Human Tryptophanyl-tRNA Synthetase Binds with Heme To Enhance Its Aminoacylation Activity†

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ABSTRACT: Mammalian tryptophanyl-tRNA synthetases (TrpRSs) are Zn^{2+}-binding proteins that catalyze the aminoacylation of $tRNA^{Trp}$. The cellular expression level of human TrpRS is highly upregulated by interferon-γ (IFN-γ). In this study, a heme biosynthesis inhibitor, succinylacetone (SA), was found to inhibit cellular TrpRS activity in IFN-γ-activated cells without affecting TrpRS protein expression. In addition, supplementation of lysates from the SA-treated cells with hemin fully restored TrpRS activity to control levels. Biochemical analyses using purified TrpRS demonstrated that heme can interact strongly with Zn^{2+}-depleted human full-length TrpRS with a stoichiometric heme:protein ratio of 1:1 to enhance the aminoacylation activity significantly. In contrast, the Zn^{2+}-bound form of TrpRS did not bind heme. Further studies using site-directed mutagenesis clarified that the Zn^{2+}-unbound human H130R mutant cannot bind heme. These results provide the first evidence of the involvement of heme in regulation of TrpRS aminoacylation activity. The regulation mechanism and its physiological roles are discussed.

Aminoacyl-tRNA synthetases are key enzymes in protein biosynthesis that catalyze the aminoacylation of their cognate tRNAs (*1*). Human tryptophanyl-tRNA synthetase (TrpRS)[1] and tyrosyl-tRNA synthetase (TyrRS) share closely similar tertiary structures, and both participate in protein synthesis and angiogenic signal transduction pathways (*2*−*8*).

Mammalian TrpRSs have an appended domain at the N-terminus, which is absent from lower eukaryotic and prokaryotic TrpRSs (*9*). It has been reported that bovine TrpRS is a Zn^{2+}-binding protein and that the Zn^{2+} is indispensable for aminoacylation by this protein (*10*). In human cells, TrpRS exists in two forms: a major form that is the full-length protein (amino acids 1−471) and a truncated form (mini TrpRS) (amino acids 48−471) in which most of the N-terminal extension (amino acids 1−47) is absent because of alternative splicing of the pre-mRNA (*9*, *11*−*13*). Proteolytic digestion of human full-length TrpRS with the extracellular protease leukocyte elastase produces two additional N-terminally truncated forms: T1 TrpRS (amino acids 71−471) and T2 TrpRS (amino acids 94−471) (*4*, *6*). Human full-length, mini, and T1 TrpRSs can catalyze the aminoacylation of $tRNA^{Trp}$, but T2 TrpRS does not (*4*, *6*, *14*). On the other hand, mini, T1, and T2 TrpRSs were shown to have angiostatic activity, whereas the full-length enzyme was inactive (*4*, *6*).

In contrast, human TyrRS is also processed by elastase into two protein fragments; however, the resulting N-terminal catalytic fragment, mini TyrRS, functions as an angiogenic factor, whereas the full-length protein is not active for cell signaling (*2*, *3*, *5*). The angiogenic activity of mini TyrRS opposes the angiostatic activities of mini, T1, and T2 TrpRSs, suggesting that the two factors could regulate angiogenesis in a coordinated manner.

The cellular expression level of human TrpRS is highly upregulated by the addition of interferon-γ (IFN-γ) (*13*, *15*, *16*). For example, previous experiments using two-dimensional gel electrophoresis showed that human TrpRS is upregulated approximately 22-fold after a 24 h IFN-γ treatment (*13*). A 42-fold increase in mRNA levels of human TrpRS was also observed (*16*). TrpRS is the only aminoacyl-tRNA synthetase whose expression is induced by IFN-γ. Another representative IFN-γ-inducible enzyme is human indoleamine 2,3-dioxygenase (IDO) (*17*, *18*). IDO is responsible for tryptophan (Trp) degradation, and it catalyzes the first as well as the rate-limiting step in the major pathway of human Trp metabolism, the kynurenine pathway (*19*). Human IDO is strongly expressed in macrophages and dendritic cells, and it is responsible for downmodulating T-cell activation and proliferation (*20*). Several lines of evidence, including studies of mammalian pregnancy and tumor resistance, support the concept that IDO expression can suppress T-cell responses and promote tolerance (*21*−*24*). It has been suggested that IDO-expressing cells avoid self-destruction resulting from Trp depletion by a concomitant increase in the level of expression of TrpRS (*25*). Overexpression of TrpRS in the cell could facilitate production of a pool of Trp-tRNA, thus providing a reservoir for Trp that would be protected from degradation by IDO and therefore available for protein synthesis.

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[1] Abbreviations: TrpRS, tryptophanyl-tRNA synthetase; IFN, interferon; hemin, ferriprotoporphyrin IX chloride; SA, succinylacetone.

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IDO has been reported to be a cytosolic heme protein (*26*, *27*). Heme is an iron protoporphyrin IX complex that serves as a prosthetic group for many proteins (*28*, *29*). Addition of ferriprotoporphyrin IX chloride (hemin) or the heme precursor, δ-aminolevulinic acid, to IFN-γ-activated cells stimulates IDO-mediated Trp metabolism, whereas inhibitors of heme synthesis block the formation of the heme−IDO complex and abolish the corresponding enzymatic activity (*30*). Furthermore, addition of hemin to cell lysates increases IDO activity 2-fold, indicating that approximately half of the IDO in these cells is present as the heme-unbound form (*30*). Therefore, the concentration of free heme within the cell might modulate IDO activity.

In this study, I have investigated whether heme regulates the aminoacylation activity of human TrpRS in IFN-γ-activated cells. I next investigated whether purified human TrpRS acts as a heme-binding protein; heme was found to bind to human Zn^{2+}-depleted full-length TrpRS and enhance its aminoacylation activity. Further insight into the heme binding properties of human TrpRS was gained by using site-directed mutagenesis.

EXPERIMENTAL PROCEDURES

Chemicals. Brewer's yeast tRNA and human IFN-γ were purchased from Roche Diagnostics (Basel, Switzerland). Succinylacetone (SA) was obtained from Sigma. Human $tRNA^{Trp}$ was synthesized using synthetic oligonucleotides and was purified by polyacrylamide gel electrophoresis (Dharmacon Research, Lafayette, CO). Iron sulfate (Wako, Osaka, Japan), hemin (Sigma, St. Louis, MO), protoporphyrin IX (MP Biomedicals, Eschwege, Germany), and its Zn^{2+} derivative (Sigma) were used as supplied without further purification.

Cell Culture. A HeLa cell line (RCB0007) was obtained from the RIKEN Cell Bank (Ibaraki, Japan). The cells were maintained in culture in Dulbecco's modified Eagle's medium (DMEM; Invitrogen-Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS) (Invitrogen-Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin, and 300 μg/mL glutamine in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed twice weekly, and the cultures were split at a 1:6 ratio once every week.

HeLa cells were incubated in the absence or presence of 250 μM SA for 30 min before being treated with IFN-γ (500 units/mL), after which they were cultured for an additional 24 h. The cells were lysed via addition of phosphate-buffered saline (PBS) supplemented with an EDTA-free complete protease inhibitor cocktail (Roche Diagnostics), and then they were subjected to three cycles of freezing and thawing. Cell lysates were then incubated on ice for 2 h in the absence or presence of 10 μM hemin, after which aminoacylation activities were assessed.

Aminoacylation Assays. Aminoacylation activities were assayed at ambient temperature (~20 °C) in buffer containing the following: 150 mM Tris-HCl (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 4 mM ATP, and 38 μM tryptophan (Trp) [5 μM [^{3}H]Trp (Amersham Biosciences, Piscataway, NJ)] or 20 μM tyrosine (Tyr) [2 μM [^{3}H]Tyr (Amersham Biosciences)]. The reactions were initiated by adding the cell lysates or purified samples (200 nM) to a buffer that included Brewer's yeast tRNA (5−500 μM) or human $tRNA^{Trp}$ (5 μM). Reaction samples were removed and spotted onto Whatman 3MM paper filters. After 1 min, the filter disks were added to cold 5% trichloroacetic acid that included 2 mM Trp or Tyr. The filters were washed three times in cold 5% trichloroacetic acid and 2 mM Trp or Tyr, twice in ethanol, and once in ether. The washed filters were then subjected to scintillation counting. Prior to the assays, the tRNA substrate was heated at 70 °C for 2 min and re-annealed at ambient temperature for 30 min.

Western Blot Analysis. Extracts of soluble proteins were prepared. Protein samples were resolved by electrophoresis on 10% polyacrylamide−SDS gels. Proteins were electroblotted onto Sequi-Blot PVDF membranes (Bio-Rad, Hercules, CA) for 1 h. The membranes were blocked with PBS and 5% skim milk (Wako Chemicals, Osaka, Japan) and incubated at 4 °C for 1 h with a primary antibody (rabbit polyclonal antibodies against human TrpRS) in PBS. After being washed three times at 4 °C with PBS containing 0.05% Tween 20, the membranes were incubated with the horseradish peroxidase (HRP)-linked $F(ab')_2$ fragment of donkey anti-rabbit Ig (Amersham Biosciences, Buckinghamshire, England) at 4 °C for 1 h. The membrane was again washed three times with the buffer at 4 °C, and the proteins were visualized via ECL Western blotting detection reagents (Amersham Biosciences).

Preparation of Proteins. cDNA fragments of human full-length TrpRS (amino acids 1−471), human mini TrpRS (amino acids 48−471), human T1 TrpRS (amino acids 71−471), human T2 TrpRS (amino acids 94−471), mouse full-length TrpRS (amino acids 1−475), zebrafish full-length TrpRS (amino acids 1−463), *Arabidopsis* full-length TrpRS (amino acids 1−402), human full-length TyrRS (amino acids 1−528), and human mini TyrRS (amino acids 1−364) were separately cloned into the pET20b (Novagen, Madison, WI) expression vector to give a gene product with a C-terminal tag of six histidine residues (six-His tag). The final constructs were sequenced with a RISA-384 multicapillary automated DNA sequencer (Shimadzu, Kyoto, Japan) to ensure no mistakes had been introduced during amplification. The expression constructs were introduced into *Escherichia coli* BL21(DE3) (Novagen). The cells were grown at 37 °C to an OD_{600} of approximately 0.8, and then heterologous gene expression was induced by the addition of 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). Cells were harvested 4 h after induction. Using the procedures described by Novagen, the recombinant six-His-tagged proteins were purified on a nickel affinity column (His·Bind resin; Novagen) from the supernatant of lysed cells.

To investigate the effect of the C-terminal six-His tag, recombinant human full-length TrpRS lacking the tag was prepared. Recombinant nontagged TrpRS was purified by conventional column chromatography. Initially, the soluble cell extract was loaded onto a DEAE-Sepharose anion-exchange column equilibrated with 20 mM Tris-HCl (pH 8.0). Recombinant TrpRS was eluted from the column with a linear gradient from 0 to 500 mM NaCl in buffer and then further purified by gel filtration chromatography (Sephacryl S-200 HR column).

After purification, recombinant TrpRS proteins were dialyzed against 50 mM Tris-HCl (pH 7.5) containing 10 mM EDTA for 5 days. EDTA was subsequently removed from the solution by further dialysis. Protein concentrations were determined by the Bradford assay using bovine serum albumin (BSA) (Sigma) as the standard (Bio-Rad, Hercules, CA). The hemin concentration was determined spectrophotometrically using an extinction coefficient of 170 mM^{-1} cm^{-1} at 403 nm in 80% dimethyl sulfoxide (*31*).

Site-Directed Mutagenesis. A QuikChange site-directed mutagenesis system (Stratagene, La Jolla, CA) was used to introduce substitutions at specific sites. The sequences were confirmed by DNA sequencing using a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences) and a RISA-384 multicapillary automated DNA sequencer (Shimadzu).

Visible Spectra. Electronic absorption spectra of purified proteins were recorded with a UV−visible spectrophotometer (model UV-2450 from Shimadzu or model Lambda from Perkin-Elmer, Boston, MA) at 20 °C. Ferrous TrpRS samples were prepared by first equilibrating TrpRS with N_2 gas and then reducing the heme with sodium dithionite. Titration experiments were performed in cuvettes by adding an aliquot of the hemin stock solution to the sample cuvette containing TrpRS. An identical aliquot of hemin was added to the reference cuvette containing only buffer.

RESULTS

Heme Regulates the Aminoacylation Activity of Human TrpRS in IFN-γ-Activated Cells. As shown in Figure 1A, addition of hemin to cell lysates prepared from IFN-γ-activated HeLa cells increased their TrpRS activity 50%, indicating that cellular heme is limiting for TrpRS activity. To examine the role of intracellular synthesis of protoporphyrin IX in TrpRS activity, SA (which inhibits protoporphyrin IX synthesis) was used. Cell lysates obtained from SA-treated cells exhibited TrpRS activity that was decreased to 35% of the activity of corresponding control lysates (Figure 1A), whereas SA had no marked effect on TrpRS protein expression (Figure 1B). Addition of hemin to lysates prepared from SA-treated HeLa cells restored TrpRS activity to control levels (Figure 1A), indicating that SA inhibited TrpRS activity by interfering with the formation of a TrpRS−heme complex. In contrast, human TyrRS activity was not inhibited by the heme synthesis inhibitor SA in IFN-γ-activated HeLa cells (Figure 1C).

Heme Binding Properties of Zn^{2+}-Depleted Human Full-Length TrpRS. It has been reported that Zn^{2+} interacts with bovine TrpRS in a stoichiometric Zn^{2+}:protein ratio of 1:1 (*10*). Recombinant human full-length TrpRS was dialyzed for 5 days to prepare the complete Zn^{2+}-depleted form. Upon addition of hemin to a solution of Zn^{2+}-depleted TrpRS, the visible absorption spectrum of TrpRS exhibited a strong Soret band at 413 nm (Figure 2A). The Soret band corresponding to free heme under the same condition is observed at 393 nm (Figure 2A). The appearance of a Soret band at 413 nm indicates the formation of a complex between TrpRS and heme. Reduction of the heme-bound TrpRS with sodium dithionite resulted in a complex that produced a Soret band at 424 nm and Q-bands at 561 and 532 nm (Figure 2B). The spectral patterns of the TrpRS−heme complexes shown in panels A and B of Figure 2 are typical of the ferric and ferrous low-spin states of the heme, respectively (*32*−*35*). Moreover, these spectra did not change even in the presence of 10 mM EDTA (data not shown).

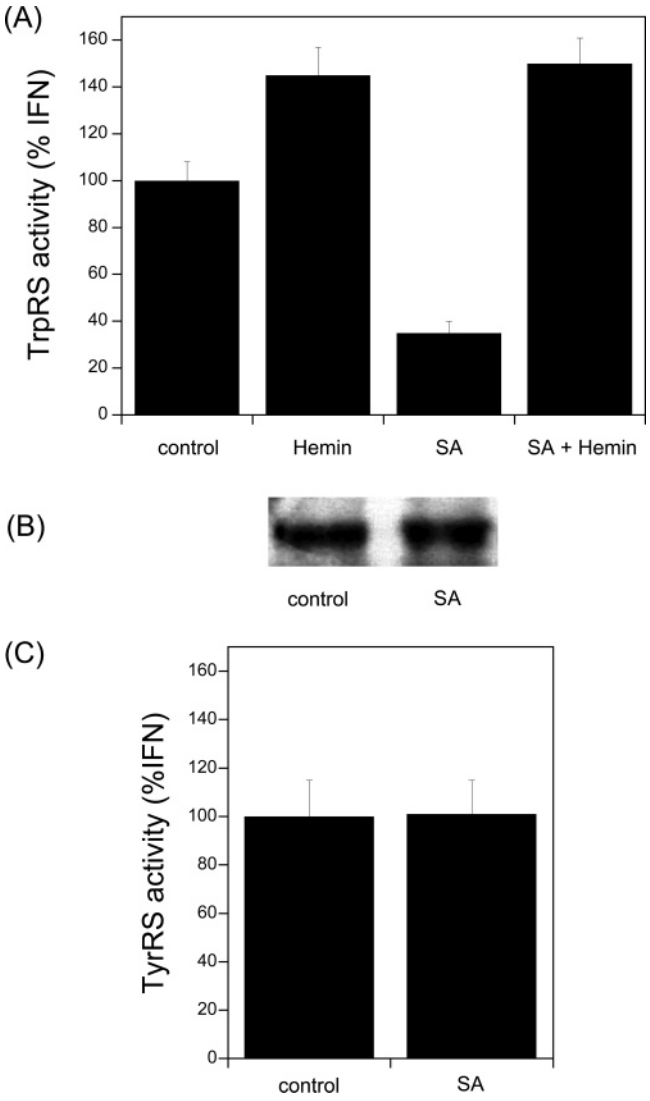

FIGURE 1: Regulation of human TrpRS activity by heme in IFN-γ-activated HeLa cells. (A) Whereas succinylacetone (SA) inhibits TrpRS activity in IFN-γ-activated HeLa cells, hemin restores TrpRS activity when added to cell lysates of SA-treated cells. Cells were incubated in the absence or presence of 250 μM SA for 30 min before being treated with IFN-γ (500 units/mL). They were then cultured for an additional 24 h. Cell lysates were then prepared and incubated in the absence or presence of 10 μM hemin on ice for 2 h, after which TrpRS activity was assessed. TrpRS activities were determined from initial rates. Results are expressed as a percentage of the values obtained using HeLa cells treated with IFN-γ alone, and they represent the mean ± standard error of three independent experiments. The 100% value for TrpRS activity was 6.3 pmol of Trp−tRNA formed per minute per microgram of cell protein. (B) SA does not inhibit TrpRS protein expression. A representative Western blot corresponding to the TrpRS protein data shown in panel A is shown. For the Western blot analyses, protein samples were resolved by electrophoresis on a 10% polyacrylamide−SDS gel and then electroblotted onto a PVDF membrane. The blot was probed with anti-human TrpRS rabbit polyclonal antibodies, followed by a HRP-linked $F(ab')_2$ fragment of anti-rabbit Ig. Proteins were visualized by ECL Western blotting detection reagents. (C) SA does not inhibit TyrRS activitiy in IFN-γ-activated cells. Experimental conditions were the same as those described for panel A. The 100% value for TyrRS activity was 1.3 pmol of Tyr−tRNA formed per minute per microgram of cell protein.

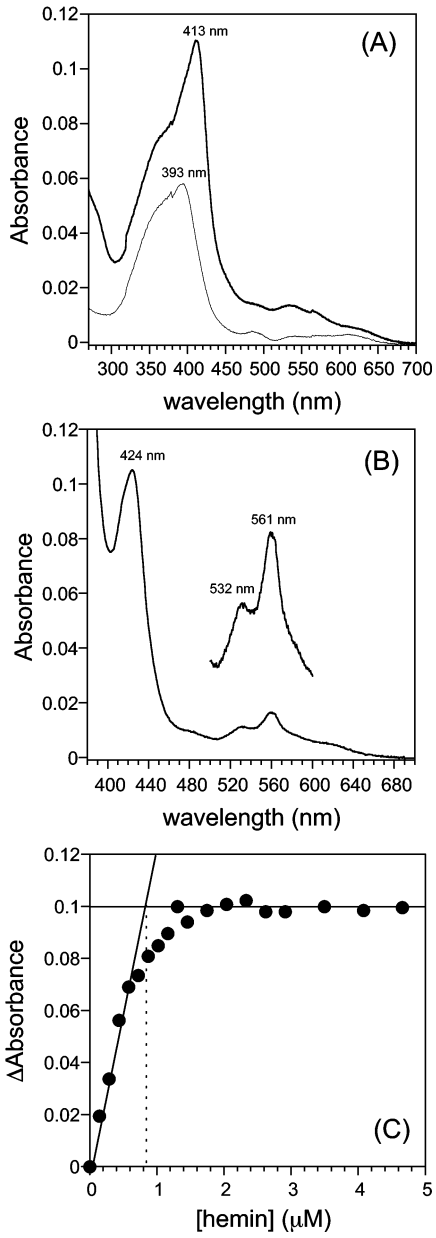

FIGURE 2: Binding of heme to human Zn^{2+}-depleted full-length TrpRS. (A) Electronic absorption spectra of the ferric forms of free heme (thin line) and heme bound to Zn^{2+}-depleted full-length TrpRS (thick line). The concentration of both TrpRS and heme was 1 μM. Spectra were recorded in 20 mM sodium phosphate buffer (pH 7.4) containing 0.1 M NaCl. (B) Electronic absorption spectra of the ferrous form of heme bound to the Zn^{2+}-depleted full-length protein. The Q-bands from 500 to 600 nm are enlarged by a factor of 5 on the perpendicular axis. The concentration of both TrpRS and heme was 1 μM. (C) Spectroscopic titration of Zn^{2+}-depleted full-length TrpRS with hemin. Titration experiments were performed in cuvettes by adding aliquots of the hemin stock solution to the sample cuvette containing 1 μM TrpRS. An identical aliquot of hemin was added to the reference cuvette containing only buffer. The difference absorption spectral changes at 413 nm were measured.

To investigate possible effects of the C-terminal six-His tag on the heme binding properties of human TrpRS, the Zn^{2+}-depleted form of nontagged human TrpRS was prepared. It bound heme, and its Soret band was observed at 413 nm; this band was shifted to 424 nm upon addition of sodium dithionite. Moreover, the Q-band at 561 nm was detected in the presence of sodium dithionite. These results

Table 1: Absorption Maxima of the Soret Band of Heme in the Presence of Zn^{2+}-Depleted TrpRS or TyrRS at a Protein:Heme Ratio of 1:0.7

sample	Soret peak (nm)
human full-length TrpRS (amino acids 1−471)	413
human full-length TrpRS (amino acids 1−471) (nontagged)	413
human mini TrpRS (amino acids 48−471)	413
human T1 TrpRS (amino acids 71−471)	413
human T2 TrpRS (amino acids 94−471)	413
human full-length H73A TrpRS	413
human full-length H129A TrpRS	*a*
human full-length H130A TrpRS	412
human full-length H140A TrpRS	413
human full-length H170A TrpRS	413
human full-length H173A TrpRS	413
human full-length H257A TrpRS	413
human full-length H336A TrpRS	*a*
human full-length H375A TrpRS	413
human full-length H387A TrpRS	413
human full-length H445A TrpRS	*a*
mouse full-length TrpRS	389
zebrafish full-length TrpRS	390
Arabidopsis full-length TrpRS	413
human full-length H130R TrpRS	392
human full-length TyrRS	390
human mini TyrRS	392
free heme	393

[a] Not determined.

using human nontagged TrpRS were identical to those obtained with human TrpRS having a six-His tag, suggesting that the tag has no effect on the heme binding properties of the protein.

The complex formed between heme and TrpRS was quantified from the spectral changes at 413 nm that were observed in the visible absorption spectra of the mixture. As shown in Figure 2C, the curve resulting from titration of hemin with TrpRS showed that hemin binds to TrpRS in a saturable manner and in a stoichiometric heme:protein ratio of 1:1. The dissociation constant (K_d) of the TrpRS−heme complex was determined to be less than 1 μM.

The visible absorption spectrum of heme in the absence or presence of either human full-length or mini TyrRS was also measured. The Soret band of heme in the presence of human full-length or mini TyrRS was observed at 390 or 392 nm, respectively (Table 1). Since the Soret band corresponding to free heme under the same condition was observed at 393 nm, these results suggest that neither human full-length TyrRS nor mini TyrRS can bind heme.

Influence of Heme Binding on TrpRS Aminoacylation Activity. Aminoacyl-tRNA synthetases catalyze the first step of protein synthesis, which consists of the aminoacylation of tRNAs (*1*). Previous studies have shown that human TrpRS can aminoacylate yeast $tRNA^{Trp}$ both in vitro and in vivo (*14*, *36*). Initially, the aminoacylation activity of Zn^{2+}-depleted human TrpRSs toward yeast $tRNA^{Trp}$ was investigated in the absence or presence of hemin. As shown in Figure 3A, hemin enhanced the aminoacylation activity of Zn^{2+}-depleted human full-length TrpRS. Although Zn^{2+} protoporphyrin also increased the enzymatic activity, it was less effective than hemin when added at an equivalent concentration (Figure 3B). Fe^{3+} and the heme precursor protoporphyrin IX (i.e., heme without iron) had no effect on the aminoacylation activity of TrpRS (Figure 3B). These

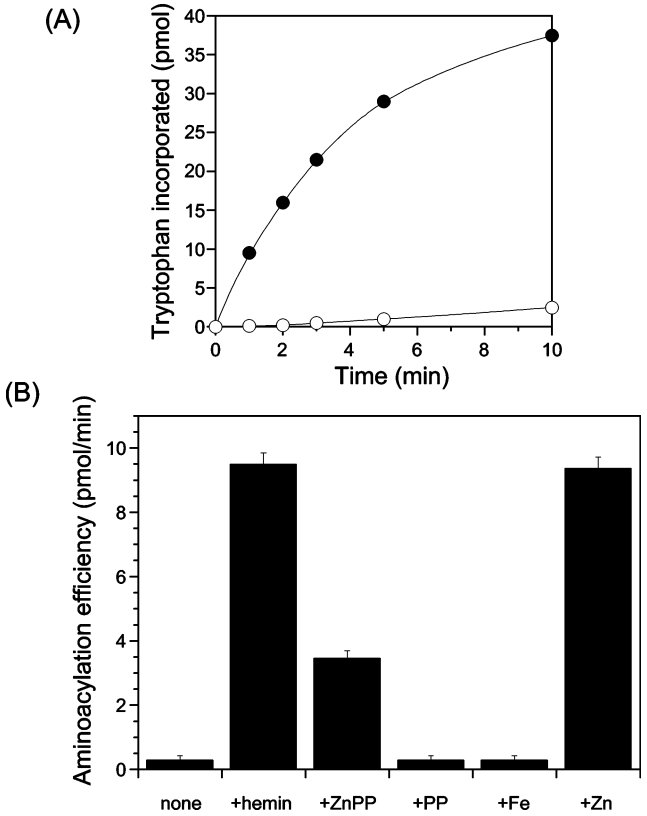

FIGURE 3: Enhancement of the aminoacylation activity of human Zn^{2+}-depleted full-length TrpRS by heme binding. (A) Aminoacylation of $tRNA^{Trp}$ by Zn^{2+}-depleted full-length TrpRS (200 nM) in the absence (○) or presence (●) of hemin (1 μM). Addition of 500 μM yeast tRNA followed. (B) Aminoacylation activity of Zn^{2+}-depleted full-length TrpRS toward yeast $tRNA^{Trp}$ in the absence or presence of hemin (1 μM), Zn^{2+} protoporphyrin IX (ZnPP) (1 μM), protoporphyrin IX (PP) (1 μM), iron (1 μM), or zinc (1 μM). Aminoacylation efficiencies were calculated as picomoles per minute of aminoacylated $tRNA^{Trp}$, which was synthesized during a 1 min incubation. The assays included 200 nM TrpRS and 500 μM yeast tRNA. Values represent the mean ± standard deviation from five experiments.

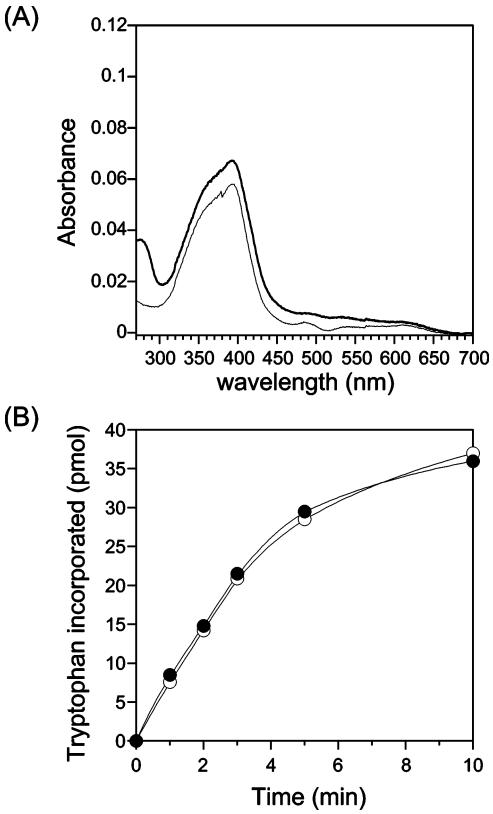

FIGURE 4: Effects of heme on human Zn^{2+}-bound full-length TrpRS. (A) Electronic absorption spectra of hemin in the absence (thin line) or presence of Zn^{2+}-bound full-length TrpRS (thick line). The concentration of both TrpRS and hemin was 1 μM. (B) Aminoacylation of $tRNA^{Trp}$ by Zn^{2+}-bound full-length TrpRS (200 nM) in the absence (○) or presence (●) of hemin (1 μM). The concentration of yeast tRNA was 500 μM.

different characteristics among hemin, Zn^{2+} protoporphyrin, Fe^{3+}, and protoporphyrin are the same as those reported previously for other heme-binding proteins (*37*, *38*). The C-terminal six-His tag does not influence the effect of hemin on the aminoacylation activity of human full-length TrpRS (data not shown). Furthermore, assays for examining the aminoacylation activity using human $tRNA^{Trp}$ gave results that were almost the same as those described above for yeast $tRNA^{Trp}$ (data not shown).

Effect of Zn^{2+} Binding on the Heme Binding Properties and Aminoacylation Activity of TrpRS. It has been reported that Zn^{2+} interacts with bovine TrpRS in a stoichiometric Zn^{2+}:protein ratio of 1:1 and that this enhances the aminoacylation activity of TrpRS (*10*). Competitive binding studies were performed to determine whether Zn^{2+} affects the binding of heme to TrpRS. No significant spectral changes in the Soret region were observed upon addition of hemin to Zn^{2+}-bound human full-length TrpRS (Figure 4A), suggesting that heme does not bind to the Zn^{2+}-bound form of the enzyme. As shown in Figure 4B, the addition of hemin to Zn^{2+}-bound TrpRS had no effect on the aminoacylation activity of the Zn^{2+}-bound form. These results may reflect a conformational change within or around the heme-binding site after Zn^{2+} is bound.

Heme Binding Properties of TrpRS. To delineate the region in human TrpRS that is responsible for binding heme, the heme binding properties of human mini, T1, and T2 TrpRSs were investigated. Zn^{2+}-depleted mini, T1, and T2 TrpRSs bound heme in a stoichiometric heme:protein ratio of 1:1, as did Zn^{2+}-depleted full-length TrpRS (data not shown). As listed in Table 1, the Soret band corresponding to the ferric heme-bound Zn^{2+}-depleted human mini, T1, or T2 TrpRS was identical to that of heme-bound Zn^{2+}-depleted full-length TrpRS. These results indicate that the N-terminal region (amino acids 1−93) of full-length TrpRS is not required to bind heme.

Which amino acid residues are important in the binding of heme to human TrpRS? Since the visible absorption spectra of the ferric and ferrous forms of human TrpRS had shift values that were almost the same as those of human neuroglobin and chimeric globins, in which histidine (His) residues bind with heme (*32*, *33*, *35*), it seemed likely that at least one of the His residues in TrpRS binds the heme iron in a manner analogous to that of these globins. Therefore, site-directed mutagenesis at each His residue in human full-length TrpRS was performed. The visible absorption spectra of H73A, H130A, H140A, H170A, H173A, H257A, H375A, and H387A mutants exhibited a strong Soret band at ~413 nm, as did that of wild-type TrpRS (Table 1), indicating that His73, His130, His140, His170, His173, His257, His375, and His387 are not crucial for heme binding in human TrpRS. In contrast, H129A, H336A, and H445A

	73	129	130	140	170	173	257	336	375	387	445
human	H	H	H	H	H	H	H	H	H	H	H
mouse	*	H	R	H	H	H	H	H	H	H	H
zebrafish	*	H	R	H	H	H	H	H	H	H	H
arabidopsis	*	H	V	H	H	H	C	E	Y	H	H

FIGURE 5: Sequence alignment of human TrpRS and mouse, zebrafish, and arabidopsis TrpRSs. The multiple-sequence alignment was performed with Clustal W followed by manual adjustments. The residues corresponding to His residues in human TrpRS are indicated. Numbers above the sequences are those of human TrpRS. In the mouse, zebrafish, or *Arabidopsis* TrpRS sequence, the residue corresponding to His73 in human TrpRS was not determined due to the low degree of amino acid sequence homology of neighboring residues.

mutants have not been purified, probably due to their low solubility.

To gain further insight into the heme binding properties of human TrpRS, the mouse, zebrafish, and *Arabidopsis* TrpRSs were expressed in *E. coli* and then purified. No red shift in the Soret band of heme was observed in the presence of mouse or zebrafish TrpRS (Table 1), indicating that these proteins cannot bind heme. Sequence alignment of the human, mouse, and zebrafish TrpRSs (Figure 5) shows that both mouse and zebrafish TrpRSs have an arginine (Arg) residue at the position corresponding to His130 of human TrpRS. To investigate the role of this Arg residue, the human H130R mutant was prepared. The H130R mutation compromised the ability of human TrpRS to bind heme. Moreover, *Arabidopsis* TrpRS, in which the residue corresponding to His130 of human TrpRS is valine, can also bind heme (Table 1).

DISCUSSION

Regulation of the Aminoacylation Activity of Human TrpRS by Heme in IFN-γ-Activated Cells. This study establishes that heme synthesis is required for the aminoacylation activity of human TrpRS, but not TyrRS, in IFN-γ-activated cells. The ~1.5-fold increase in human TrpRS activity upon addition of hemin to the IFN-γ-activated cells indicates that a significant portion of TrpRS expressed in these cells is present as the apoprotein, which does not contain Zn^{2+}. Moreover, the in vitro experiments indicated that heme binds to human Zn^{2+}-unbound TrpRS. When human TrpRS is strongly expressed in the presence of IFN-γ, some of the newly produced TrpRS proteins are in the Zn^{2+}-unbound form, and their aminoacylation activities can therefore be regulated by heme.

A database of human tissue and cell lines for the gene expression profile of TrpRSs showed striking overexpression of human TrpRS in three tissues: placenta, lung, and spleen (*39*, *40*). In contrast, significantly different levels of expression of mouse TrpRS were not detected among any of the tissues tested (*39*, *40*). These expression patterns, which differ between human and mouse TrpRSs, might be related evolutionarily to the results presented here which show that mouse TrpRS does not bind heme.

Relationship between Human TrpRS and Oxidative Stress. Human TrpRS was reported to be upregulated during the maturation of human monocytes to macrophages (*41*). Moreover, human TrpRS expression was found to be upregulated during maturation of immature dendritic cells from peripheral blood monocytes (*42*). It also appeared to be a good marker of the final stage of maturation and differentiation of monocyte-derived dendritic cells (*42*). Both macrophages and dendritic cells are known to be antigen-presenting cells. Immune system proteins that mediate immune activation, e.g., human major histocompatibility complex (MHC) class I and class II, β_2-microglobulin, and complement factor B, are all induced by IFN-γ and are all exceptionally enriched in Trp residues, as compared to human proteins in general (*43*).

Immature dendritic cells spontaneously express heme oxygenase-1 (HO-1), whereas the level of HO-1 expression is drastically decreased when human dendritic cell maturation is induced in vitro (*44*). HO-1 is an intracellular enzyme that degrades heme and inhibits immune responses and inflammation in vivo. The induction of HO-1 expression decreases the levels of reactive oxygen species (ROS) and inhibits dendritic cell maturation (*44*). In contrast, inhibition of HO-1 expression during dendritic cell maturation leads to the accumulation of free heme and produces ROS, and oxidative stress induces the maturation of human dendritic cells (*45*). Conversely, antioxidants inhibit DC activation (*46*).

Taken together, these results suggest human TrpRS that is overexpressed during dendritic cell maturation binds to accumulated free heme to enhance its aminoacylation activity. It should also be noted that the concentration of intracellular free Zn^{2+} decreases during dendritic cell maturation (*47*), implying that heme, but not Zn^{2+}, regulates aminoacylation activity under conditions of oxidative stress. Recently, I reported that an oxidized form of human glyceraldehyde-3-phosphate dehydrogenase interacts with TrpRS and stimulates the aminoacylation potential of mini TrpRS (*14*). This result may be another example of oxidative stress-responsive regulation of human TrpRS activity. Further studies will be necessary to clarify the relationship between human TrpRS and oxidative stress.

Possible Regulation Mechanism of Human TrpRS. Previous studies comparing the structures of ligand-free and tryptophanyl-5′-AMP-complexed forms of prokaryotic TrpRS showed that significant repacking occurs between the Rossmann fold domain and the anticodon-binding domain and that domain movement has a significant impact on the active site architecture, especially in the ATP binding site, necessary for the enzymatic activity of TrpRS (*48*, *49*). The "induced-fit" mechanism responsible for TrpRS activity implies that the binding sites for heme and Zn^{2+} might be located at boundaries between the Rossmann fold domain and the anticodon-binding domain.

Bovine TrpRS contains Zn^{2+}, which is required for its enzymatic activity (*10*). The bond between Zn^{2+} and the enzyme is rather labile; indeed, even dialysis in the absence of a chelating agent leads to a significant decrease in Zn^{2+} content (*10*). Previous experiments using chemical modification of bovine TrpRS suggested that exposed His residue(s) and carboxylic group(s) of the enzyme are involved in the Zn^{2+} binding and that the SH groups of cysteine residues are not involved in Zn^{2+} binding (*10*). On the basis of these previous findings and recently published X-ray structural data (*50*), I propose a possible Zn^{2+} binding site consisting of Glu167, Asp221, and His445. As shown in Figure 6, these residues are also located at boundaries between the Rossmann fold domain and the anticodon-binding domain.

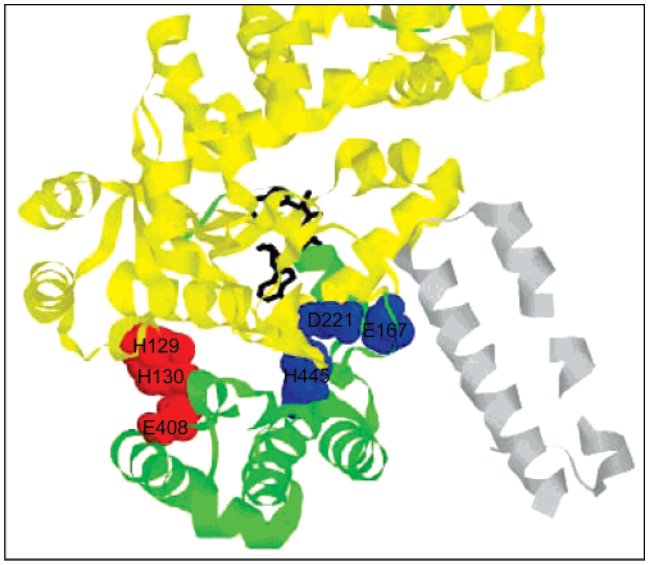

FIGURE 6: Crucial residues for heme or Zn^{2+} binding in human full-length TrpRS. The crystal structure of human full-length TrpRS (Protein Data Bank entry 1R6T) with bound tryptophanyl adenylate (black) shows that TrpRS consists of an N-terminal appended domain (gray), a Rossmann fold catalytic domain [Glu82−Ser353 (yellow)], and an anticodon-binding domain [Asp354−Ala467 (green)]. Glu167, Asp221, and His445 residues, which form a possible Zn^{2+} binding site, are represented by blue space-filling balls. His129, His130, and Glu408 are represented as red space-filling balls. Glu408 (red) may interact with Arg130 (red) in the H130R mutant, thereby inhibiting heme binding. Mutation of His129 (red) or His445 (blue) to Ala would likely cause large structural changes, as H129A and H445A mutants have not been purified.

This study demonstrates that the H130A, but not the H130R, TrpRS mutant binds heme. X-ray structural analyses of human TrpRS suggest that Arg130 may interact with Glu408 in the H130R mutant (Figure 6). Since residues 130 and 408 are located in the Rossmann fold domain and the anticodon-binding domain, respectively, an interaction between Arg130 and Glu408 may block crucial interdomain conformational changes necessary for heme binding.

Since H129A, H336A, and H445A mutants have not been purified, it remains unclear whether the His129, His336, and His445 residues are crucial for heme binding of human TrpRS. However, *Arabidopsis* TrpRS, in which the residue corresponding to His336 of human TrpRS is glutamic acid, can also bind heme. Therefore, His129 and His445 are potentially crucial residues for heme binding in human TrpRS. His129 and His445 are also located at boundaries between the Rossmann fold domain and the anticodon-binding domain (Figure 6).

Conclusion. The results presented in this paper demonstrate that heme binds to Zn^{2+}-depleted human TrpRS, causing an increase in its aminoacylation activity. This is the first example indicating the possible involvement of heme in the regulation of aminoacylation activity. Human TrpRS in IFN-γ-activated cells exists in both a Zn^{2+}-unbound form and a Zn^{2+}-bound form. The Zn^{2+}-unbound TrpRS binds with heme, and this enhances its aminoacylation activity. Because Trp is the least abundant amino acid in humans, regulation of TrpRS activity by heme could serve as a mechanism for protecting the cell against Trp starvation due to heme-mediated IDO activation. The Trp−tRNA complex provides a reservoir of Trp in a form that is protected from IDO-mediated degradation and that is directly available for protein synthesis. Studies aimed at identifying the heme binding site in human TrpRS and investigating whether the up- and downregulation of heme-bound TrpRS in cells affect cell proliferation, apoptosis, and other cell function are now in progress.

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