Activation of Heme-regulated Eukaryotic Initiation Factor 2α Kinase by Nitric Oxide Is Induced by the Formation of a Five-coordinate NO-Heme Complex

OPTICAL ABSORPTION, ELECTRON SPIN RESONANCE, AND RESONANCE RAMAN SPECTRAL STUDIES*

Received for publication, September 16, 2003, and in revised form, December 28, 2003
Published, JBC Papers in Press, January 29, 2004, DOI 10.1074/jbc.M310273200

Heme-regulated eukaryotic initiation factor 2α kinase (HRI) regulates the synthesis of hemoglobin in reticulocytes in response to heme availability. HRI contains a tightly bound heme at the N-terminal domain. Earlier reports show that nitric oxide (NO) regulates HRI catalysis. However, the mechanism of this process remains unclear. In the present study, we utilize in vitro kinase assays, optical absorption, electron spin resonance (ESR), and resonance Raman spectra of purified full-length HRI for the first time to elucidate the regulation mechanism of NO. HRI was activated via heme upon NO binding, and the Fe(II)-HRI complex displayed 5-fold greater eukaryotic initiation factor 2α kinase activity than the Fe(III)-HRI complex. The Fe(III)-HRI complex exhibited a Soret peak at 418 nm and a rhombic ESR signal with g values of 2.49, 2.28, and 1.87, suggesting coordination with Cys as an axial ligand. Interestingly, optical absorption, ESR, and resonance Raman spectra of the Fe(II)-NO complex were characteristic of five-coordinate NO-heme. Spectral findings on the coordination structure of full-length HRI were distinct from those obtained for the isolated N-terminal heme-binding domain. Specifically, six-coordinate NO-Fe(II)-His was observed but not Cys-Fe(II) coordination. It is suggested that significant conformational change(s) in the protein induced by NO binding to the heme complex led to HRI activation. We discuss the role of NO and heme in catalysis by HRI, focusing on heme-based sensor proteins.

Jotaro Igarashi, Akira Sato, Teizo Kitagawa, Tetsuhiko Yoshimura, Seigo Yamauchi, Ikuo Sagami, and Toru Shimizu

From the Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Sendai 980-8877, Japan, the Center for Integrative Bioscience, Okazaki National Research Institutes, Okazaki 444-8585, Japan, and Institute for Life Support Technology, Yamagata Public Corporation for the Development of Industry, Yamagata 990-2473, Japan

* This work was supported in part by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to T. S.) and by the Joint Studies Program (2002–2003) of the Institute for Molecular Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Toru Shimizu, Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577, Japan. Tel.: 181-22-217-6560 ×5605; Fax: 181-22-217-6560 ×5390; E-mail: shimizu@tagen.tohoku.ac.jp.

‡ The abbreviations used are: HRI, heme-regulated eIF2α kinase or eIF2α kinase family, binds heme as a prosthetic group (1–3). The family of eIF2α kinases (including HRI, double-stranded RNA-activated protein kinase (PKR), PKR-like endoplasmic reticulum-related kinase (PERK), and general control of amino acid biosynthesis kinase (GCN2) (4)) catalyzes phosphorylation of eIF2α. Phosphorylated eIF2α binds eIF2B and inhibits the guanine nucleotide exchange factor required for protein synthesis. HRI regulates globin synthesis in reticulocytes in response to heme availability. Earlier studies show that HRI contains two heme-binding sites. One heme is tightly bound to the N-terminal domain, whereas the other interacts weakly with the catalytic domain (5). Under conditions of heme deficiency, HRI becomes active and phosphorylates the α-subunit of eIF2 at Ser51. It is proposed that interaction of the second heme with the catalytic domain suppresses catalysis, whereas heme deficiency leads to dissociation of heme from the catalytic domain and initiation of catalysis. In iron-deficient HRI−/− mice, globins are devoid of the heme aggregate within red blood cells and precursors, resulting in hyperchromic, normocytic anemia with decreased red cell blood count, compensatory erythropoiesis hyperplasia, and accelerated apoptosis in bone marrow and spleen (6).

HRI is additionally activated by other environmental and chemical stimuli, including nitric oxide (NO), heat shock, oxidative stress, denatured proteins, and sulfhydryl-reactive reagents (3). Specifically, NO affects reticulocyte HRI in situ and in vitro complexed with heat shock protein (HSP90) and its partner proteins (7, 8). To determine the mechanism of NO-induced regulation of HRI, the heme environment of the isolated N-terminal heme-binding domain (HRI-NTD) was investigated using physicochemical methods and site-directed mutagenesis (9–11). Ishikawa et al. (10) demonstrated that NO-bound HRI-NTD forms a six-coordinate NO-heme complex and concluded that the molecular mechanism of NO-induced regulation of HRI is distinct from that of soluble guanylate cyclase (sGC), whereby formation of the five-coordinate NO-heme complex is essential for activity. However, to date, there have been no reports on the biochemical and physicochemical characterization of the HRI holoenzyme. Elucidation of the roles of heme and NO in catalysis as well as the structure of full-length HRI in the absence of the second heme should be particularly useful to determine the molecular mechanism of globin synthesis in reticulocytes.

In the present report, we examine, for the first time, the effects of the redox state of heme and NO on catalysis by full-length HRI enzyme in the absence of the second heme,
using an in vitro kinase assay. Various spectroscopic methods (including optical absorption, ESR, and resonance Raman) are applied to the system. Our data show that one of the axial ligands of the Fe(III) complex is thiolate and that NO-induced activation is accompanied by the formation of a five-coordinate NO-heme complex in HRI. These findings are distinct from the results obtained for HRI-NTD. We further discuss the heme environment of HRI and the possibility that HRI is a novel heme-based sensor protein (12–14).

EXPERIMENTAL PROCEDURES

Materials—The mouse liver Uni-ZAP cDNA library (C57BL/6) was purchased from Stratagene (La Jolla, CA). ExTaq DNA polymerase was obtained from Takara Bio (Otsu, Japan). Restriction and modification enzymes were acquired from Takara Bio, Toyobo (Osaka, Japan), New England Biolabs (Beverly, MA), and Nippon Roche (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Plasmid Construction—To construct a plasmid coding for His
tagged HRI comprising residues 1–619 (His	nMRI), mouse liver cdNA fragment was amplified by PCR using primers 5’-CTTCGAAATTCTATATG-3’ and 5’-GGGATGACACCACCTACTTTAGAAGC-3’. The PCR product was digested with EcoRI and subcloned into the pBluescript II SK+ cloning vector (Stratagene). The nucleotide sequence was confirmed using an automatic Shimadzu DSQ-2000L sequencer (Kyoto, Japan). The vector was digested with NdeI and EcoRI, and the isolated fragment was inserted into pET-28a (Novagen, Madison, WI). Next, the HRI expression vector, pET-28a/HRI, was transformed into Escherichia coli strain BL21(DE3) Codon Plus RIL (Stratagene).

Protein Expression and Purification—His6-tagged eIF2α containing amino acid residues 1–315 (His6-eIF2α), the above strategy was employed using the primers 5’-ACACATGAAATTCATATGCTGGG-3’ and 5’-CCGTGTTGCTAGCTTTAGTCGATTAAGGTTGT-3’. Protein Expression and Purification—His6-HRI was expressed from E. coli BL21 (DE3) Codon Plus harboring pET-28a/HRI (15) and purified as described previously (16, 17) with some modifications. Cell lysates containing apo-His6 HRI were reconstituted with 50 μM hemin dissolved in 0.1 M NaOH for 30 min at 4 °C (except for preparation of the apo form), fractionated with ammonium sulfate, and subjected to DEAE-cellulose (DE32, Whatman, Maidstone, UK) and Ni2+-nitrilotriacetic acid-agarose (Qiagen K.K., Tokyo, Japan) chromatography. Concentrations were determined using the Coomassie Brilliant Blue dye binding method for protein (Nakalai Tesque, Kyoto, Japan) and the pyridine hemochromogen method for heme. His6-eIF2α was expressed and purified according to a previous report (18).

In Vitro Protein Kinase Assay—The in vitro protein kinase assay was conducted as described previously, with some modifications (19). The kinase reaction mixture (20 μl) containing 20 mM Tris-HCl, pH 7.7, 2 mM Mg(OAc)2, 60 mM KCl, 2 μg of His6-eIF2α, 500 ng of His6-HRI, and 50 μM ATP was incubated at 15 °C for 10 min. This process was performed under anaerobic conditions by monitoring absorption spectra. NO solutions were prepared using the NO donor (NOC7, Sigma) in kinase reaction buffer. The reaction was stopped by adding Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% 2-mercaptoethanol, 2% SDS, 0.002% bromphenol blue) for 10 min at 95 °C and subjected to 10% SDS-PAGE. Proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad). Phosphorylated proteins were detected by immunoblotting with an anti-phosphorylated eIF2α antibody. Primary mouse anti-His6 monoclonal IgM (H-3), rabbit anti-eIF2α IgG (FL-315), and goat anti-phosphorylated eIF2α IgG (Ser52) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For immunoblotting, the membrane was blocked for 1 h with 5% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at room temperature with primary antibody diluted in Tris-buffered saline containing 0.1% Tween 20. After washing with Tris-buffered saline containing 0.1% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG, donkey anti-rabbit IgG (Amersham Biosciences), or donkey anti-goat IgG (Santa Cruz Biotechnology) for 1 h. Immunoreactive protein bands were visualized using an ECL reagent (Amersham Biosciences).

Optical Absorption Spectra—Spectral experiments under aerobic conditions were performed on a Shimadzu UV-1650 spectrophotometer maintained at 25 °C. Anaerobic spectral experiments were conducted on a Shimadzu UV-1650 spectrophotometer at 15 °C in an anaerobic glove box (Hirasawa, Tokyo, Japan).

ESR Spectra—Fe(III)-HRI complexes were prepared in 50 mM Tris-HCl, pH 8.0, containing 12% glycerol at 25 °C for ESR measurements. ESR spectra were recorded on a JEOL FE-3X spectrometer (Tokyo, Japan) at 20 K. The magnetic field was calibrated using an NMR gauss meter (Echo Electronics; model EFM-2000), and temperature was controlled with an Oxford ITC4 cryostystem.

The Fe(II)-NO complex was formed by adding NO gas to the sodium dithionite-reduced anaerobic Fe(II)-HRI sample (360 μM) in 50 mM Tris-HCl, pH 8.0. NO gas was formed under anaerobic conditions by reducing NaNO2 with an aqueous solution of sodium ascorbate in a Thunberg-type tube, with a side arm on a vacuum line. ESR spectra were recorded on a JEOL JES-TE200 spectrometer at 77 K, using a liquid nitrogen Dewar.

ESR Resonance Measurements—The Fe(III)-HRI complex (25 μM, in 50 mM Tris-HCl, pH 8.0) was placed in an air-tight spinning cell with a rubber septum and reduced by the addition of sodium dithionite at a final concentration of 10 mM. 15NO and 15CO (Shoko, Tokyo, Japan) gas was collected from a gas cylinder.

Raman scattering was performed by excitation at 413.1 and 406.7 nm with a krypton ion laser (Spectra Physics; model 206). Excitation light was focused into the cell at laser power of 5 mW for the Fe(II)-HRI and Fe(III)-HRI complexes, and 0.1–0.2 mW for the Fe(II)-HRI(CO) and Fe(II)-HRI(NO) complexes to avoid photolysis. Raman spectra were detected with a CCD camera attached to a single polychromator (Ritsu Okyakagaku; model DG-1000). Raman shifts were calibrated with indene.

RESULTS

Protein Purification—Purified His6 HRI was more than 95% homogeneous, as determined by SDS-PAGE followed by staining with Coomassie Brilliant Blue R250 (Fig. 1A). Purified HRI reconstituted with hemin displayed an apparent molecular mass of 91,700 Da and contained 0.84 mol of heme/mol of protein, indicating a stoichiometry of one heme at the N-terminal domain per subunit. Gel filtration analysis using Superose 6 (Amersham Biosciences) revealed that heme-reconstituted His6 HRI has a molecular mass of nearly 560 kDa (data not shown), similar to previously described His6 HRI (640 kDa) and native HRI (420 kDa) (17). It is well established that native HRI from reticulocyte forms a homodimer with elongated shape, although the molecular mass estimated by gel filtration appears variable, depending on experimental conditions. The difference in molecular mass (80 kDa) between the present and previous results may be due to different conditions of gel filtration column chromatographies. The yield of HRI was low at 0.2 mg/liter of culture.

Optical Absorption Spectra—We examined the absolute absorption spectra of highly purified HRI under various conditions. Absorption spectra of the reconstituted Fe(III), Fe(II), and Fe(II)-CO complexes of HRI are depicted in Fig. 1B. Spectral parameters are compared with those of other heme proteins in Table I. Soret absorption maxima of the Fe(III), Fe(II), and Fe(II)-CO complexes of HRI are depicted in Fig. 1B. Spectral parameters are compared with those of other heme proteins in Table I. Soret absorption maxima of the Fe(III), Fe(II), and Fe(II)-CO forms of HRI were observed at 418, 426, and 421 nm, respectively. Spectral data on the Fe(III) complex revealed characteristic six-coordinate low spin heme complexes similar to those of CooA (a CO-sensing transcription factor from Rhodospirillum rubrum) (20, 21), CBS (22–24), and P450cam (25). The Soret absorption of the Fe(III) complex appeared comparatively broad with a shoulder at around 360 nm, distinct from that of the isolated heme binding domain, HRI-NTD (Fig. 1, B and C). The optical absorption spectrum of the Fe(II)-HRI complex displayed peaks at 426, 531, and 560 nm, signifying six-coordinate low spin heme. The spectrum of the Fe(II)-HRI complex is similar to those of CooA (20, 21) and the acidic form (pH 6.0) of CBS (23) that contains neutral Cys thiol and His (Table I). The peak of the Fe(II)-HRI(CO) complex in the Soret region was located at around 420 nm, compared with 450 nm for cytochrome P450, suggesting that the proximal ligand is unlikely to be thiolate as in HRI-NTD (11).
Complexes were obtained in 50 mM Tris-HCl, pH 8.0. The Fe(II) complex (thick solid line), and Fe(II)-NO, Fe(II)-CO (dashed line) of Fe(III) and HRI-NTD, Fe(II)

curves of HRI (apparent molecular weight was calculated as 91,700. Optical absorption at 415–425 nm (Table I). The spectral pattern of HRI in response to NO is different from that of HRI-NTD, which displays a Soret band at 422 nm characteristic of a six-coordinate NO-heme (Fig. 1C). Despite the loss of both endogenous axial ligands following NO binding to the Fe(II)-HRI complex, the five-coordinate NO-heme did not dissociate from HRI on a gel filtration column.

A spectrum of the Fe(II)-O_{2} complex was not obtained due to its high autoxidation rate.

**In Vitro eIF2α Kinase Assay**—A previous study on HRI in rabbit reticulocyte lysates showed that NO binds to the heme iron and stimulates kinase activity, whereas binding of CO to the heme iron suppresses kinase activity (7). It is possible that in this system, regulation of HRI by NO or CO is mediated through interaction with HSP90 and its partner protein or modification of cysteine by NO. The effects of NO and CO on eIF2α kinase activities of highly purified HRI require examination to confirm these hypotheses. In this report, we investigate whether CO and NO directly affect eIF2α kinase activity of HRI via interactions with heme.

To determine the effects of NO and CO on HRI activity, we employed highly purified protein (expressed in E. coli at 15 °C) in an in vitro kinase assay, using His_{6} eIF2α as a substrate. As shown above, purified HRI contains only one heme in the N-terminal domain and releases the heme from the second binding site in the catalytic domain. Heme redox and coordination states were simultaneously monitored with a spectrophotometer. The Fe(II)-HRI complex displayed 2.4-fold higher kinase activity than the Fe(III)-HRI complex oxidized with potassium ferricyanide (Fig. 2A). Upon binding of NO to the Fe(II)-HRI complex, kinase activity was increased by 2-fold and eventually up to 5-fold, compared with the Fe(III)-HRI complex, at a concentration of 10 μM NOC9. NO-induced activation was not observed in the heme-deficient apo form of HRI (Fig. 2C). Rather, NO inhibited eIF2α activity in a dose-dependent manner. Therefore, it is evident that NO-induced activation is closely associated with the heme prosthetic group. The Fe(II)-HRI(CO) complex displayed 1.2-fold higher activity than the CO-free Fe(III)-HRI complex (Fig. 2B).

Notably, purified HRI did not incorporate ^{32}P, in the in vitro kinase assay using [γ-^32P]ATP as a substrate, suggesting that the protein is already hyperphosphorylated in E. coli (15). Therefore, in comparison with previous reports, basal activity on eIF2α was high and not significantly down-regulated upon the addition of heme (7). This finding indicates that purified HRI is less sensitive to inhibition induced by binding of the second heme. An earlier investigation shows that HRI expressed in rabbit reticulocyte lysates was high and not significantly down-regulated upon the addition of heme (7). This finding indicates that purified HRI is less sensitive to inhibition induced by binding of the second heme. An earlier investigation shows that HRI expressed in E. coli at 13 °C is hyperphosphorylated (particularly at Thr^{485} in the activation loop (15).

We additionally examined HRI activity on eIF2α following digestion of the His tag with thrombin. No significant differences in HRI activity were observed between substrate proteins with and without the tag, thus eliminating the possibility of His tag interaction.

**ESR Spectra**—The ESR spectrum of the Fe(III)-HRI-NTD complex displayed major signals at g = 3.05, 2.20 and 1.46, implying that low spin heme has His/His as axial ligands, analogous to bovine liver cytochrome b_{5} (g = 3.03, 2.23, and 1.43) (10). As shown in Fig. 3A, the ESR spectrum of the Fe(III)-HRI complex contained signals at g = 2.49, 2.28, and 1.87, characteristic of low spin heme. However, these g values are similar to those of CBS (23, 32) and CooA (33–35), which contain cysteine thiolate and nitrogenous ligands as axial ligands to the heme (Table II). Crystal field analyses of g values for low spin Fe(III) hemoproteins and model complexes are useful to identify heme axial ligands. Crystal field data ob-

---

**Fig. 1. Characterization of purified HRI from E. coli.** A, SDS-PAGE of HRI stained with Coomassie Brilliant Blue R250. Lane 1, molecular weight markers; lane 2, 1 μg of purified His_{6} HRI. The apparent molecular weight was calculated as 91,700. Optical absorption spectra of HRI (B) and HRI-NTD (C) of Fe(III) (thin solid line), Fe(II) (dotted line), Fe(II)-CO (dashed line), and Fe(II)-NO (thick solid line) complexes were obtained in 50 mM Tris-HCl, pH 8.0. The Fe(II) complex was prepared by the addition of sodium dithionite to the Fe(III) complex.

Upon the addition of NO to the Fe(II)-HRI complex, the Soret band shifted from 426 to 398 nm, indicating reductive nitrosylation of the complex, leading to the formation of a Fe(II)-HRI(NO) complex (data not shown), as reported for Hb (26). The Soret band of the Fe(II)-HRI(NO) complex at around 400 nm is characteristic of a five-coordinate NO-heme complex, analogous to sGC (27), CBS (28), CooA (29), and cytochrome c′ from denitrifying bacteria (30, 31), and is distinct from spectra of the six-coordinate NO-heme, which contain Soret bands at around 415–425 nm (Table I). The spectral pattern of HRI in response to NO is different from that of HRI-NTD, which displays a Soret band at 422 nm characteristic of a six-coordinate NO-heme (Fig. 1C). Despite the loss of both endogenous axial ligands following NO binding to the Fe(II)-HRI complex, the five-coordinate NO-heme did not dissociate from HRI on a gel filtration column.

A spectrum of the Fe(II)-O_{2} complex was not obtained due to its high autoxidation rate.

**In Vitro eIF2α Kinase Assay**—A previous study on HRI in rabbit reticulocyte lysates showed that NO binds to the heme iron and stimulates kinase activity, whereas binding of CO to the heme iron suppresses kinase activity (7). It is possible that in this system, regulation of HRI by NO or CO is mediated through interaction with HSP90 and its partner protein or modification of cysteine by NO. The effects of NO and CO on eIF2α kinase activities of highly purified HRI require examination to confirm these hypotheses. In this report, we investigate whether CO and NO directly affect eIF2α kinase activity of HRI via interactions with heme.

To determine the effects of NO and CO on HRI activity, we employed highly purified protein (expressed in E. coli at 15 °C) in an in vitro kinase assay, using His_{6} eIF2α as a substrate. As shown above, purified HRI contains only one heme in the N-terminal domain and releases the heme from the second binding site in the catalytic domain. Heme redox and coordination states were simultaneously monitored with a spectrophotometer. The Fe(II)-HRI complex displayed 2.4-fold higher kinase activity than the Fe(III)-HRI complex oxidized with potassium ferricyanide (Fig. 2A). Upon binding of NO to the Fe(II)-HRI complex, kinase activity was increased by 2-fold and eventually up to 5-fold, compared with the Fe(III)-HRI complex, at a concentration of 10 μM NOC9. NO-induced activation was not observed in the heme-deficient apo form of HRI (Fig. 2C). Rather, NO inhibited eIF2α activity in a dose-dependent manner. Therefore, it is evident that NO-induced activation is closely associated with the heme prosthetic group. The Fe(II)-HRI(CO) complex displayed 1.2-fold higher activity than the CO-free Fe(III)-HRI complex (Fig. 2B).

Notably, purified HRI did not incorporate ^{32}P, in the in vitro kinase assay using [γ-^32P]ATP as a substrate, suggesting that the protein is already hyperphosphorylated in E. coli (15). Therefore, in comparison with previous reports, basal activity on eIF2α was high and not significantly down-regulated upon the addition of heme (7). This finding indicates that purified HRI is less sensitive to inhibition induced by binding of the second heme. An earlier investigation shows that HRI expressed in E. coli at 13 °C is hyperphosphorylated (particularly at Thr^{485} in the activation loop (15).

We additionally examined HRI activity on eIF2α following digestion of the His tag with thrombin. No significant differences in HRI activity were observed between substrate proteins with and without the tag, thus eliminating the possibility of His tag interaction.

**ESR Spectra**—The ESR spectrum of the Fe(III)-HRI-NTD complex displayed major signals at g = 3.05, 2.20 and 1.46, implying that low spin heme has His/His as axial ligands, analogous to bovine liver cytochrome b_{5} (g = 3.03, 2.23, and 1.43) (10). As shown in Fig. 3A, the ESR spectrum of the Fe(III)-HRI complex contained signals at g = 2.49, 2.28, and 1.87, characteristic of low spin heme. However, these g values are similar to those of CBS (23, 32) and CooA (33–35), which contain cysteine thiolate and nitrogenous ligands as axial ligands to the heme (Table II). Crystal field analyses of g values for low spin Fe(III) hemoproteins and model complexes are useful to identify heme axial ligands. Crystal field data ob-
Comparison of the optical absorption spectral parameters between HRI complexes and other heme proteins

| Proteins | Coordination | Soret | Visible | Reference/Source |
|----------|--------------|-------|---------|------------------|
| Fe(III) complexes | | | | |
| HRI | Cys? | 418 | 538 | This work |
| HRI-NTD | His/His | 415 | 533 | Ref. 11 |
| CBS | His/Cys | 428 | 550 | Ref. 25 |
| CooA | Cys/Pro | 424 | 541, 566 | Ref. 21 |
| CooA | Cys/Pro | 418 | broad | Ref. 20 |
| P450cam | Cys/H2O, OH | 417 | 536, 569 | Ref. 25 |
| P450cam(Im) | Cys/Im | 425 | 542, 574 | Ref. 68 |
| sGC | His | 393 | 555 | Ref. 27 |
| Cytochrome c' | His | 401 | 500, 843 | Ref. 31 |
| Fe(II) complexes | | | | |
| HRI | His? | 426 | 531, 569 | This work |
| HRI-NTD | His/His? | 428 | 530, 569 | Ref. 11 |
| CBS, pH 8.0 | His/Cys | 448 | 540, 571 | Ref. 23 |
| CBS, pH 6.0 | His/CysH | 425 | 530, 558 | Ref. 23 |
| CooA | His/Pro | 426 | 529, 569 | Ref. 21 |
| P450cam(py) | Cys/py | 444 | 538, 566 | Ref. 69 |
| sGC | His | 431 | 555 | Ref. 27 |
| Cytochrome c' | His | 424 | 550 | Ref. 31 |
| Fe(II)-CO complexes | | | | |
| HRI(CO) | His | 421 | 539, 565 | This work |
| HRI-NTD(CO) | His | 422 | 537, 569 | Ref. 11 |
| CBS(CO) | His | 420 | 540, 570 | Ref. 23 |
| CooA(CO) | His | 422 | 540, 568 | Ref. 21 |
| P450cam(CO) | Cys | 446 | 551 | Ref. 69 |
| sGC(CO) | His | 423 | 541, 567 | Ref. 27 |
| Cytochrome c'(CO) | His | 418 | 534, 565 | Ref. 31 |
| Five-coordinate Fe(II)-NO complexes | | | | |
| HRI(NO) | His | 398 | 538 | This work |
| CBS(NO) | His | 390 | 538, 572 | Ref. 28 |
| CooA(NO) | His | 399 | 544, 572 | Ref. 29 |
| sGC(NO) | His | 398 | 537, 572 | Ref. 27 |
| Cytochrome c'(NO) | His | 397 | 541, 565 | Refs. 30 and 31 |
| Six-coordinate Fe(II)-NO complexes | | | | |
| HRI-NTD(NO) | His | 422 | 546, 577 | This work |
| Mb(NO) | His | 420 | 548, 579 | Ref. 70 |
| Hb(NO), R-state | His | 418 | 545, 573 | Ref. 71 |
| P450cam(NO) | Cys | 438 | 557 | Ref. 70 |

*P450cam, cytochrome P450 from d-camphor-grown Pseudomonas putida; Im, imidazole; py, pyridine.

tained using Bohan’s method (convention I) (36) applied to HRI and other related hemoproteins are listed in Table II. A crystal field diagram based on data from Table II is depicted in Fig. 3B. It is clear that HRI belongs to a family with His/Cys ligands (area i), whereas HRI-NTD is part of a family with His/His ligands (area ii). Cytochrome P450 proteins with Cys and water/hydroxyl anion as axial ligands are identified in area iii.

The Fe(II)-HRI(NO) complex exhibited a classical axially symmetric three-line spectrum characteristic of the five-coordinate NO-heme complex (Fig. 4), supporting optical absorption spectral data. The hyperfine triplet arises from coupling to a single \( ^{14}N \) nucleus of bound NO. The g values and hyperfine coupling constants measured for the Fe(II)-HRI(NO) complex (\( g_3 = 2.010 \) and \( A_3 = 1.7 \) milliteslas) were comparable with those of the NO adducts of sGC (37, 38), CBS (28), CooA (29), cytochrome c’ (30), and Fe(II)-TPP(NO) complexes (39).

Resonance Raman Spectra—To further clarify the nature of the heme environment of HRI, resonance Raman spectra of the Fe(III), Fe(II), Fe(II)-CO, and Fe(II)-NO complexes were analyzed. Spectra of the Fe(II)-HRI and Fe(III)-HRI complexes in the high frequency region are depicted in Fig. 5. Bands at 1360 and 1374 cm\(^{-1} \) for the Fe(II)-HRI and Fe(III)-HRI complexes, respectively, were assigned as redox-sensitive \( \nu_4 \) bands. Spin-and coordination state marker bands (\( \nu_3 \)) were observed at 1470 and 1493 cm\(^{-1} \) for the Fe(II)-HRI complex, which represent the five-coordinate high spin and six-coordinate low spin states, respectively (Fig. 5a). On the other hand, in the Fe(III)-HRI complex, \( \nu_3 \) was located at 1503 cm\(^{-1} \), signifying the six-coordinate low spin state (Fig. 5b). A shoulder at 1494 cm\(^{-1} \) ascribed to a five-coordinate high spin complex was observed as a minor component in Fe(III)-HRI. Upon binding of CO and NO to the Fe(II)-HRI complex, \( \nu_4 \) bands shifted to 1496 and 1507 cm\(^{-1} \), respectively (data not shown).

To characterize the proximal ligand in the Fe(II)-HRI complex, a resonance Raman spectrum of photolyzed Fe(II)-HRI(NO) was obtained with 435.7-nm 10-ns pulses. The spectrum of the photolyzed Fe(II)-HRI(NO) complex displayed features characteristic of a transient five-coordinate high spin Fe(II)-HRI complex. The iron-His stretching band (\( \nu_{\text{Fe-His}} \)) was observed at 219 cm\(^{-1} \) in Fe(II)-HRI (Fig. 5, inset) and 226 cm\(^{-1} \) in Fe(II)-HRI-NTD (data not shown). The \( \nu_{\text{Fe-His}} \) band (corresponding to iron-His bond tension) in this case is compared with those of other heme proteins in Table III. The data imply that iron-His bond tension of HRI is lower than those of sGC (204 cm\(^{-1} \)) and Mb (220 cm\(^{-1} \)).

Since the Fe-CO and C-O stretching frequencies are sensitive to the electrostatic and steric interactions of surrounding groups, resonance Raman spectra of the CO adducts of heme proteins provide valuable information of the heme environment. Low and high frequency regions of the resonance Raman spectrum of the Fe(II)-HRI(NO) complex are shown in Fig. 6. Isotope-sensitive lines were observed at 492 cm\(^{-1} \) (482 cm\(^{-1} \) for \( ^{13}C^{18}O \)) and 1967 cm\(^{-1} \) (1880 cm\(^{-1} \)) for \( ^{13}C^{18}O \) in the Fe-CO and C-O stretching regions, respectively. Accord-
HRI regulates activity in response to heme redox and coordination states with NO. However, this regulation is not observed for apo-HRI in the absence of heme. Since HRI is activated upon NO binding to heme, protein conformational changes at the heme-binding domain induced by the formation of five-coordinate NO-heme and/or dissociation of both endogenous axial ligands may be transduced to the kinase domain, leading to increased kinase activity. Based on these findings, we propose that HRI is a novel heme-based NO sensor protein.

**Heme Coordination Structure and Heme Environment of HRI**

**Fe(III)-HRI Complex**—Optical absorption, ESR, and resonance Raman spectra reveal that the Fe(III)-HRI complex is a six-coordinate low spin heme with Cys as one of axial ligands. In the crystal field diagram (Fig. 3B), HRI is located within the solid line area (i), HRI-NTD is present within the dotted line area (ii), and cytochrome P450 proteins are observed in the dashed line area (iii). The diagram clearly shows that HRI and CBS have the same coordination structure, which is distinct from that of CooA. CooA contains Cys and an N-terminal Pro residue as axial ligands, whereas P450 has Cys and water at the resting state. Therefore, we speculate that the Fe(III)-HRI complex has His and Cys residues as axial ligands, similar to CBS. The coordination structure differences between HRI (His/Cys) and HRI-NTD (His/His) appear to be a result of deletion of the C-terminal catalytic domain in HRI-NTD. Truncation of the C terminus may lead to different heme environments, due to loss of interactions between NTD and other domains.

The Soret band of HRI is broad, with a peak at 418 nm. The effect of CO on catalysis by heme-free HRI. Kinase activities were assayed using the conditions employed in lane 11–18 in C correspond to lanes 1–8 in A. Note that heme-free HRI has basal eIF2α kinase activity, since hyperphosphorylated HRI was used in the present study (cf. in vitro eIF2α kinase assay under “Results”).
concentrated enzymes (100 μM) in 50 mM Tris-HCl, pH 8.0, containing 12% glycerol was measured at 20 K. Spectra were accumulated 64 times. Modulation frequency and amplitude, 100 kHz and 1 millitesla; microwave frequency and power, 9.14874 GHz and 2 mW. B, crystal field diagram for low spin Fe(III) heme proteins. The numbers refer to the systems in Table II. The areas surrounded by the solid line (i), dashed line (ii), and dotted line (iii) contain His/Cys, His/His, and Cys/water or hydroxyl anion as the axial ligands, respectively.

spectra of low spin P450 complexes (Cys and water: axial ligands) with a 417-nm peak (Table I) (25). Binding of 2-phenylimidazolone, benzimidazolone, or indole to P450 leads to optical absorption spectra characteristic of low spin complexes coordinated with “abnormal nitrogen” (25). Soret bands of low spin complexes bound to abnormal nitrogen are observed between 416 and 420 nm. However, the crystal structure of 2-phenylimidazolone-bound P450 reveals that water is bound to the heme iron (91). An additional shoulder is observed at around 395 nm in the Soret region of the Fe(III)-HRI complex, which is ascribed to five-coordinate Cys-Fe(III) (44) (Fig. 1a). Moreover, resonance Raman spectra indicate that a small portion of a five-coordinate Fe(III)-HRI complex at 1494 cm⁻¹ is mixed with the major six-coordinate Fe(III)-HRI complex (Fig. 5b). Thus, the possibility that the peak at 418 nm is observed as a result of mixing an absorption peak of the Cys-Fe(III)-His complex at around 220 cm⁻¹, and a geometrical change of imidazole was observed concomitantly in that dihedral angles (ϕ) between the projection of imidazole plane and the nearest N(pyrrole)–Fe-N(pyrrole) axis altered from 0° (wild type) to 45° (H93G), the exogenous imidazole acts as the proximal ligand, and thus the strain on coordinated imidazole should be absent. The υFe-His frequency of the cavity mutant shifted to 225 cm⁻¹, and a geometrical change of imidazole was observed concomitantly in that dihedral angles (ϕ) between the projection of imidazole plane and the nearest N(pyrrole)–Fe-N(pyrrole) axis altered from 0° (wild type) to 45° (H93G) (54–56). Therefore, the geometry of His bound to the heme iron is also an important factor in the origin of the υFe-His frequency associated with hydrogen-bonded imidazole coordination (49). We propose that the higher frequency of the υFe-His mode in Fe(II)-HRI-NTD (226 cm⁻¹) compared with Fe(II)-HRI (219 cm⁻¹) is collectively imposed by several factors that affect the Fe-His bond upon isolation of the N-terminal heme-binding domain.

The υFe-His frequency at 219 cm⁻¹ in the Fe(II)-HRI complex is comparable with that of Mb (220 cm⁻¹). Interestingly, Mb forms a six-coordinate NO-heme, whereas HRI forms a five-coordinate NO-heme complex upon NO binding. Among the heme proteins that form the five-coordinate NO-heme complex, the υFe-His frequencies of sGC (203 and 204 cm⁻¹), CoxA (211 cm⁻¹), and SmFixLα (209 and 212 cm⁻¹) are relatively low, whereas those of Alcaligenes xylosoxidans cytochrome c’ and ovine prostaglandin endoperoxide H synthase (pGHS) are observed at 231 and 222 cm⁻¹, respectively. X-ray crystallographic analyses of A. xylosoxidans cytochrome c’ reveal that
the imidazole ring of His\(^{120}\) is oriented such that \(\phi \) is \(-33^\circ\) in the Fe(II) state (57). In the crystal structure of ovine prostaglandin endoperoxide H synthase, the imidazole ring of His\(^{388}\) is oriented such that \(\phi \) is \(-26^\circ\) in the Fe(III) state (50). Therefore, it is speculated that iron-His bond cleavage of HRI upon NO binding is associated, at least in part, with the geometry of the proximal His residue. However, a contribution of the hydrogen bond with the proximal His N\(_3\) proton and protein strain to iron-His cleavage cannot be ruled out. Structural differences between the heme distal sides trans to His of HRI, Mb, \(A.\) \textit{xylosoxidans}\(\) cytochrome \(c\), and ovine prostaglandin endoperoxide H synthase should also be considered. Specifically, a distal mutant, G117L, of CooA (with \(v_{\text{Fe-His}}\) frequency at 220 cm\(^{-1}\)) forms a five-coordinate Fe(II) complex, whereas the wild-type protein (\(v_{\text{Fe-His}}\) frequency at 211 cm\(^{-1}\)) forms a six-coordinate Fe(II) complex (41, 58, 59).

**Fe(II)-HRI(CO) Complex**—The correlation between \(v_{\text{Fe-CO}}\) and \(v_{\text{C-O}}\) frequencies is shown in Fig. 6B. The Fe(II)-HRI(CO) complex contains a neutral His as the proximal ligand trans to CO (60). The CO molecules of Fe(II)-CO complexes, which contain resonance Raman bands in the central part of the \(v_{\text{Fe-CO}}\) versus \(v_{\text{C-O}}\) plot, do not interact strongly with nearby amino acid residues. Therefore, bound CO of HRI is unlikely to interact directly or strongly with distal amino acids, despite the observed dissociation of Cys from the heme upon CO binding. The Cys residue should dissociate from the heme plane following CO binding. The observed \(v_{\text{C-O}}\) stretching frequency (1967 cm\(^{-1}\)) implies that the CO molecule is located in a somewhat hydrophobic environment.

**Fe(II)-HRI(NO) Complex**—The six-coordinate Fe(II)-NO complex shows weak back-bonding correlation, as observed from resonance Raman spectra of \(v_{\text{Fe-NO}}\) and \(v_{\text{N-O}}\) since steric interactions alter the Fe-N-O angle (61). On the other hand, the five-coordinate Fe(II)-NO complex displays strong back-bonding.

---

**Table II**

| No. \(^a\) | Proteins\(^b\) | g-values | Crystal field parameters | Reference/Source |
|---|---|---|---|---|
| 1 | HRI | 2.49 | 2.28 | 1.87 | 5.27 | 0.455 | This work |
| 2 | CBS, pH 8.5 | 2.5 | 2.3 | 1.86 | 5.03 | 0.436 | Ref. 32 |
| 3 | CBS, pH 8.0, glycerol | 2.51 | 2.33 | 1.87 | 5.05 | 0.415 | Ref. 23 |
| 4 | CBS, pH 6.0 | 2.51 | 2.33 | 1.87 | 5.05 | 0.415 | Ref. 23 |
| 5 | CooA | 2.48 | 2.26 | 1.90 | 5.84 | 0.479 | Refs. 33-35 |
| 6 | CooA, H77A | 2.42 | 2.25 | 1.92 | 6.33 | 0.448 | Ref. 33 |
| 7 | P450 | 2.41 | 2.25 | 1.92 | 6.12 | 0.479 | Ref. 72 |
| 8 | P450\(_{\text{AKO}}\) | 2.40 | 2.25 | 1.92 | 6.39 | 0.404 | Ref. 72 |
| 9 | P450\(_{\text{MTR}}\) | 2.42 | 2.26 | 1.91 | 6.08 | 0.403 | Ref. 72 |
| 10 | P450\(_{\text{M}}\)(N-MeIm) | 2.54 | 2.26 | 1.88 | 5.46 | 0.602 | Ref. 72 |
| 11 | P450\(_{\text{M}}\)(n-Oc amine) | 2.49 | 2.25 | 1.90 | 5.85 | 0.573 | Ref. 72 |
| 12 | P450\(_{\text{M}}\)(metyrapone) | 2.48 | 2.26 | 1.89 | 5.72 | 0.517 | Ref. 72 |
| 13 | Hb(Am), pH 6.5 | 2.92 | 2.23 | 1.46 | 3.17 | 0.571 | Ref. 74 |
| 14 | Mb(Im), pH 9.6 | 2.93 | 2.22 | 1.52 | 3.55 | 0.527 | Ref. 75 |
| 15 | Cytochrome \(b\), pH 6.2 | 3.05 | 2.22 | 1.41 | 3.22 | 0.503 | Ref. 76 |
| 16 | LHb(Im), pH 7.0 | 2.93 | 2.27 | 1.49 | 3.11 | 0.599 | Ref. 77 |

\(^a\) The numbers correspond to Fig. 3B.

\(^b\) P450\(_{\text{AP}}\), rat liver microsomal P450 induced by phenobarbitone; P450\(_{\text{AKO}}\), rat liver microsomal P450 induced by Aroclor 1254; P450\(_{\text{MTR}}\), P450 in bovine adrenal cortex submitochondrial particles, \(N\)-MeIm, \(N\)-methylimidazole; n-Oc amine, n-octylamine; Im, imidazole; LHb, leghemoglobin.
ing character similar to the Fe(II)-CO complex (62). The resonance Raman spectral correlation between the νFe-CO and νN-O frequencies is shown in Fig. 7B. The correlation line was obtained by Fe(II)-TPP(NO) complexes with electron-withdrawing and -donating substituents on the phenyl groups (62). It is suggested that the Fe(II)-HRI(NO) complex contains a five-coordinate NO-heme complexes of proteins listed in Table III; closed squares, phenyl-modified derivatives of the Fe(II)-TPP(NO) complexes (62).

### Table III

| Proteins | νFe-His | νFe-CO | νC=O | νFe-NO | νN-O | Reference/Source |
|----------|---------|--------|------|--------|------|-----------------|
| HRI      | 219<sup>a</sup> | 492    | 1967 | 524    | 1677 | This work       |
| HRI-NTD  | 226<sup>a</sup> | 494    | 1963 | NR     | NR   | This work, Ref. 11 |
| sSC      | NR      | 493    | 1960 | NR     | NR   | Ref. 10         |
| sSC<sub>2</sub> | 204     | 473    | 1987 | 521    | 1681 | Ref. 78         |
| CBS      | NR      | 497    | 1959 | 520    | 1660 | Refs. 79 and 80 |
| CooA     | 211<sup>a</sup> | 496    | 1969 | NR     | NR   | Ref. 82         |
| SmFixL<sup>a</sup> | 209, 212 | 498    | 1962 | 525    | 1676 | Refs. 42, 84, and 85 |
| Cytochrome c<sup>c</sup> | 231     | 491    | 1966 | 526    | 1661 | Ref. 31         |
| sPGHS    | 206, 222 | 502, 529 | 1952 | 526    | 1667 | Refs. 50 and 88 |
| Ec-Dos   | 214<sup>a</sup> | 486    | 1973 | 563    | 1632, 1576 | Refs. 87 and 88 |
| HemAT    | 225     | 494    | 1964 | NR     | NR   | Ref. 89         |
| Mb       | 220     | 507    | 1947 | NR     | 1614 | Refs. 61 and 90 |

<sup>a</sup> EcDos, E. coli direct oxygen sensor protein.

<sup>a</sup> Transiently observed immediately after CO photodissociation.

<sup>c</sup> NR, not reported.

---

Fig. 6. Resonance Raman spectra of the Fe(II)-HRI(CO) complex in low frequency and high frequency regions excited at 413.1 nm. A, spectra of Fe(II)-HRI complexes of $^{12}$C$^{16}$O (a) and $^{13}$C$^{16}$O (b) and the difference ($^{12}$C$^{16}$O $-$ $^{13}$C$^{16}$O) (c). B, inverse correlation between the 2ν<sub>CO</sub> and ν<sub>C=O</sub> frequencies. Closed circles, HRI and HRI-NTD; open circles, various hemoglobin, myoglobin, peroxidases, and proteins listed in Table III that contain histidine as the proximal ligand. Open triangles are specific for cytochrome P450s and nitric-oxide synthases that contain cysteine as a proximal ligand (60).

Fig. 7. Resonance Raman spectra of the Fe(II)-HRI(NO) complex in the low frequency and high frequency regions excited at 406.7 nm. A, spectra of the Fe(II)-HRI complexes of $^{14}$N$^{16}$O (a) and $^{15}$N$^{16}$O (b) and the difference ($^{14}$N$^{16}$O $-$ $^{15}$N$^{16}$O) (c). B, inverse correlation between the 2ν<sub>NO</sub> and ν<sub>N-O</sub> frequencies. Closed circles, HRI; open circles, the five-coordinate NO-heme complexes of proteins listed in Table III; closed squares, phenyl-modified derivatives of the Fe(II)-TPP(NO) complexes (62).
coordinate NO-heme. Moreover, NO does not have strong polar interactions with nearby amino acid residues and may be in a hydrophobic environment, similar to Fe(II)-TPP(NO) and Fe(II)-TPP(p-tolyl)(NO) complexes. In contrast, the NO molecules of A. xylosoxidans cytochrome c/H11032 and ovine prostaglandin endoperoxide H synthase are located in basic environments, whereby Arg124 in cytochrome c/H11032 and Fe(II)-TPP(NO) complex with electron-donating substituents on the phenyl groups (such as Fe(II)-TPP(4-OH)(NO) and Fe(II)-TPP(4-OCH3)(NO) complexes) are present.

**Kinase Activity and Coordination Structure**

Our in vitro eIF2α kinase assay data indicate that the Fe(II)-HRI complex has higher activity than the Fe(III)-HRI complex, and formation of the five-coordinate NO-heme complex further enhances catalysis. The heme coordination structures proposed in this study are shown in Fig. 8. In the Fe(III)-HRI complex, the heme iron is possibly coordinated with endogenous Cys and His or water/hydroxyl anion. When the Fe(III)-HRI complex is reduced to Fe(II)-HRI, His and neutral Cys thiol appear to coordinate to the heme iron and to form a six-coordinate low spin complex. In Fe(II)-HRI(CO), Cys is dissociated from the heme iron. Upon binding to the Fe(II)-HRI complex, NO interacts with the heme iron either distal or proximal to the heme plane, resulting in the formation of a five-coordinate NO-heme complex.

To identify the axial ligand for heme, we prepared Ala mutants of His 75, His 78, and His 80 (His75, His81, and His83 for rabbit, respectively) and performed preliminary in vitro kinase experiments. The H75A protein was expressed similarly to the wild-type protein but did not display kinase activity, whereas the H80A mutant exhibited activity comparable with that of the wild-type enzyme. The H75A mutant was degraded at the expression stage. In view of the data, we speculate that His75 is an axial ligand of HRI. To identify the axial ligands of the Fe(III)-HRI and Fe(II)-HRI complexes, further site-directed mutagenesis and spectroscopic studies are currently under way.

**Mechanism of NO-induced Activation of HRI**

Conformational changes induced by ligand binding to the heme are crucial for effector sensing and signal transduction in heme-based sensor proteins. Previous spectral studies on HRI-NTD show that the five-coordinate NO-heme complex formed via cleavage of the Fe-His bond is not necessary for HRI activation (10). In this study, we use full-length HRI for in vitro kinase assay and spectral measurements and demonstrate that HRI is activated by NO, forming the five-coordinate NO-heme complex. These results imply a similar activation mechanism to that of sGC.

The activation mechanism of binding of NO to sGC has been discussed for A. xylosoxidans cytochrome c', in view of crystallographic and spectroscopic data (57, 63, 64). One proposal is that NO binds to the vacant distal side of the heme trans to proximal His ligand, forming a five-coordinate NO-heme complex. Due to the repulsive trans effect of bound NO, proximal His dissociates from the heme, forming a five-coordinate NO-heme with 300-fold increased activity (63, 65). Therefore, it is
HRI activity than the Fe(III) complex, and 4) the Fe(II)-

Eur.

Bio.

Chem.

Biochem.

J. Biol.

Chem.

J. Biol.

Chem.

Biochim.

Biophys.

Res.

Commun.

Biochemistry

Mol.

Biol.

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochim.

Biophys.

Acta

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

Biochemistry

J. Biol. Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.

J. Biol.

Chem.
Activation of Heme-regulated Eukaryotic Initiation Factor 2α Kinase by Nitric Oxide Is Induced by the Formation of a Five-coordinate NO-Heme Complex: OPTICAL ABSORPTION, ELECTRON SPIN RESONANCE, AND RESONANCE RAMAN SPECTRAL STUDIES
Jotaro Igarashi, Akira Sato, Teizo Kitagawa, Tetsuhiko Yoshimura, Seigo Yamauchi, Ikuko Sagami and Toru Shimizu

J. Biol. Chem. 2004, 279:15752-15762.
doi: 10.1074/jbc.M310273200 originally published online January 29, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310273200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 90 references, 31 of which can be accessed free at http://www.jbc.org/content/279/16/15752.full.html#ref-list-1