Identification of Crucial Histidines for Heme Binding in the N-terminal Domain of the Heme-regulated eIF2α Kinase*

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Takayuki Inuzuka‡, Bo-Geon Yun§, Haruto Ishikawa§‡, Satoshi Takahashi‡, Hiroshi Hori**, Robert L. Matts§†‡, Koichi Ishimori§‡‡, and Isao Morishima§¶¶

From the ‡Department of Molecular Engineering, Graduate School of Engineering, Kyoto University, Kyoto 615-8510, Japan, the §Department of Bioengineering, Graduate School of Engineering Science, Osaka University, Osaka 560-8531, Japan, and the ¶¶Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, Oklahoma 74078

The heme-regulated eukaryotic initiation factor-2α (eIF2α) kinase (HRI) regulates the initiation of protein synthesis in reticulocytes. The binding of NO to the N-terminal heme-binding domain (NTD) of HRI positively modulates its kinase activity. By utilizing UV-visible absorption, resonance Raman, EPR and CD spectroscopies, two histidine residues have been identified that are crucial for the binding of heme to the NTD. The UV-visible absorption and resonance Raman spectra of all the histidine mutants constructed were similar to those of the unmutated NTD. However, the change in the CD spectra of the NTD construct containing mutation of His78 to Ala (H78A) indicated loss of the specific binding of heme. The EPR spectrum for the ferric H78A mutant was also substantially perturbed. Thus, His78 is one of the axial ligands for the NTD of HRI. Significant changes in the EPR spectrum of the H123A mutant were also observed, and heme readily dissociated from both the H123A and the H78A NTD mutants, suggesting that His123 was also an axial heme ligand. However, the CD spectrum for the Soret region of the H123A mutant indicated that this mutant still bound heme specifically. Thus, while both His78 and His123 are crucial for stable heme binding, the effects of their mutations on the structure of the NTD differed. His78 appears to play the primary role in the specific binding of heme to the NTD, acting analogously to the “proximal histidine” ligand of globins, while His123 appears to act as the “distant” heme ligand.

The heme-regulated eukaryotic initiation factor-2α (eIF2α) kinase (HRI) is a member of a family of kinases that regulate initiation of protein synthesis in eukaryotic cells. HRI functions, in part, to coordinate the synthesis of globin chains in reticulocytes with heme availability (1). A deficiency of heme, which is required for the assembly of α- and β-globin chains into hemoglobin, induces the activation of HRI, which subsequently phosphorylates the α-subunit of eIF2, leading to the inhibition of polypeptide chain initiation and the arrest of protein synthesis.

HRI is a multidomain hemoprotein that contains two distinct heme-binding sites (2). One site, which has been tentatively assigned as being located in the “kinase insertion domain” of HRI, appears to bind heme “reversibly” and regulates HRI activity in response to changes in heme concentration (3). The other heme-binding site is located in the N-terminal domain (NTD) of HRI, which consists of ∼165 amino acids (4). This domain is responsible for the stable “constitutive” binding of heme to HRI (4) and appears to be the active center for nitric oxide (NO)-induced activation of HRI (5). The isolated NTD stably binds heme but shows no kinase activity (4). The kinase activity of HRI is coded by the C-terminal region (2). The isolated C-terminal region has the “reversible” heme-binding site and phosphorylation activity, but it does not respond to NO. Although axial ligands for the heme-binding sites have not yet been determined, sequence alignment of the NTD to globins (5) and preliminary spectroscopic analyses of the NTD (6) suggested that histidine was the axial ligand of the heme-binding site in the NTD.

In the presence of NO, heme in the NTD forms a six-coordinate NO complex (5, 6). The formation of this six-coordinate NO-Fe-His complex is of interest, because it differs from what occurs in the prototypical NO-responsive sensor protein, soluble guanylate cyclase. In soluble guanylate cyclase, the binding of NO to the heme iron induces cleavage of the iron-histidyl bond to form a five-coordinate NO complex, which is postulated to be the primary trigger for the activation of its cyclase activity (7). However, we have shown that the iron-histidyl bond is retained in the NO adduct of the NTD (6). This observation has led us to propose that changes in the conformation of amino acid residues adjacent to the NO binding site (the “distant” heme ligand), not cleavage of the axial histidine ligand at the “proximal” side of the heme-binding site, would be crucial for the NO-induced activation mechanism in HRI (6).

To determine the axial ligands in the NTD, we prepared point mutants substituting Ala for His at each of the seven histidine residues present in the NTD of rabbit HRI (His78, His81, His83, His122, His129, and His149) (5). Two of the residues (His78 and His123) are invariant amino acids from zebrafish to human and the other four histidines (His81, His83, His122, and His129) are conserved in mammalian HRIs (Table I). In addition, we prepared another mutant in which Gln58, which molecular modeling suggested might be equivalent to...
the distal histidine ligand of globins (4), was replaced with Ala. Using a combination of these mutations in the NTD with UV-visible absorption, resonance Raman, EPR and CD spectroscopies, His78 and His223 were identified as being two crucial residues for the ligation of heme within the NTD of HRI.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant NTD and Its Mutants—Ala to His mutants within the NTD were constructed as described previously (4). All of the mutants were expressed in Escherichia coli and isolated as holoproteins. The unmutated and mutated NTDs of HRI were purified as described previously (6) with some modifications. To avoid nonspecific heme binding, the (His)5-tag was cleaved by enterokinase (4), and the cleavage of the (His)5-tag was confirmed by the N-terminal amino acid analysis and SDS-PAGE. The spectroscopic data obtained with the proteins from which the (His)5-tag was cleaved were identical to the data obtained from (His)5-tag fused proteins, as reported previously (5).

The heme concentration was determined by the pyridine hemochromogen assay (8), and the heme content was estimated by the absorption ratio of the Soret peak to 280 nm (A493/A280 nm). We observed that the A493/A280 nm for the unmutated NTD is about 4.5. However, heme dissociated from the His78 and His223 mutants during the purification. The A493/A280 nm for these mutant proteins were at least 1.5, indicating that more than 30% of the heme binding sites were occupied by heme. The destabilized protein structure and weak heme affinity of these mutants prevented further purification.

Spectroscopy—The UV-visible absorption and CD spectra of the purified NTD and its mutants were obtained using a Perkin-Elmer lambda 19 and a Jasco model 720 spectrometers, respectively, at room temperature. Proteins at a concentration of 5–10 µM in 50 mM Tris-HCl (pH 7.5) were placed in a cuvette (1-cm cell length). The ellipticity in the CD spectra was normalized by the heme concentration, and the presence of the apoprotein did not affect the CD spectra for the Soret region. Resonance Raman spectra were measured using the system and the method reported previously (6). The EPR spectra of the NTD and its mutants were measured at 15 K with a Varian E-12 spectrometer (X-band; 9.22 GHz) equipped with an Oxford ESR-900 liquid helium flow cryostat. The sample concentration for the EPR measurement was 200 µM in 50 mM Tris-HCl at pH 7.5.
distinct and the mutation at His123 significantly perturbed the heme environment of the ferric NTD. However, the effects of the His123 mutation were different from those of His78. The intense peak in the CD spectrum of the His123 mutant indicated that the mutation did not abolish the specific binding of heme. Thus, His123 might be the other axial ligand to the heme iron with the contribution of His123 to the specific binding of heme to the NTD being less significant than that of His78.

The differences between the effects of the His78 and His123 mutations were more enhanced in the ferrous state of the NTD. In the ferrous state, the recombinant NTD gave a positive peak at 418 nm and a negative peak at 427 nm (Fig. 1B). The CD spectrum of the His78 mutant also had no intense signals in the Soret region, as observed for the ferric state, indicating that His78 was ligated to the ferrous heme iron in the NTD. However, the spectral changes in the ferrous His123 mutant were less drastic than those in the ferric state. The spectral pattern of the ferrous His123 mutant is essentially the same as that of the unmutated NTD. The structural changes induced by the mutation at His123 appear to be relieved by the reduction of the heme. Thus, the ligation of His123 to the ferrous heme iron was maintained without the ligation of His123. However, during the purification of the His123 mutant, we noted that dissociation of heme from its heme-binding site was enhanced, indicating that the affinity of the His123 mutant for heme was decreased to the same extent as was found for the His78 mutant. Thus, the ligation of heme by His123 appears to be required for stabilizing the specific binding of heme to the NTD.

Such nonequivalent contributions of two axial ligands to the structural environment of the heme-binding site are also encountered in cytochrome b5 (12). Mutation of one axial ligand, His99, resulted in minor spectral changes and formation of a rather stable hemoprotein, while the replacement of the other axial ligand, His321, induced severe destabilization of the protein structure and decreased the heme binding affinity of the protein (12). Based on the heme binding and spectroscopic data for the two axial mutants of cytochrome b5, we had proposed that His99 plays a primary role for the binding of heme to cytochrome b5 and acts in a manner analogous to the proximal histidine in globins, On the other hand, the similarities between the CD spectra of the ferrous-CO state of the unmutated and the His123-mutated NTD (Fig. 1C) indicated that specific binding of heme to the NTD, corresponding to the proximal histidine in globins. On the other hand, the similarities between the CD spectra of the ferrous-CO state of the unmutated and the His123-mutated NTD (Fig. 1C) indicated that specific binding of heme to the NTD, corresponding to the proximal histidine in globins.

### Table II

| Protein | Peak positions (nm) |
|---------|---------------------|
| Fe(III) | Fe(II) | Fe(II)-CO |
| 415 | 534 | 565 | 429 | 532 | 562 | 424 | 539 | 565 |
| H78A | 414 | 531 | 562 | 424 | 533 | 565 | 425 | 532 | 562 | 424 | 539 | 566 |
| H81A | 414 | 531 | 562 | 424 | 533 | 565 | 425 | 532 | 562 | 424 | 539 | 566 |
| H83A | 415 | 534 | 565 | 429 | 532 | 562 | 424 | 539 | 564 |
| H122A | 414 | 531 | 562 | 424 | 532 | 562 | 425 | 532 | 562 | 424 | 539 | 567 |
| H123A | 414 | 534 | 565 | 429 | 532 | 562 | 424 | 539 | 563 |
| H129A | 415 | 534 | 565 | 429 | 532 | 562 | 424 | 539 | 571 |
| H148A | 415 | 534 | 565 | 429 | 532 | 562 | 424 | 539 | 571 |
| Cytochrome b5 | 412 | 533 | 562 | 423 | 525 | 556 | 424 | 539 | 571 |

* Ref. 6.  
* Shoulder peak.  
* Ref. 9.  
* —, not formed.

### Table III

#### Positions of marker lines in resonance raman spectra of the unmutated and mutated NTDs of HRI

The intensity of these peaks are very low.

| Protein | Line positions (cm⁻¹) |
|---------|----------------------|
| Fe(III) | Fe(II) | Fe(II)-CO |
| 344 | 375 | 415 | 1371 | 1469 | 1506 | 1580 | 1624 |
| H78A | 344 | 376 | 413 | 1371 | 1468 | 1503 | 1582 | 1620 |
| H129A | 342 | 374 | 419 | 1374 | 1469 | 1506 | 1581 | 1624 |

| Protein | Line positions (cm⁻¹) |
|---------|----------------------|
| Fe(II) | Fe(II)-CO |
| 343 | 381 | 415 | 1360 | 1468 | 1492 | 1582 | 1613 |
| H78A | 345 | 380 | 415 | 1360 | 1470 | 1492 | 1583 | 1605 |
| H129A | 345 | 381 | 415 | 1360 | 1468 | 1493 | 1583 | 1609 |

| Protein | Line positions (cm⁻¹) |
|---------|----------------------|
| Fe(II)-CO | Fe(II)-CO |
| 350 | 376 | 419 | 494 | 1961 |
| H78A | 347 | 376 | 414 | 494 | 1964 |
| H129A | 347 | 377 | 419 | 493 | 1965 |
histidine”, with ligation of His39 to the heme iron being critical for the electron transfer reaction catalyzed by cytochrome b5 and not for heme binding (12).

To further examine the roles of the axial histidines in determining the structural environment of the heme-binding site, the resonance Raman spectra were measured. Unexpectedly, most of the stretching modes of the porphyrin ring in the spectra of the His78 and His123 mutants could be superimposed on those of the unmutated NTD (Table III). Compared with the spectral changes reported for the axial ligand mutants in other hemoproteins (13–15), one might conclude that such small spectral changes would not reflect the substitution of the axial ligands for the heme iron of the NTD. However, it should be noted here that the NTD has histidine residues adjacent to the positions of His78 and His123. His81 and His83 are located near His78, and His122 is located next to His123. These histidines may coordinate to the heme iron instead of His78 or His123, resulting in the slight changes in the resonance Raman spectra. With respect to the His122 mutant, heme was also observed to dissociate during its purification, indicating that this residue plays some role in maintaining the integrity of the heme-binding site. For the His78 mutant, loss of the intrinsic axial ligand would severely perturb the structural environment of the heme-binding site. Ligation of an alternate axial ligand may allow variations in the orientation of the bound heme, leading to the loss of the “specific binding” of heme and no clear peaks in the CD spectrum of mutant. Mutation of His83 to Gln has previously demonstrated that this residue plays a crucial role in maintaining the integrity of the heme binding site (4). This observation further supports the hypothesis that the region of the NTD containing His78 corresponds to the proximal side of the heme-binding site.

The EPR spectrum for the ferric state of the mutants also supports the notion that His78 and His123 are ligated to the heme iron. As illustrated in Fig. 2A, the EPR spectrum for the ferric state of the unmutated NTD showed g values at 3.07, 2.20, and 1.46, which is typical of the low spin bishistidine-ligated heme (16), although a low intensity signal at approxi-
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Heme ligand, mutation of His^{78} was found to have limited effects on the NO-induced activation of HRI. The kinase activity of the His^{78} mutant of HRI was somewhat less sensitive to activation by NO, particularly at low NO concentrations (70% less active in the presence of 0.01 mM NOC-9, a NO generator), but the His^{78} mutant still showed NO-dependent phosphorylation activity. The low sensitivity to NO in the His^{78} mutant would reflect some structural changes induced by the replacement of His^{78} with another histidine, indicating that the proper orientation of the heme moiety, which requires His^{78}, might be also important in the mechanism by which NO activates HRI. Such limited effects of the mutation at His^{78} correspond to our previous suggestion (6) that the NO-induced activation of HRI is primarily based on the conformational changes in the ligand binding site, not in the cleavage of the axial ligand from the protein.

Previous results (6) have led us to propose that changes in the conformations of amino acid residues on the distal side of the heme-binding site, which are induced by the binding of NO to the ferrous heme iron, trigger the NO-induced activation of HRI. Despite the unambiguous identification of His^{78} as the proximal ligand of the NTD, the identity of the amino acid residue at the NO/CO binding site, which can be referred to as the distal ligand, in the ferrous state is still uncertain. Molecular modeling based on the sequence alignment of the NTD with α-globin suggested that Gln^{58} might act as the distal heme-binding ligand (4). However, mutation of Gln^{58} to Ala did not induce significant spectral changes in the UV-visible absorption, CD, and EPR spectra. Comparison of the EPR spectra of the ferric unmutated NTD and the His^{123} mutant (Fig. 2) implies the ligation of His^{123} to heme in the ferric state, suggesting that the distal axial ligand in the ferrous state is His^{123}, not Gln^{58}. Systematic mutational studies to identify critical amino acid residues on the distal side of the heme binding site in the NTD are now in progress.

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