Elucidation of the Heme Binding Site of Heme-regulated Eukaryotic Initiation Factor 2α Kinase and the Role of the Regulatory Motif in Heme Sensing by Spectroscopic and Catalytic Studies of Mutant Proteins*1

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Heme-regulated eukaryotic initiation factor 2α (eIF2α) kinase functions in response to the heme iron concentration. At the appropriate heme iron concentrations under normal conditions, HRI function is suppressed by binding of the heme iron. Conversely, upon heme iron shortage, HRI auto-phosphorylates and subsequently phosphorylates the substrate, eIF2α, leading to the termination of protein synthesis. The molecular mechanism of heme sensing by HRI, including identification of the specific binding site, remains to be established.

In the present study we demonstrate that His-119/His-120 and Cys-409 are the axial ligands for the Fe(III)-protoporphyrin IX complex (hemin) in HRI, based on spectral data on site-directed mutant proteins. Cys-409 is part of the heme- regulatory Cys-Pro motif in the kinase domain. A P410A full-length mutant protein displayed loss of heme iron affinity. Surprisingly, inhibitory effects of the heme iron on catalysis and changes in the heme dissociation rate constants in full-length His-119/His-120 and Cys-409 mutant proteins were marginally different to wild type. In contrast, heme-induced inhibition of Cys-409 mutants of the isolated kinase domain and N-terminal-truncated proteins was substantially weaker than that of the full-length enzyme. A pulldown assay disclosed heme-dependent interactions between the N-terminal and kinase domains. Accordingly, we propose that heme regulation is induced by interactions between heme and the catalytic domain in conjunction with global tertiary structural changes at the N-terminal domain that accompany heme coordination and not merely by coordination of the heme iron with amino acids on the protein surface.

Eukaryotic cells decrease their overall rates of protein synthesis for survival in response to a variety of stress conditions, such as shortage of amino acids, UV light illumination, virus infection, and accumulation of denatured proteins. Much of the decrease in protein synthesis is caused by phosphorylation of eukaryotic initiation factor 2α (eIF2α) at Ser-51 by eIF2α kinases that respond specifically to stress (1–4). Heme-regulated eIF2α kinase or heme-regulated inhibitor (HRI) is a member of the eIF2α kinase family that controls globin synthesis in response to the heme concentration in reticulocytes (5–8). HRI is inactive at normal heme concentrations. Under conditions of heme deficiency, the enzyme is activated by autophosphorylation and subsequently phosphorylates eIF2α at Ser-51. In addition to globin, HRI controls the synthesis of tryptophan 2,3-dioxygenase and cytochrome P450 2B in liver upon acute porphyria (9, 10). Thus, HRI is possibly critical for heme binding by heme-sensing proteins and heme sensor protein families for eukaryote survival in response to cell emergency states.

Heme-responsive/sensing proteins, also known as “heme sensor proteins” are a current focus of investigation. In these proteins heme association (or dissociation) per se regulates various important physiological functions, such as transcription, proteolysis, and kinase activity (11). The heme-sensing (or binding) sites of most heme sensor proteins constitute the Cys thiolate group. Cys thiolate coordination to the Fe(III)-protoporphyrin IX complex (hemin) is weaker than that of the well characterized His coordination, as observed for hemoglobin. Because axial ligand switching from thiolate to nitrogen occurs easily upon heme reduction from Fe(III) to Fe(II) complexes, redox-dependent ligand switching, in addition to response to the Fe(III) complex concentration, appears to regulate the physiological functions of heme sensor proteins (11). A heme regulatory motif containing a Cys-Pro sequence (CP motif) is possibly critical for heme binding by heme-sensing proteins (11).

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4 The abbreviations used are: eIF2α, eukaryotic initiation factor 2α; HRI, heme-regulated eIF2α kinase or heme-regulated inhibitor; Fe(III) complex, hemin or Fe(III)-protoporphyrin IX complex; Fe(II) complex, Fe(II)-protoporphyrin IX complex; Δ145, an N-terminal-truncated mutant where amino acids 1–145 of the full-length HRI were truncated, and amino acids 146–619 remained; KD, isolated kinase domain with amino acids 146–243 linked with those 361–619; NTD, isolated N-terminal domain with amino acids 1–138.
The heme-sensing mechanism of HRI hitherto reported is briefly summarized as follows. Under normal conditions, heme iron blocks the kinase active site (including the ATP binding and activation segment) of HRI to inhibit activity. Conversely, under heme deficiency conditions, the heme iron dissociates from the kinase active site, which is consequently exposed to the solvent, promoting catalytic activity to phosphorylate the Ser-51 residue of eIF2α. Spectroscopic evidence on truncated HRI mutant proteins indicates that the nitrogen atom of a His residue in the N-terminal domain and the sulfur atom of a Cys residue in the C-terminal domain are the heme axial ligands for HRI under normal conditions (7, 8). Hg²⁺ strongly suppresses HRI catalysis with an IC₅₀ value of 0.6 μM, which is restored by NO, supporting the theory that the Cys thiolate is involved in catalytic regulation (12). An earlier study shows that two His residues are heme axial ligands of the isolated N-terminal domain of HRI (13), distinct from the His and Cys residues proposed for full-length HRI (7, 8). The mechanism proposed for NO-induced catalytic activation of the isolated N-terminal domain is also inconsistent with that for the full-length enzyme (7, 8, 13). Thus, the molecular mechanisms of catalytic activation and inhibition of full-length HRI by heme require clarification, in particular interactions between the heme iron and full-length HRI, the mechanism by which full-length HRI senses the heme iron and the amino acids acting as axial ligands for the heme iron.

To elucidate the heme-sensing mechanism of HRI, it is necessary to identify the axial ligands for the heme iron complex and determine their roles in catalysis and heme inhibition. In the present study we generated three N-terminal-truncated mutant proteins along with seven single and two double His mutant proteins in the N-terminal domain and six single Cys mutant proteins in the C-terminal domain of the full-length HRI enzyme and conducted spectroscopic and catalytic analyses. Spectroscopic data indicate that His-119/His-120 and Cys-409 are the axial ligands for Fe(III) complex bound to full-length HRI, and the Pro residue adjacent to Cys-409 is critical for Fe(III) complex binding. The inhibitory effects of the heme iron on the C499S mutant proteins for the N-terminal-truncated and -isolated kinase domains were significantly lower than that for wild-type full-length HRI. Based on these results we suggest that heme sensing is conducted via heme-protein interactions, including those between the N-terminal and kinase domains, accompanied by global structural changes induced by coordination of the heme iron. Thus, it appears that heme iron coordination with the side chains of surface amino acids is not the only essential requirement for heme sensing to inhibit full-length HRI activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phos-tag acrylamide was acquired from the Phos-tag consortium. Highly purified oligopeptides were purchased from Greiner Bio-One (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemicals (Osaka, Japan).

**Site-directed Mutagenesis, Protein Expression and Purification**—Mutagenesis was conducted using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) and the PrimeSTAR mutagenesis basal kit from Takara Bio (Otsu, Japan). The desired mutations were confirmed by DNA sequencing.

*Escherichia coli* strain BL21(DE3) Codon Plus RIL (Stratagene) harboring the His-tagged HRI expression vector was grown at 37 °C until absorbance at 600 nm reached 0.6. Protein expression was induced by 50 μM isopropyl-β-d-galactoside. *E. coli* was harvested after 20 h of incubation at 15 °C and stored at −80 °C until use.

The protein purification procedures were used to those described previously, with some modifications (7, 8). *E. coli* pellets were dissolved in buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 10 mM 2-mercaptoethanol) containing 20 mM imidazole and protease inhibitors. Cells were disrupted using a sonicator, and cell debris was removed by ultracentrifugation at 100,000 × g for 30 min. Supernatant fractions were loaded on a nickel-nitrilotriacetic acid-agarose column (Qiagen KK, Tokyo, Japan), washed in buffer A containing 20 mM imidazole, and eluted with a linear gradient of 20–200 mM imidazole in buffer A. HRI fractions were pooled and loaded on a HiTrap Q-Sepharose column (GE Healthcare) equilibrated with buffer A. The column was washed with buffer A, and HRI was eluted with a linear gradient of 150–500 mM NaCl containing 20 mM Tris-HCl, pH 8.0, and 10 mM 2-mercaptoethanol. The His tag was cleaved using human rhinovirus 3C protease (Novagen, Darmstadt, Germany) at 4 °C for 12 h. Finally, the sample was loaded on a HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated with buffer A. SDS-PAGE analyses disclosed that the purified protein was more than 95% homogenous. The typical yield of HRI was 1 mg from 1 liter of culture medium. N-terminal-truncated mutant proteins (Δ85 (an N-terminal-truncated mutant where amino acids 1–85 of the full-length HRI were truncated), and amino acids 86–619 remained), Δ127 (an N-terminal-truncated mutant where amino acids 1–127 of the full-length HRI were truncated, and amino acids 128–619 remained), and Δ145) and the isolated kinase domain were purified in a similar manner to the full-length enzyme. Notably, the isolated kinase domain did not absorb to the HiTrap Q-Sepharose column. His₁₀⁻N-terminal domain (NTD) was expressed and purified as described elsewhere (27).

For heme reconstitution, a slight excess of Fe(III) complex dissolved in 90% ethylene glycol was mixed with apoprotein in buffer A and incubated overnight at 4 °C. Unbound heme was removed using the HiTrap Q-Sepharose column under similar conditions as above. The protein concentration was determined using the Quick start protein assay kit (Bio-Rad) using bovine serum albumin as a standard.

**Optical Absorption and CD Spectra**—Optical absorption spectra were obtained for HRI in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl with a Shimadzu UV-2500 spectrophotometer maintained at 25 °C under aerobic conditions. To ensure the appropriate solution temperature, the reaction mixture was incubated for 10 min before spectroscopic measurements. CD spectra were obtained for 10 μM Fe(III)-HRI in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl with a Jasco J-720 CD spectrometer at room temperature. Molar ellipticity was normalized to the heme concentration determined using the pyridine hemochromogen assay. Heme dissociation kinetics values from HRI
to apoH64Y/V68F mutant were obtained as described previously (8, 31).

Enzyme Assay—In vitro kinase assays were performed as described previously (7, 8). Briefly, the reaction mixture consisting of 20 mM Tris-HCl, pH 7.7, 60 mM KCl, 2 mM magnesium acetate, 0.35 μM HRI, and 2–20 μg of eIF2α was incubated at 15 °C for 5 min, and the reaction was initiated by adding 50 μM ATP at 15 °C. At the indicated times the reaction was terminated by adding sample buffer and heat-denatured at 95 °C for 5 min. Samples were loaded on a 7.5% SDS gel containing 50 μM Phos-tag acrylamide and 100 μM manganese chloride. Phosphorylated eIF2α protein interacted with the Phos-tag manganese complex so that the mobility of the phosphorylated protein was slower than that of phosphate-free eIF2α. Proteins were visualized with Coomassie Brilliant Blue R350 staining. Gel images were acquired using LAS-3000 (Fujifilm, Tokyo, Japan), and quantified using MultiBlue R350 staining.

RESULTS

Amino Acid Alignments—A His residue of the N-terminal domain and Cys residue of the C-terminal catalytic domain are possible axial ligands for Fe(III)-complexed full-length HRI (7, 8). Amino acid alignments of selected N-terminal and C-terminal kinase domain regions of HRI are shown in Fig. 1. His-75 and -120 in the N-terminal domain are highly conserved, whereas His-78, -80, and -119 are present only in mammals. Cys-208, -385, and -464 in the kinase domain are highly conserved from mammals to Xenopus and zebrafish, whereas Cys-409, -491, and -550 are identified only in mammals. We targeted all His residues in the N-terminal domain and Cys residues in the kinase domain and generated N-terminal truncated and full-length single mutant proteins.

Optical Absorption Spectra of N-terminal Single His Mutants of Full-length HRI—Sequencing of HRI revealed the presence of seven His residues at positions 75, 78, 80, 86, 119, 120, and 126 in the N-terminal domain (Fig. 1). We replaced the individual His residues of the N-terminal domain with Ala. H75A and H78A mutant proteins were not properly expressed, as observed from SDS-PAGE analyses, suggesting that bulkiness at these positions is critical to maintain the global structure of the full-length HRI protein. Accordingly, we generated H75L and H78L mutants of full-length HRI. Surprisingly, optical absorption spectra in the Soret and visible regions for the Fe(III), Fe(II), and Fe(II)-CO complexes of all single mutant (His-75, -78, -80, -86, -119, -120, and -126) full-length proteins were similar to those of wild-type full-length HRI (Table 1, Fig. 2).

Optical Absorption Spectra of N-terminal His Double Mutant Proteins of Full-length HRI—An earlier study reports that ligand switching to the neighboring amino acid residue(s) possibly occurs upon single mutations at the His residues (21). In view of the amino acid sequence, ligand switching within the His-75/His-78 and His-119/His-120 couples appears to occur upon single mutations at one of the sites. Accordingly, we generated double mutants, H75L/H78L and H119A/H120A mutants of full-length HRI protein. The H119A/H120A mutant exhibited an unusual spectrum where the Fe(III) complex was not bound to the crystal structure of the full-length HRI protein. Accordingly, we generated double mutants, H75L/H78L and H119A/H120A, of full-length HRI. The H119A/H120A mutant exhibited an unusual spectrum where the Fe(III) complex was not bound properly to the protein (Fig. 2D, Table 1). In contrast, the H75L/H78L mutant protein displayed essentially similar optical absorption spectra as the wild-type full-length protein. Based on these findings, we propose that either His-119 or His-120 is essential for Fe(III) binding.
an axial ligand for the Fe(III) complex in full-length HRI. The data are in accordance with the spectral characteristics of N-terminal-truncated mutant proteins in that a His residue between positions 86 and 127 of the N-terminal domain should be one of the axial ligands for the Fe(III) complex in the full-length HRI protein (supplemental Fig. 1S) (8).

CD Spectra of Full-length His Mutant Proteins—Because it was implied that CD spectrum in the Soret region of the heme-binding affinity.

These spectra were obtained in the absence of 2-mercaptoethanol. However, time-dependent spectral changes were observed for the H119A/H120A mutant protein in the presence of 10 mM 2-mercaptoethanol (see supplemental Fig. 8S).
protein sensitively reflects the protein structure around the heme binding site, even optical absorption spectra do not manifest any changes upon mutations at the heme binding site (13). To further identify the amino acid(s) that acts as the axial ligand for Fe(III) complex in full-length HRI, we obtained CD spectra of all His mutant proteins. Specifically, the CD intensity at 425 nm of the H119A mutant protein was significantly larger (on the minus side) than those (tough at 423 nm) of other mutant and wild-type proteins (Fig. 3 and supplemental Fig. 2S). In addition, the CD spectrum of H120A showed the peak at 386 nm and the trough at 423 nm, and these spectral positions are also different from those of the wild-type protein. CD spectra of other full-length mutant proteins were essentially similar to that of the wild-type protein (Fig. 3 and supplemental Fig. 2S). These spectral findings indicate that either His-119 or His-120 is the axial ligand trans to Cys for the Fe(III) complex in the full-length HRI protein.

**Optical Absorption Spectra of Single Cys Mutants of the Kinase Domain of Full-length HRI**—Spectroscopic characterization of full-length single mutant and N-terminal-truncated mutant HRI proteins suggests that a Cys residue in the C-terminal kinase domain is an axial ligand of the Fe(III) complex in full-length HRI protein. The kinase domain of HRI contains six Cys residues (Fig. 1). We generated six Cys-Ser mutant proteins and examined their optical absorption spectra. The maxima of the Soret and visible absorption spectra are summarized in Table 1. Selected absorption spectra are shown in Fig. 4. Evidently, only the C409S mutant protein lost heme binding ability in terms of the ratio of absorbance at the Soret peak to that at 280 nm and spectral features, whereas other mutants displayed similar heme binding ability to wild type. Here, we suggest that Cys-409 is the axial ligand for the Fe(III) complex in full-length HRI protein. Complete loss of heme binding ability of the C409S mutant additionally supports the theory that there is only one heme binding site per HRI monomer. These results are consistent with a 1:1 stoichiometry of heme binding to full-length HRI protein (8), although earlier studies on the isolated domain indicate the presence of two heme binding sites (23).

**Role of the Heme-regulatory CP Motif in Cys Binding to the Fe(III) Complex**—There are two heme regulatory or CP motifs (identified at residues 409 and 550) located in the kinase
domain of HRI (Fig. 1). The CP motif may be critical in heme sensing in several heme-associated proteins, including HAP1, IRP2, Bach1, ALAS1, and Irr (11, 18, 19) (supplemental Fig. 3). To address the role of Pro residues adjacent to the two Cys residues, Cys-409 and Cys550, we generated P410A and P551A mutant proteins and examined their heme binding affinities. As shown in Fig. 5, the P410A mutant protein lost heme binding properties, whereas the P551A protein maintained heme binding affinity (Table 1). Evidently, the Pro residue next to Cys residue plays an important role in Cys binding to the Fe(III) complex. The results strongly suggest that Cys-409 is an axial ligand to the Fe(III) complex in full-length HRI.

To further address the general role of Pro adjacent to Cys in binding to the Fe(III) complex, we additionally obtained optical absorption spectra of the Fe(III) complex in the presence of oligopeptides containing the HRI CP motif and non-CP Cys (supplemental Fig. 4S). Oligopeptides with non-CP Cys residues did not bind free Fe(III) heme (supplemental Fig. 5S). In contrast, two oligopeptides containing the CP motif of the HRI sequence (Cys-409—Pro-410 and Cys-550—Pro-551) bound tight to free Fe(III) heme with equilibrium dissociation constants of less than 5 μM (supplemental Fig. 6S). Additionally, the binding affinities of the oligopeptides to the Fe(III) complex were significantly lowered when the Pro residue next to Cys was mutated to Ala (supplemental Fig. 6S). Molecular modeling investigations with classical force fields and, using the small peptide AAA-CP-AAA, suggest that Pro adjacent to Cys favors the coordination of Cys to the heme iron center with the aid of H-bonds (supplemental Fig. 7S). Additionally, hydrophobic interactions between the heme plane, proline, and a protrusive Cys residue, with the aid of the adjacent Pro, cannot be ruled out.

**Heme Binding Properties of Single Mutants of Full-length HRI**—To determine the stability of heme binding, we measured the heme dissociation rate constants for selected mutant full-length HRI proteins. The Fe(III) complex dissociation from mutant proteins, except H119A and C409S full-length HRI proteins, by transferring to apomyoglobin was composed of two phases. The rate constants of the phases were not higher than those of the wild-type protein (supplemental Table 1S). However, dissociation of H119A and C409S mutant proteins was composed of one phase. These findings suggest that coordination of the Fe(III) complex with surface amino acids is not solely responsible for association or dissociation of the Fe(III) complex in full-length HRI. Moreover, association or dissociation of the Fe(III) complex may occur over two steps, one involving the N-terminal domain and other the kinase domain.

**Catalytic Activities of the Fe(III) Complex on the Activities of the C409S Full-length Mutant Proteins**—After the identification of potential candidates for the axial ligands of the Fe(III) complex from spectroscopic studies, we examined the catalytic activities and effects of the Fe(III) complex on mutant proteins that lost heme binding ability. An *in vitro* eIF2α kinase assay was conducted using a Phos-tag acrylamide gel (Fig. 6). Slower migrating upper bands represented phosphorylated eIF2α protein interacting with the Phos-tag manganese complex, whereas the lower bands signified non-phosphorylated eIF2α protein. Wild-type and C409S mutant enzymes displayed similar kinetic parameters for eIF2α. Specifically, $k_{cat}$ values for the wild-type and C409S mutant were 11.0 and 8.7 min$^{-1}$, and $K_m$ values were 9.3 and 7.6 μM, respectively. Half-inhibition constants (IC$50$) of enzyme activity upon binding to the Fe(III) complex were evaluated as 2.1 and 5.1 μM for wild-type and C409S mutants, respectively (Table 2, Fig. 6). These data clearly demonstrate that wild-type and C409S mutant enzymes display similar enzymatic activities and responses to heme inhibition. Thus, catalytic regulation may occur via interactions caused by global structural changes induced by heme binding and not simply coordination of the heme iron with amino acid side chains on the protein surface.

**Catalytic Activities of the Wild-type and C409S Mutant Proteins of the Isolated Kinase Domain and the Truncated Mutant, Δ145**—To address the role of global protein structural changes in heme-regulated catalysis, we generated an isolated kinase domain composed of amino acids 146–243 and 361–619 based on x-ray crystal structures of other eIF2α kinases with different sensing domains (22). The center-deleted mutants were used probably because the protein folding or stabilities of the center-deleted kinase domain should be better than the whole isolated kinase domain. We additionally generated a C409S mutant of the N-terminal-truncated mutant, Δ145, to establish whether the N-terminal domain is involved in inhibition by the Fe(III) complex. The IC$50$ values of wild-type Δ145 and isolated kinase domain upon binding of the Fe(III) complex were 0.25 and 0.23 μM, respectively (Fig. 7, Table 2). In contrast, the IC$50$ values of the C409S mutants of Δ145 and isolated kinase domain were...
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FIGURE 6. Catalytic regulation of the full-length wild-type and C409S mutant enzymes by the Fe(III) complex. The eIF2α protein exhibits reduced mobility on SDS-PAGE gels after phosphorylation (P) (see “Experimental Procedures”) (upper panel). An aliquot (5 µl) of the 40-µl solution containing 10 µg of eIF2α in sample solution was loaded on each lane. Time-dependent phosphorylation was inhibited by increasing the amount of the Fe(III) complex. No significant differences in catalytic regulation by the Fe(III) complex were observed between the wild-type (open circle) and C409S mutant (filled circle) proteins (lower panel). Experiments were conducted at least three times and the averaged values are shown. WT, wild-type protein. FL, full-length protein.

TABLE 2
Effects of the Fe(III) complex on catalysis by HRI mutant enzymes expressed as IC₅₀ values of inhibition

| Proteins | IC₅₀ (µM) |
|----------|----------|
| Full-length enzyme | |
| Wild type | 2.1 |
| C409S | 5.1 |
| H119/H120 | 3.7 |
| Δ145-truncated mutant | |
| Wild type | 0.25 |
| C409S | >10* |
| Isolated kinase domain | |
| Wild type | 0.23 |
| C409S | >1* |

* For Δ145 and the isolated kinase domain with the C409S sequence, the heme sensitivities were substantially reduced in that the addition of a large excess of hemin (more than a 10-fold excess, or 1 µM) caused only a marginal decrease in activity (Fig. 7). We, therefore, temporarily varied the maximal hemin concentration added in experiments examining the interactions of Δ145 and the isolated kinase domain mutant with the C409S sequence shown in this table. Note that we could not determine the exact IC₅₀ value for the C409S mutant because of very low assay sensitivity when the Fe(III) complex was present.

greater than 10 and 1 µM, respectively. Note that we could not determine the exact IC₅₀ value for the C409S mutants because of very low sensitivity for the Fe(III) complex. Therefore, we conclude that conformational changes or structural factors involving the N-terminal domain are critical in sensing the Fe(III) complex in the full-length wild-type HRI protein.

Catalytic Activity and Effects of the Fe(III) Complex on Activity of the H119A/H120A Full-length HRI Protein—The catalytic activity and effects of heme on the activity of the H119A/H120A double mutant, which lost heme binding affinity, were comparable with those of the wild-type protein (not shown, Table 2). Evidently heme coordination is not important for inhibition of catalysis by the heme iron complex.

Pulldown Assay for Determining Interactions between the N-terminal and Catalytic Domains—Interactions between the NTD and C-terminal catalytic domains (Δ145) appear to be a key factor in catalysis regulation by the Fe(III) heme. Thus, we conducted a pulldown assay to examine these interactions (Fig. 8). A solution containing both His₆-tagged NTD and Δ145 proteins (lane 0 in Fig. 8) was applied to a Ni-Sepharose column. In the absence of the Fe(III) heme, NTD with the wild-type sequence and Δ145 were separated into flow-through (data not shown) and eluted fractions (Fig. 8 lane 3), respectively. In the presence of the Fe(III) heme, NTD with the wild-type sequence and Δ145 were co-eluted with buffer containing 200 mM imidazole. However, when C409S of the Δ145 protein with no heme binding affinity was used, the eluted solution only contained NTD (lanes 1 and 2). These findings imply that interactions between NTD and Δ145 occur only in the presence of the Fe(III) heme.

On the other hand, mutations in the NTD also affected the interaction between the NTD and Δ145 in the presence of the Fe(III) heme. The H119A, H120A, and H119A/H120A mutants of the NTD were mixed with Δ145 (with wild-type sequence) and applied to a Ni-Sepharose column. The interactions between the NTD with the H119A/H120A sequences and Δ145 with the wild-type sequence were completely eliminated (Fig. 8, lane 4), as was observed for the interaction between the NTD with wild-type sequence and Δ145 with the C409S sequence (lane 2). The interactions between NTDs with single mutations at His-119 (lane 6) or His-120 (lane 8) and Δ145 with wild-type sequence, however, remained 90 and 50% that of the NTD with the wild-type sequence (Fig. 8, bottom). Because the effect of the His-120 mutation in the NTD on the protein-protein (NTD-Δ145) interaction was greater than that of the His-119 mutation, His-120 appeared to play a crucial role in the interaction between the NTD and Δ145. Note that even with 20 mM imidazole in the equilibration buffer, imidazole could not increase interactions between NTDs with H119A, H120A, or H119A/H120A sequences on the one hand and Δ145 with wild-type sequence on the other. These results indicated that imidazole had no effect on the interaction between the NTD and Δ145.

DISCUSSION

Heme is an essential prosthetic group for oxygen transport and storage (hemoglobin, myoglobin), electron transfer (cytochromes), catalysis using the activated oxygen molecule (peroxidase, cytochrome P450, and catalase), and gas sensor proteins (Ec DOS, soluble guanylate cyclase, FixL, and CooA) (15–17). Heme per se also plays a regulatory role in various important physiological functions in organisms. For example, Bach1 controls the transcription of β-globin and heme oxyge-
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nase 1 genes in response to the heme concentration. NPAS2, IRP2, E75, DGCR8, Slo BK channel, Rev-erba, and arginine transferase, ATE1 (Refs. 11 and 26 and references therein), additionally conduct crucial physiological functions as heme sensor proteins in response to the heme concentration in that association or dissociation of heme iron from protein regulates catalysis or transcription (11).

For heme sensor proteins the heme sensing site is important for heme-dependent regulation. The heme regulatory motif or CP motif, comprising a Cys-Pro dipeptide, is proposed as the heme sensing site for ALAS, IRP2, bacteria, yeast, and mammalian transcription factors, including Irr, HAP1, and Bach1 (18, 19). Direct evidence for heme binding to the CP motif as the heme regulatory site was recently demonstrated for IRP2 (19). HRI contains two CP motifs in the kinase domain (Fig. 1). A simple classical molecular modeling using the small peptide, AAA-CP-AAA, suggests that Pro adjacent to Cys aids in coordination of Cys to the heme iron with the aid of H-bonds (supplemental Fig. 7S). Additionally, hydrophobic interactions between the heme plane, proline, and a protrusive Cys residue with the aid of the adjacent Pro cannot be ruled out.

Autophosphorylation at the initial stages of catalytic regulation is a key step in HRI activation. However, we could not examine the differences in phosphorylation status because the purified overexpressed HRI protein is already phosphorylated when the heme regulatory site was recently demonstrated for IRP2 (19). HRI contains two CP motifs in the kinase domain (Fig. 1). A simple classical molecular modeling using the small peptide, AAA-CP-AAA, suggests that Pro adjacent to Cys aids in coordination of Cys to the heme iron with the aid of H-bonds (supplemental Fig. 7S). Additionally, hydrophobic interactions between the heme plane, proline, and a protrusive Cys residue with the aid of the adjacent Pro cannot be ruled out.

Sequence alignments of HRI (Fig. 1) revealed that some of the Cys and His residues mutated in this study are not highly conserved throughout animals. In particular, His-119 and Cys-409, the proposed heme axial ligands, are conserved only in mammals. Because the HRI homolog from Schizosaccharomyces pombe is not controlled by the heme iron (24), it is assumed that the heme-regulated HRI function is specific to mammals. In addition, erythroid differentiation in mammals is different from that in other vertebrates.

The Soret CD band arises because of coupled oscillator interaction between the heme transitions and π–π* transitions in nearby aromatic side chains and was well rationalized by theoretical calculations on heme rotational strengths in myoglobin and hemoglobin (29, 30). Thus, subtle structural changes in heme surroundings would significantly influence the Soret CD spectral bands of the heme proteins even if such changes do not affect other spectroscopies (13). The ratio (RZ value) of the Soret peak absorbance to that at 280 nm of the heme protein is used to measure heme binding to the apoprotein. Because the RZ values of both the H119A and H120A mutants are comparable to that of the wild-type protein, the Fe(III) complex would be adequately incorporated into the mutant proteins. The unusual Soret CD bands of both H119A and H120A

FIGURE 7. Catalytic regulation of the N-terminal-truncated mutant protein, Δ145 (left panel), and the isolated kinase domain (KD, right panel) with the wild-type (WT) and C409S (C409S) sequences by the Fe(III) complex. Reactions were terminated at 4 and 5 min for Δ145 and isolated kinase domain proteins, respectively. Reaction conditions were similar to those described in Fig. 6. Experiments were conducted at least three times, and the averaged values are shown. WT, wild-type protein; KD, isolated kinase domain containing residues 146–243 linked with those at positions 361–619.

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FIGURE 8. Pulldown assay to detect interactions between the His-tagged isolated N-terminal domain (His\(_6\)-NTD) and Δ145 N-terminal-truncated mutant protein. His\(_6\)-NTD proteins with the wild-type (WT), H119A, H120A, and H119A/H120A sequences were examined, whereas Δ145 with the wild-type and C409S sequences were investigated. The mixtures of His-tagged-NTDs and Δ145 proteins were applied to Ni-Sepharose columns. These input solutions (lane 0) were eluted with 200 mM imidazole, and the eluates analyzed by SDS-PAGE. In the presence of the Fe(III) heme, NTD with the wild-type sequence bound to Δ145 with the wild-type sequence (lane 4), whereas in the absence of the Fe(III) heme, NTD with the wild-type sequence did not bind to Δ145 with the wild-type sequence (lane 3). The NTD with the wild-type sequence did not interact with Δ145 containing the C409S sequence, however, even in the presence of the Fe(III) heme (lane 2). As Δ145 with the C409S sequence lost heme binding affinity, heme-dependent interactions between the NTD and Δ145 were not detected. On the other hand, the interaction between the NTD with the H119A sequence and Δ145 with the wild-type sequence was almost the same as that between the NTD with the wild-type sequence and Δ145 with the wild-type sequence (lanes 4 and 6). Consistent with the spectroscopic results, the NTD with the H119A/H120A sequence completely lost heme-induced interaction with Δ145 (lane 10). The mutation at His-120 substantially decreased the interaction between the NTD and Δ145 (lane 8), which is in contrast with the interaction seen between the NTD with the wild-type sequence and Δ145 with the wild-type sequence. Experiments were repeated three times, and averaged values are shown. Lane 0, 10% of input mixture before application to Ni-Sepharose.

Mutations do not arise because of improper binding of the Fe(III) complex to the mutant proteins.

Mutations at either His-119 or His-120 of the full-length protein caused ligand switching to the next His position, resulting in an optical absorption band similar to that of the wild-type protein. The double mutant (H119A/H120A) protein presented an absorption spectrum distinct from that of heme-bound wild-type full-length HRI protein (Table 1, Fig. 2D), whereas all other single and double mutant proteins displayed similar absorption spectra (Table 1, Figs. 2, A–C). The spectrum of the Fe(III) complex-bound double mutant protein at the initial stage of heme association in the presence of 10 mM 2-mercaptoethanol was typical of the 5-coordinated S\(^-\)-Fe(III) complex with Cys-409 as the axial ligand (supplemental Fig. 85). Time-dependent structural change(s) would allow switching of the axial ligand from the thiolate group to an unknown nitrogen atom to partially coordinate with the Fe(III) complex. It is evident that Cys-409 is the axial ligand trans to His-119/His-120, since the optical absorption spectrum of the C409S mutant reveals loss of heme binding ability, in contrast to other Cys-Ser full-length mutant proteins where the heme binding affinities were not altered.

Facile ligand switching to adjacent amino acids upon mutations is observed for a heme sensor protein, Irr (21). Irr has a characteristic motif consisting of three consecutive His residues, His-117/His-118/His-119, which may be potential axial ligands for the heme iron complex. Only the triple His mutant lost heme affinity, whereas none of single or double mutants displayed heme absorption spectral changes. These ligand switching phenomena induced by mutation of the heme axial ligand are not generally observed for globin proteins. Protein structural flexibility may, thus, be an intrinsic characteristic of heme sensor proteins.

According to the prediction of protein disorder server (DISOPRED2) (25), part of the N-terminal domain of HRI (amino acids 117–164) is disordered. His-119 and His-120 are located in the disordered region where single site mutations accompany ligand switching.

Notably, HRI enzyme activity is not simply controlled by axial ligand coordination but also by other interactions generated upon heme iron binding. In this regard the phosphorylation state of the enzyme should additionally play a critical role in heme sensing. Moreover, the CP motif itself is not always a requirement for heme sensing, as our data show that Cys-409—Pro-410, but not Cys-550—Pro-551, comprises the heme sensing site for the full-length enzyme (Table 1, Figs. 4 and 5). Therefore, the heme binding property of the heme regulatory motif enzyme is not intrinsic but is generated during evolution in response to the necessity for heme sensing.

Based on the protein structures of two eIF2α kinases, PKR (double-stranded RNA-dependent protein kinase) and GCN2 (general control nonrepressible 2), we generated a homology model of the kinase domain in HRI. The existence of Cys-409 is unique for HRI, as there are no counterparts in the PKR and GCN2 sequences (22). However, in view of the PKR and GCN2 structures, it is likely that Cys-409 is located between helices αD and αE. The heme binding site of HRI may be situated away from the catalytic center and ATP binding site. Fig. 9 depicts the hypothetical structure derived from our proposed allosteric control model of HRI. In the presence of sufficient heme iron (normal conditions), the heme iron is coordinated to two axial ligands, His-119/His-120 and Cys-409, and the disordered N-terminal domain interacts with the kinase domain, leading to a closed and inactive conformation (Fig. 9, left). In the absence of heme iron (heme shortage conditions), the inhibitory effect of the heme iron is released, and the kinase domain forms an open and active conformation (Fig. 9, right). Heme-dependent interactions between NTD and the catalytic domain were dem-
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FIGURE 9. A hypothetical model of the heme coordination structure of full-length HRI. Heme association/dissociation at the heme-sensing site of HRI regulates the eIF2a kinase reaction. Heme association with full-length HRI blocks catalysis (left), whereas heme dissociation opens the active site and allows catalysis (right). It is assumed that the heme binding site of HRI is situated away from the catalytic center and ATP binding site. Because the axial ligand(s) appears changed upon heme reduction, the redox-dependent ligand switch may additionally modulate catalysis. KD, kinase dead.

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