRS mutant with Cys-115 replaced by alanine (hcArgRS-C115A) was also inhibited by hemin, suggesting that hemin binding to Cys-115 is not responsible for the inhibition of enzymatic activity and that the specific binding may participate in other biological functions.

Aminoacyl-tRNA synthetase (aaRS) plays an essential role in the first step of protein biosynthesis by catalyzing the attachment of amino acids to the 3’-end of their cognate tRNAs. Based on the architecture of their catalytic sites, the 20 aaRSs can be classified into two groups (1). Class I aaRSs have “HIGH” and “KMSKS” signature sequences located in the Rossmann fold active site. Class II aaRSs are characterized by three homologous motifs (1). Usually, the reaction catalyzed by aaRSs occurs in two steps: amino acid activation followed by aminoacylation of the cognate tRNA. The amino acid activation of aaRSs does not require the presence of the cognate tRNA, except for arginyl-tRNA synthetase (ArgRS), glutamyl-tRNA synthetase (GluRS) and glutaminyl-tRNA synthetase (GlnRS), which
activate their cognate amino acids in a tRNA-dependent manner (2, 3).

ArgRS is a class I aaRSs and tRNA binding is a prerequisite for activating arginine. The crystal structures of ArgRS from several species have been solved (4-6). *S. cerevisiae* cytoplasmic ArgRS (yArgRS) is a monomeric protein with five domains: the N-terminal additional domain (Add1), the catalytic domain including two insertion domains (Ins-1 and Ins-2) and the C-terminal additional domain (Add2). The tRNA^Arg serves as the activator of ArgRS in the reaction of amino acid activation and as the substrate in aminoacylation. Three regions of ArgRS are involved in recognizing tRNA^Arg: the active center, the anticodon binding domain and the Add1 domain. The recognition of tRNA^Arg by the three regions is essential for activation of mammalian ArgRS (7, 8). The Add1 domain containing a two-layer α/β unit, is unique since it is missing in other class I aaRSs, and it is involved in arginine activation by interacting with the D-loop of tRNA^Arg (4).

Two forms of ArgRS exist in the mammalian cytoplasm. One form is a component of the multiple-synthetase complex (MSC), playing a critical role in protein translation and the other exists free in the cytoplasm with the N-terminal 72 amino acids truncated (ΔNhcArgRS) (9). Both forms of ArgRS exhibit similar catalytic characteristics in vitro (10). Compared to the free enzyme, mammalian ArgRS in MSC has an extended N-terminus and interacts with other components of MSC. Guigou *et al.* reported that hamster ArgRS with a 73 amino acid extension at its N-terminus interacts with the p43 factor in MSC (11); Ling, *et al* in our lab demonstrated that human cytoplasmic ArgRS (hcArgRS) with an N-terminal 72-amino acid extension binds to the C-terminus domain of human cytoplasmic leucyl-tRNA synthetase (hcLeuRS) in MSC (12). Our previous investigations have shown that both forms of human cytoplasmic ArgRS (hcArgRS) are produced from two translational initiations of a single mRNA (13). The hcArgRS in MSC is essential for normal protein synthesis (14). Interestingly, in addition to the critical role in protein synthesis, ArgRS also participates in the ubiquitin-dependent “N-end rule” pathway of protein degradation by providing Arg-tRNA^Arg as a substrate for arginyl-tRNA transferase (ATE1) (15). It was hypothesized that the free form of ArgRS is probably responsible for protein arginylation and degradation (16). Recently, it was found that the “N-end rule” pathway is a sensor of heme and several key proteins in the pathway are regulated by heme (17). ArgRS is also a component of the “N-end rule” pathway, so we ask whether heme interacts with ArgRS.

Heme is a protoporphyrin IX derivative containing an iron atom in the center, in either the reduced ferrous (heme) or the oxidized ferric state (hemin). When heme binds to protein, a cysteine or histidine residue usually serves as the axial ligand of heme by coordinating the iron center of heme (18). Heme absorbs in the ultraviolet-visible region and the absorption peak around 400 nm is known as the Soret band. The Soret band results from Π–Π* transition of the porphyrin ring. Binding of heme to proteins may change the energetic gap of the Π–Π* transition, which shifts the Soret band to shorter or longer wavelengths (19). Heme participates in oxygen and carbon dioxide transport, electron transfer and other biological functions by behaving as a cofactor for proteins such as hemoglobin and cytochrome c, etc (20). Accumulating evidence shows that heme
plays other important roles by working as an active signal molecule (21). Eukaryotic initiation factor 2α (eIF2α) kinase (HRI) regulates the initiation of protein biosynthesis in eukaryotic cells and the activity of the kinase is regulated by heme binding (22, 23). Heme induces polyubiquitination and degradation of some heme-sensor proteins, implying heme may be another class of molecular signature for recognition by an E3 ubiquitin ligase (17, 24, 25). Transcription of some key genes in the human circadian cycle is regulated by heme (26, 27). Heme is also involved in processing of microRNAs by promoting dimerization of DGCR8 (28). Tryptophanyl-tRNA synthetase (TrpRS) and GluRS bind to and are regulated by heme (29, 30), which prompted us to investigate the effects of heme on another aaRS — human cytoplasmic ArgRS.

In the present work, we showed that both forms of hcArgRS can bind hemin in vitro and that hemin specifically binds to the Add1 domain of hcArgRS by an axial ligand, Cys-115, a conserved residue in eukaryotic ArgRSs. Hemin also induces oligomerization of hcArgRS and inhibits the catalytic activity of hcArgRS. Our results suggest that hcArgRS might be regulated by hemin in the “N-end rule” pathway in human cells.

EXPERIMENTAL PROCEDURES

Materials — Hemin, hemin-agrose, L-arginine, dithiothreitol, tetrasodium pyrophosphate, ATP, Tris-HCl, magnesium chloride, sodium chloride, potassium chloride, isopropyl-1-thio-β-D-galactopyranose side (IPTG), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and activated charcoal were purchased from Sigma (USA). Ni²⁺-NTA Superflow was purchased from Qiagen Inc (Germany). [³H]-L-arginine, tetrasodium [³²P]pyrophosphate, Superose-12 column, Superdex-75 column and glutathione Sepharose 4B beads were obtained from Amersham Biosciences (Sweden). E. coli tRNA Arg (ACG) with an accepting activity of 1200 pmole/A₂₆₀ was isolated from an E. coli strain overproducing the tRNA constructed in our lab as described previously (31).

Preparation of hemin solution — Hemin solution was freshly prepared in 0.1M NaOH and then diluted into the appropriate buffer. Stock solutions were kept on ice in the dark until use. The concentration of hemin was determined at 385 nm using an extinction coefficient of 5.84 × 10⁴ cm⁻¹M⁻¹.

Purification of hcArgRS and ΔNhcArgRS and mutants from E. coli transformants — pMFT7H₆-hcArgRS was constructed by inserting the full length hcArgRS gene into the gap between NcoI and BamHI of vector pMFT7H₆ which was constructed previously in our lab (32). ΔNhcArgRS was constructed by Ling et al. in our lab (12). E. coli BL21-CodonPlus (DE3)-RIL was transformed with the two plasmids, pMFT7H₆-hcArgRS and pET15-ΔNhcArgRS, to produce N-terminal His₆-tagged hcArgRS and ΔNhcArgRS, respectively. The transformants containing each plasmid were grown at 37 °C to reach A₆₀₀= 0.6 in 500 mL of 2×YT with 100 μg/mL ampicillin and 60 μg/mL chloramphenicol and were induced by adding IPTG to a final concentration of 200 μM. After 5-hour induction at 22 °C, the cells were harvested and suspended in buffer A (10 mM imidazole, 300 mM NaCl, 10% glycerol, 20mM, β-mercaptoethanol, 0.5mM phenylmethylsulfonyl fluoride(PMSF), 50mM Na₂HPO₄, pH 8.0). After sonication
and centrifugation, the supernatant was gently mixed with 1.0 ml of Ni\textsuperscript{2+} NTA Superflow resin for 1 hour. The mixture was loaded on a minicolumn for gravity flow chromatography. The resin was washed with 25 ml buffer B (except for 20 mM imidazole, other components were as for buffer A) to remove nonspecifically bound contaminants. Then the enzyme was eluted with 10 ml buffer C (except for 250 mM imidazole, other components were as for buffer A). The eluted His\textsubscript{6}-hcArgRS or ΔNhcArgRS fractions were pooled and dialyzed against storage buffer containing 50 mM Tris–HCl (pH 7.5) with 100 mM NaCl and concentrated using an Amicon Ultra-15 filter (Millipore). Site-directed mutagenesis was performed by the protocol provided by the KOD-Plus-Mutagenesis kit (TOYOBO, Japan) and the mutants were purified by Ni\textsuperscript{2+}-NTA affinity chromatography. Protein concentrations were determined by the Bradford method (33). The purified enzymes had over 95% purity by SDS-PAGE assay.

Purification of three other ArgRSs — ArgRSs from E. coli and B. stearothermophilus were purified as described previously by our lab (31, 34). The gene of ArgRS from S. cerevisiae was a gift from Dr. Eriani (Institute of Molecular and Cellular Biology, CNRS, France). The gene was inserted into the gap between Nco\textsubscript{I} and Sal\textsubscript{I} of vector pMFT7H\textsubscript{6} to produce the plasmid pMFT7H\textsubscript{6}-yArgRS and expressed in E. coli BL21-CodonPlus (DE3)-RIL. yArgRS was purified by affinity chromatography on a Ni\textsuperscript{2+}-NTA column as described earlier. The three purified ArgRSs were over 95% homogeneous as determined by SDS-PAGE (data not shown).

Expression and purification of Add1 domain of ΔNhcArgRS — Alignment of ArgRS sequences shows that the Add1 domain of ΔNhcArgRS is equivalent to that of yArgRS. A DNA fragment encoding 110 amino acid residues of the N-terminus of ΔNhcArgRS linked to a glutathione (GST) tag was inserted into pGEX-4T-1 between BamHI and EcoRI sites to produce the pGEX-Add1 recombinant plasmid. The BL21-CodonPlus (DE3)-RIL E. coli cells harboring the plasmid were grown at 37 °C to reach A\textsubscript{600} = 0.6 and induced by 100 μM IPTG. After incubation at 16 °C for 12 hours, the E. coli transformant cells were harvested, dissolved in phosphate buffered saline (PBS) buffer containing PMSF and lysed completely on ice using a sonicator. By ultracentrifugation at 150,000 g for 1 hour cell debris was removed and supernatant was mixed with appropriate amounts of GST Sepharose 4B beads at 4 °C for 1 hour. The mixture was loaded onto a mini column for gravity flow chromatography and the resin was washed with PBS. Thrombin was incubated with the resin in PBS for 16 hours at 22 °C to cleave the Add1 domain from the GST-Add1 fusion protein. The isolated Add1 domain was harvested and concentrated using an Amicon-ultra 15 centrifugal filter.

Gel filtration chromatography — ΔNhcArgRS preincubated with hemin at a molar ratio of 1:1 was applied to a Superose-12 column for high-performance liquid chromatography (HPLC) and eluted at a flow rate of 0.5 mL/min by using a buffer containing 50 mM Tris–HCl (pH 7.5) and 100 mM NaCl. UV-absorbing fractions that contained ΔNhcArgRS were collected and concentrated with an Amicon-ultra 15 centrifugal filter. A Superdex-75 column was used for HPLC analysis of the isolated Add1 domain. The same assay without hemin was performed as a control. Alcohol dehydrogenase (150 kDa), BSA (67 kDa),
ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and α-lactalbumin (14 kDa) were used as protein markers to calculate the relative molecular weights of protein oligomers.

UV-visible absorption spectroscopy — The absorption spectrum properties of hemin were determined by recording the absorption spectra of hemin in the absence or presence of proteins (ΔNhcArgRS, the Add1 domain and mutants) in 50 mM Tris–HCl (pH 7.5) with 100 mM NaCl. The molar ratio of hemin to proteins was 1:1. The spectra were recorded between 200 and 600 nm on a NanoDrop ND1000 spectrophotometer (Thermo Scientific).

Determination of the binding affinity — 50 μM hemin was incubated with ΔNhcArgRS at various concentrations from 0 μM to 90 μM in 50 mM Tris–HCl (pH 7.5) with 100 mM NaCl. After 1-hour incubation in the dark at room temperature, the absorption at 420 nm was measured. ΔA420 was obtained by subtracting the absorbance of hemin in the absence of protein. The data were fit to the equation $Y = \frac{B_{\text{max}} \times X}{K_d + X}$ using GraphPad Prism 5.0 software. $B_{\text{max}}$ refers to the maximum specific binding in the same units as Y and $K_d$ is the equilibrium binding constant in the same units as X.

Direct chemiluminescent imaging — ΔNhcArgRS was mixed with hemin (molar ratio = 1:1) incubated on ice for 30 min in the dark and analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) using a loading buffer without a reducing agent. After SDS-PAGE, the gel was washed with deionized water and Super Signal Chemiluminescent Substrate (Thermo Scientific) was sprayed onto the gel for 30 seconds. An image was acquired using a Fujifilm LAS-4000 System configured for chemiluminescence. ΔNhcArgRS alone without hemin served as a control.

Hemin-agarose pull down assays — 100 μg hcArgRS or ΔNhcArgRS was dissolved in binding buffer containing 50 mM Tris-HCl (pH 7.5), 80 mM KCl, 12 mM MgCl2 and 0.5 M NaCl. 20 μl hemin-agarose beads were equilibrated with the same buffer and incubated with the protein solutions for 2 hours at 4°C in a rotator. The mixture was centrifuged at 5000g for 1 min and the agarose pellets were washed three times with binding buffer to remove unbound material. Sepharose-CL 4B beads (Amersham Biosciences) were used as a control to rule out nonspecific interactions between the protein and agarose. Hemin-agarose and Sepharose beads were then treated with 2×SDS loading buffer (containing 50 mM DTT) and incubated at 95°C for 5 min. The mixture was subjected to 12% SDS-PAGE. Proteins on the gel were visualized by immunoblot analysis using polyclonal antibody for ΔNhcArgRS.

DTNB titration — Cysteine residues on the protein surface of ΔNhcArgRS were determined using DTNB according to the method described previously (31). The reaction was performed at 25 °C in 50 mM potassium phosphate buffer (pH 7.5) containing 5 μM enzyme and 2 mM DTNB. The change in absorbance at 412 nm was recorded and total SH content was calculated assuming a molar absorption coefficient of 14150. Protein concentrations were determined by the Bradford method.

Assays of aminoacylation and ATP-PPi exchange — The aminoacylation activity of ArgRS was determined at 37°C in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 12 mM MgCl2, , 80 mM
KCl, 0.1 mM EDTA, 0.05 mg/mL BSA, 20 μM isolated E. coli tRNA^Arg_{(ACG)} , 20 μM [³H]arginine (450 cpm/pmol) and 3 nM ∆NhcArgRS in the absence or presence of hemin at various concentrations. After incubation on ice for 30 min, the reaction was initiated by adding 4 mM ATP. The kinetic constants of enzymes were determined using various concentrations of the relevant substrates in the reaction mixture in the absence or presence of 10 μM hemin. The data were analyzed by Hyper1.1 software. Effects of hemin on the activity of amino acid activation were determined by assaying the rate of ATP-PPi exchange in the absence or presence of 10 μM hemin. The reaction was performed in 50 mM Tris-HCl (pH 7.5), 12 mM MgCl₂, 4 mM ATP, 80 mM KCl, 0.1 mM EDTA, 0.05 mg/mL BSA, 20 μM E. coli tRNA^Arg, 2 mM arginine, 2 mM tetrasodium [³²P] pyrophosphate(10 cpm/pmol), and 3 nM ∆NhcArgRS.

RESULTS

**HcArgRS is a hemin-binding protein —**

HcArgRS and ∆NhcArgRS were purified from E. coli BL21-Codon Plus (DE3)-RIL transformants by Ni²⁺-NTA affinity chromatography. Although both hcArgRSs could be obtained from overproducing E. coli strains, ∆NhcArgRS was much easier to purify than the full-length hcArgRS. The differences of primary sequences and enzymatic characteristics between hcArgRS and ∆NhcArgRS were slight except for the 72 amino acid residues at the N-terminus(10, 12) and therefore, ∆NhcArgRS was mainly used in this work. Hemin is a protoporphyrin containing a ferric iron in the center and we tested whether hemin could interact with ∆NhcArgRS in vitro. We incubated ∆NhcArgRS and hemin at a molar ratio of 1:1 and performed gel filtration to remove unbound molecules. The concentrated ∆NhcArgRS after incubation with hemin presented a red color, suggesting hemin bound to the protein, however ∆NhcArgRS without hemin did not show an obvious color (Fig.1A). Soret band shift is a sensitive method to detect interaction between heme/hemin and proteins (19). Here the UV-visible absorption of ∆NhcArgRS bound with hemin after gel filtration exhibited obvious hyperchromic and bathochromic effects (Fig. 1B). The absorption peak of the Soret band of hemin was shifted from 385 nm to 420 nm, which is a typical spectrum of hemin binding protein. Full-length hcArgRS showed similar spectral characteristics with ∆NhcArgRS in the presence of hemin (data not shown). A novel direct chemiluminescent (CL) imaging method has been developed for the detection of a hemin-binding proteins in polyacrylamide gels. The method is based on the fact that metalloporphyrins can bind to proteins and catalyze the CL reaction of the luminol–H₂O₂ system (35). The band corresponding to ∆NhcArgRS was observed in a gel by direct CL imaging for the protein incubated with hemin, suggesting that hemin formed a complex with the enzyme (Fig.1C). The result of hemin-agarose affinity chromatography also showed that ∆NhcArgRS/hcArgRS could be pulled down by hemin-agarose (Fig.1D) but could not bind to the control Sepharose beads (data not shown). These data indicated that hemin formed a stable complex with ∆NhcArgRS or hcArgRS. In order to test whether heme (ferrous protoporphyrin IX) also binds to ∆NhcArgRS, 2mM dithionite was added to the complex of the enzyme and hemin to reduce hemin to Fe²⁺-heme and the absorption spectra were recorded. The peak
site of the Soret band was red-shifted to a longer wavelength around 430 nm, which is a characteristic of a heme-binding protein (data not shown).

**Hemin binds to the Add1 domain of hcArgRS with Cys-115 as an axial ligand** — It’s well documented that the Heme regulatory motif (HRM) is usually involved in heme binding by a conserved Cys-Pro dipeptide found in some heme-responsive proteins (23-25). However, no similar sequence to the HRM motif was observed in hcArgRS. The majority of known heme-protein interactions are mediated by coordination bonds between the heme center and the SH group of Cys or side chain of His (23-25, 36). There are total of 14 Cys residues (3 at N-terminus) and 12 His residues (1 at N-terminus) in full length hcArgRS. In order to determine the axial ligand of hemin in hcArgRS, we mutated each cysteine and histidine residue to alanine (single or double site mutant) and purified the mutants. Because WT hcArgRS/ΔNh hcArgRS with hemin exhibited a typical absorption peak of the Soret band around 420 nm, the absorption spectra of these mutants with hemin were all measured. All mutants had similar spectral characteristics to hcArgRS with the Soret peak around 420 nm, except for hcArgRS-C115A (Table 1). The Soret peak of hcArgRS-C115A with hemin at 420 nm disappeared completely as shown in Fig. 2A, suggesting that Cys-115 may serve as the axial ligand for hemin. When Cys-115 was changed to Ser to construct another mutant, C115S, the Soret peak at 420 nm for C115S with hemin was also not observed (data not shown). These data indicated that Cys-115 of hcArgRS is crucial to its binding with hemin. DTNB titration was performed to determine whether the thiol group of Cys-115 is located on the protein surface (Fig. 2B). Seven SH groups were modified by DTNB for ΔNh hcArgRS and six thiol groups were titrated by DTNB for ΔNh hcArgRS-C115A, suggesting that Cys-115 is located at the protein surface under these experimental conditions. Generally hemin interacts with the surface thiol group of a protein, so the thiol group of Cys-115 of hcArgRS coordinates with the iron in the hemin center.

Available crystal structures of ArgRSs from *S. cerevisiae*, *T. thermophilus* and *P. horikoshii* show that ArgRS is a structurally conserved protein (4-6). According to sequence alignment and conservation analysis, the Cys-115 of hcArgRS is located in the Add1 domain, the most characteristic domain in the ArgRS structure. As shown in Fig. 3, the Add1 domain of yArgRS is involved in tRNA<sup>Arg</sup> recognition by interacting with the D-loop region of tRNA<sup>Arg</sup>. It has been shown that the Add1 domain of mammalian ArgRS also plays an essential role in anchoring tRNA and participating in tRNA-induced amino acid activation (8). Sequence alignments confirm that Cys-115 is unique to human and other higher eukaryotic species (supplemental Fig. S1A). Other ArgRSs lacking the cysteine, for example ArgRSs from *B. stearothermophilus*, *E. coli* and *S. cerevisiae* didn’t show a shift of the Soret band of hemin (supplemental Fig. S1B).

The Add1 domain of ΔNh hcArgRS containing N-terminal 110 amino acid residues was isolated and purified using the GST fusion technique. The purified polypeptide was 90% pure and had a molecular mass of 12.5 kDa (Fig. 4A). The Soret band of hemin was shifted to 420 nm in the presence of the isolated Add1 domain, further confirming that Cys-115 located within the Add1 domain is a specific axial
ligand for hemin binding. (Fig. 4B). Titration of the absorption change at 420 nm by adding ΔNhcArgRS to a limiting amount of hemin indicated a dissociation constant of 8.6±1.7 μM for binding of hemin to the axial ligand of the enzyme (Fig. 5).

**Hemin inhibits the catalytic activity of hcArgRS** — Hemin inhibited the aminoacylation activity of ΔNhcArgRS in a dose-dependent manner (Fig. 6A). The 50% inhibitory concentration of hemin under the experimental conditions was estimated as lower than 5 μM. The ATP-PPi exchange assay showed that arginine activation was also inhibited in the presence of hemin (Fig. 6B), suggesting that the inhibitory effect of hemin on the catalytic activity of the enzyme results from blocking amino acid activation. The same effect of hemin on the catalytic activity of full-length ArgRS was also observed (data not shown). In the aminoacylation reaction, the kinetic parameters of ΔNhcArgRS for various substrates in the absence or presence of hemin were measured and are summarized in Table 2. The apparent $K_m$ for tRNA, arginine and ATP remained almost unchanged in the presence of hemin while the $k_{cat}$ values for all three substrates all decreased substantially, suggesting that hemin was a non-competitive inhibitor. The inhibitor constant ($K_i$) for the non-competitive inhibition was 2 μM. The effect of hemin on other ArgRSs was also determined. The activity of prokaryotic ArgRSs from *E. coli* and *B. stearothermophilus* was not sensitive to inhibition by hemin (Fig. 7 and supplemental Fig. S2). However, the aminoacylation activity of yArgRS was inhibited (supplemental Fig. S2). It seems that eukaryotic ArgRSs are more sensitive to hemin than prokaryotic ArgRSs.

However, the aminoacylation activity of ΔNhcArgRS-C115A was also inhibited by hemin (Fig. 8A), suggesting that hemin binding to Cys-115 is not involved in inhibition of the catalytic activity of the enzyme. A direct chemiluminescent assay demonstrated that hemin was still bound to ΔNhcArgRS-C115A(Fig. 8B), suggesting that Cys-115 wasn’t the only binding site and that other interactions existed, which might contribute to the inhibition of the catalytic activity.

High concentrations of heme/hemin can cause oxidative damage to cellular macromolecules, which accounts for inhibition of the aminoacylation activity of GluRS (28). In our case, the inhibitory effect of hemin on ΔNhcArgRS was not altered in the presence of NADPH or NADP$^+$ (supplemental Fig. S3). Similarly, catalase could not reverse the decrease of activity induced by hemin (data not shown). The secondary structure of ΔNhcArgRS wasn’t noticeably altered in the presence of hemin (supplemental Fig. S4), suggesting that the inhibition of activity was not due to structural changes of the protein. Some porphyrin compounds can bind to some DNA or RNA molecules and disrupt their structures (37, 38). In particular, porphyrins can bind to the D-loop/T-loop of *E. coli* tRNAval and *E. coli* pre-tRNA$^{Gly}$ (39). UV absorbance melting curves of *E. coli* tRNA$^{Arg}$ suggested hemin didn’t change the melting profile (supplemental Fig. S5). Gel filtration chromatography was also used to investigate the effect of hemin on the isolated Add1 domain. One major peak was observed in the absence of hemin; when hemin was added, another smaller peak emerged (Fig. 9A). SDS-PAGE analysis showed that the major peak of the Add1 domain corresponded to a monomer (Fig. 9B) while the additional small peak in
presence of hemin corresponded to an SDS-resistant dimer (Fig. 9C). These data suggested that hemin induced oligomerization of the isolated Add1 domain. In the gel filtration assay reference proteins indicated that the oligomer ran as a tetramer. Similarly, oligomerization of ΔNhcArgRS occurred in the presence of hemin (Fig. 10). Aminoacylation activity of the oligomeric fraction was dramatically reduced relative to the monomeric fraction, suggesting that oligomerization induced by hemin inactivated the enzyme (Fig. 11). Oligomerization of ΔNhcArgRS-C115A was also induced by hemin (data not shown), which explains the inhibition of the activity of ΔNhcArgRS-C115A in the presence of hemin.

Oligomerization of ΔNhcArgRS through the Add1 domain (not Cys-115) might interfere with recognition of tRNAArg by this domain and inhibit arginine activation and thus eliminating aminoacylation activity.

DISCUSSION

ArgRS participates in both protein synthesis and protein degradation, playing significant roles in the protein quality control system of mammalian cells. Therefore, it is necessary to identify regulatory factors of the enzyme. Here we investigated the effects of hemin on hArgRS and found that hemin binds to the enzyme and inhibits its activity in vitro.

By investigating the spectral changes of mutant proteins in the absence and presence of hemin, we identified Cys-115 as an axial ligand for hemin binding, which is located in the unique Add1 domain of ArgRS, a domain missing in other class I aaRS (4). However, our data demonstrated that ΔNhcArgRS-C115A still interacts with hemin probably through other sites. Hemin-induced oligomerization of ΔNhcArgRS and ΔNhcArgRS-C115A and inhibited their activities, indicating that the presence of Cys-115 is not required for inhibition of enzymatic activity. Consistent with this interpretation is yArgRS, which is inhibited by hemin even though it lacks the homologous cysteine. Soret band shift is unique to Cys-115 of hArgRS, indicating that the cysteine is the single axial ligand of hemin binding to the enzyme. Cys115 appeared in higher eukaryotic ArgRSs during evolution and may play a biological role in the response to a more complicated intracellular environment. However, the specific role of the binding of hemin to Cys-115 remains to be explored. For many heme-containing proteins, the heme moiety facilitates the binding of diatomic gases, such as NO, CO and O2 (20). Soluble guanylate cyclase (sGC) is a heme sensor protein that selectively binds NO at the heme iron, triggering the enzyme to convert GTP to cGMP (35). Heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase (HRI) regulates protein synthesis in red blood cells and its kinase activity is strongly inhibited by hemin (22, 23). HRI contains two distinct heme-binding sites: one site located in the kinase insertion domain seems to bind heme dynamically and regulates HRI kinase activity; the other binding site is located in the N-terminal domain. It was shown that NO can activate the kinase activity of HRI by binding to the heme iron in the N-terminal heme-binding domain (40). The “N-end rule pathway” is also a sensor of nitric oxide and oxygen (41, 42). Therefore, it will be interesting to determine whether the hemin bound to Cys-115 of human ArgRS can bind diatomic gases.

Although the specific binding of hemin to the Cys-115 of human ArgRS is not
responsible for the inhibition of catalytic activity, the enzyme is still sensitive in response to the environmental hemin concentration with a 50% inhibitory concentration of less than 5 μM. The Add1 domain plays a crucial role in recognizing the D-loop region of tRNA^{Arg}. Oligomerization of the Add1 domain induced by hemin may damage the interaction between the domain and tRNA^{Arg}, which is a prerequisite for amino acid activation. Without correct recognition of tRNA^{Arg} by the enzyme, the aminoacylation of ArgRS is inhibited followed by the blockage of amino acid activation. That is to say, the monomer is the active form while the oligomer is in an inactive form. In our study, the changes of kinetic constants in the absence and presence of hemin can be explained by this model. The reaction mixture contains both oligomers and monomers in the presence of a low concentration of hemin. The monomers are active while the oligomers are inactive. The effective concentration of the active enzyme (monomer) is decreased, which accounts for the obvious decline of $V_{\text{max}}$ and $k_{\text{cat}}$. The $K_m$ value is independent of the enzyme concentration, and therefore $K_m$ remained unaltered.

We have identified that hemin can bind to both forms of human ArgRS and inhibit their enzymatic activities in vitro. Whether hemin affects both enzymes in vivo remains unknown. If this is the case, it will need to be determined whether hemin affects both forms of ArgRS simultaneously. The full-length hcArgRS is involved in the formation of MSC via its N-terminal domain. HcArgRS interacts with several proteins in the complex (11, 12). It’s likely that the tight associations of hcArgRS with other proteins in MSC protect the enzyme from attack by hemin. The free form of human ArgRS is more likely to be the effector of hemin, which is hypothesized to participate in the “N-end rule” pathway.

The “N-end rule pathway” is a well-established ubiquitin-dependent proteolysis pathway (43). The pathway is tRNA-dependent, arising from the involvement of arginyl-tRNA synthetase, which provides Arg-tRNA^{Arg} as the substrate to ATE1. Hemin has been shown to specifically inhibit the ATP and dependent ubiquitin-dependent proteolytic system of rabbit reticulocytes (44, 45). Recently, the “N-end rule pathway” was shown to act as a sensor of hemin. Hemin inhibits the activity of ATE1 and blocks the function of E3 ubiquitin ligase (17). ArgRS was used in the arginylation assay of ATE1 to produce Arg-tRNA^{Arg} as a substrate. Hu et al. reported that ArgRS is not significantly affected by hemin (17). In their arginylation assay system, E. coli ArgRS was used. Our data showed that prokaryotic ArgRSs aren’t sensitive to hemin, which is consistent with their result. However, we have demonstrated that eukaryotic ArgRSs are very sensitive to hemin in vitro. The “N-end rule” pathway only functions in eukaryotic species and therefore it is likely that human ArgRS is regulated by hemin in the degradation pathway.

Oligomerization of proteins induced by hemin has been observed for other proteins. Neuronal nitric-oxide synthase (nNOS) is a homodimer with heme as one of the prosthetic groups. It was reported that heme is the sole prosthetic group controlling the quaternary structure of nNOS by promoting subunit dimerization (46). The RNA-binding protein DGCR8 is essential for the processing of microRNA and association with heme promotes oligomerization of DGCR8, allowing it to trigger pri-miRNA cleavage (28). Oligomerization induced by
hemin provides these proteins with active functions. It's known that novel functions beyond aminoacylation have been observed for many aaRSs (47, 48). It remains to be determined whether the oligomers of hcArgRS induced by hemin exhibit other novel functions. In addition, hemin usually induces the ubiquitin-dependent degradation of some hemin-sensor proteins in vivo (17, 24, 25) and oligomerized proteins tend to be easily recognized by E3 ubiquitin ligase (49). It remains to be determined whether oligomerized hcArgRS is susceptible to ubiquitin-dependent degradation. In this study, we have demonstrated that hemin acts as an inhibitor of hcArgRS species in vitro, which has prompted us to explore the physiological effects of hemin on hcArgRS in vivo. This investigation is currently underway.

REFERENCES

FOOTNOTES
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The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; ATE1, arginyl-tRNA transferase; CL, chemiluminescent; GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-l-tRNA synthetase; hcArgRS, human cytoplasmic ArgRS; ΔNhcArgRS, N-terminal 72-amino acid deletion mutant of hcArgRS; hcleuRS, human cytoplasmic leucyl--tRNA synthetase; MSC, multiple-synthetase complex TrpRS, tryptophanyl-tRNA synthetase; WT, wide type; yArgRS, S. cerevisiae ArgRS.
FIGURE LEGENDS

Figure 1: Binding of hemin to purified human cytoplasmic ArgRS.
(A) ΔNhcArgRS was incubated with hemin first (1:1) and then gel filtration was performed to remove unbound hemin. The fractions containing ΔNhcArgRS after gel filtration were collected and concentrated using an Amicon Ultra centrifugal filter 30 kDa (right). The left is a control without hemin. (B) Soret band shift monitored by UV absorption. Black line, 50 μM hemin alone; green line, 50 μM ΔNhcArgRS alone; red line, 50 μM ΔNhcArgRS bound with hemin (unbound hemin removed). (C) Direct CL imaging to detect the binding of hemin to ΔNhcArgRS. (D) Both hcArgRS and ΔNhcArgRS were pulled down by hemin-agarose in vitro.

Figure 2: Cys-115 is an axial ligand for hemin binding.
(A) Absorption spectral changes of hemin in the presence of ΔNhcArgRS and ΔNhcArgRS-C115A. The molar ratio of hemin to protein was 1:1. (B) Titration of cysteine residues in ΔNhcArgRS (■) and ΔNhcArgRS-C115A (○) by monitor absorption at 412 nm in a solution containing 100 mM potassium phosphate buffer (pH 7.5), 10 μM enzyme and 2 mM DTNB.

Figure 3: Crystal structure of yArgRS showing the residue corresponding to Cys-115 of hcArgRS.
The Add1 domain is colored in yellow, which interacts with the D-loop region of tRNA. The residue in yArgRS corresponding to Cys-115 of hcArgRS is highlighted in magenta (4).

Figure 4: Characterization of the purified isolated Add1 domain of ΔNhcArgRS.
(A) SDS-PAGE showing the purity of the isolated Add1 domain of ΔNhcArgRS. Lane 1, Protein standard markers; lane 2, isolated Add1 domain purified by GST fusion method. The isolated Add1 domain contains 110 amino acids from the N-terminus of ΔNhcArgRS, with a molecular mass 12.5 kDa. (B) Spectral change of the Soret band of hemin when complexed with the Add1 domain of ΔNhcArgRS. Solid line, 50 μM hemin alone; dashed line, 50 μM isolated Add1 domain bound with hemin (unbound hemin removed).

Figure 5: Spectrophotometric determination of the binding affinity of hemin to ΔNhcArgRS.
Hemin (50 μM) was incubated with ΔNhcArgRS ranging from 0 μM to 90 μM and the absorption at 420 nm of the mixture was measured. The data was fit using a one site–specific binding model in GraphPad Prism 5.0 software. The calculated $K_d = 8.6\pm1.7$ μM.

Figure 6: Inhibition of the catalytic activity of ΔNhcArgRS by hemin binding.
(A) The aminoacylation assay of ΔNhcArgRS was initiated by adding ATP after 30-min incubation of enzyme (3 nM) and hemin at different concentrations on ice. (B) The ATP-PPi exchange reaction in the absence (−) or presence (+) of 10μM hemin after 30 min incubation.
3nM ΔNhcArgRS and 20 μM *E. coli* tRNA\(^{\text{Arg}}\) (ACG) were used for both reactions.

**Figure 7: Comparison of the aminoacylation activity of *E. coli* ArgRS and ΔNhcArgRS in the presence of hemin.**

The aminoacylation assay of *E. coli* ArgRS and ΔNhcArgRS was initiated separately by adding ATP after 30-min incubation of enzyme (3 nM) and hemin at different concentrations on ice.

**Figure 8: Inhibition of the aminoacylation activity of ΔNhcArgRS-C115A induced by hemin binding.**

(A) The aminoacylation assay of WT ΔNhcArgRS (■) and ΔNhcArgRS-C115A (○) was carried out in the presence of hemin under the same condition described as before. (B) Direct CL imaging to detect the binding of hemin to ΔNhcArgRS-C115A.

**Figure 9: Oligomerization of the isolated Add1 domain of ΔNhcArgRS induced by hemin.**

(A) Gel filtration of the isolated Add1 domain in the absence and presence of hemin. The isolated Add1 domain was preincubated in the absence (black line) or presence (red line) of hemin (1:1) for 30 min on ice in the dark and then loaded onto a Superdex-75 column. Small peaks (*) indicate contaminants. SDS-PAGE stained by Coomassie blue was used to examine fractions obtained in the absence (B) or and presence of hemin (C). Dimers of the isolated Add1 domain are highlighted by a dashed rectangle in (C).

**Figure 10: Oligomerization of ΔNhcArgRS in the presence of hemin.**

ΔNhcArgRS with (red line) or without (black line) hemin was incubated for 30 min on ice in the dark and then loaded onto a Superose-12 column.

**Figure 11: Comparison of the aminoacylation activity of oligomeric and monomeric ΔNhcArgRS induced by hemin.**

After ΔNhcArgRS was treated with hemin on ice, gel filtration (Superose-12) was used to recover the oligomeric (○) monomeric (■) fractions (10 nM each).
Table 1: Mapping the axial ligand of hemin binding by visible absorption of Soret band

<table>
<thead>
<tr>
<th>Cys mutants</th>
<th>Soret band at 420 nm</th>
<th>His mutants</th>
<th>Soret band at 420 nm</th>
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<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>Wild type</td>
<td>+</td>
</tr>
<tr>
<td>C8A</td>
<td>+</td>
<td>H 56A</td>
<td>+</td>
</tr>
<tr>
<td>C32A/C34A</td>
<td>+</td>
<td>H144A</td>
<td>+</td>
</tr>
<tr>
<td>C115A</td>
<td>−</td>
<td>H165A</td>
<td>+</td>
</tr>
<tr>
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<td>H208A/H211A</td>
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<td>H237A</td>
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<td>+</td>
<td>H568A</td>
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<tr>
<td>C615A/C617A</td>
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<td>H595A</td>
<td>+</td>
</tr>
<tr>
<td>C638A</td>
<td>+</td>
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+: Presence of Soret band at 420 nm
−: Absence of Soret band at 420 nm.
Table 2: Kinetic constants of ΔNhcArgRS in the arginylation reaction at 37°C

<table>
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<th>$K_m$ (μM)</th>
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<th>$k_{cat}/K_m$ (s⁻¹ μM⁻¹)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$k_{cat}/K_m$ (s⁻¹ μM⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>E. coli tRNA$^{Arg}$</td>
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<td>8.1±0.8</td>
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<tr>
<td>Arginine</td>
<td>6.5±0.3</td>
<td>17.9±0.8</td>
<td>2.8</td>
<td>7.8±0.7</td>
<td>3.8±0.5</td>
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<tr>
<td>ATP</td>
<td>501±23</td>
<td>15.5±0.2</td>
<td>0.03</td>
<td>529±14</td>
<td>3.4±0.1</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 4
Figure 5

\( \Delta A_{420} \)

Protein concentration (\( \mu \text{M} \))
Figure 6
Figure 7
Figure 8
Figure 9
Figure 11
Hemin binds to human cytoplasmic arginyltRNA synthetase and inhibits its catalytic activity
Fang Yang, Xian Xia, Hui-Yan Lei and En-Duo Wang

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