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Mechanism of Heme-Dependent Protein Regulation for Intracellular Heme Metabolism

(細胞内ヘム代謝におけるヘム依存的な蛋白質の機能制御機構)

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2. Yuta Watanabe, Takeshi Uchida, Kazuhiro Iwai, and Koichiro Ishimori
“Characterization of the heme-dependent regulation mechanism of Iron Regulatory Protein (IRP) by fluorescence anisotropy”
The 23rd Symposium on Role of Metals in Biological Reactions, Biology and Medicine (Tokyo, Japan) June 21-22, 2013
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“Characterization of the heme effect on the interaction between Iron Regulatory Protein (IRP) and the targeted mRNA by fluorescence anisotropy”
The 24th Symposium on Role of Metals in Biological Reactions, Biology and Medicine (Kyoto, Japan) June 14-15, 2014
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“Heme-dependent Regulation Mechanism of the Interaction between Iron Regulatory Protein (IRP) and the Target mRNA”
7th Asian Biological Inorganic Chemistry Conference (AsBIC-VII) (Gold Coast, Australia) November 30-December 5, 2014
6. Yuta Watanabe, Yukiko Takeda, Takeshi Uchida, Kazuhiro Iwai, and Koichiro Ishimori
“Heme-dependent Regulation Mechanism of Iron Regulatory Proteins (IRPs) by Cell-based Reporter Assay”
Biochemistry and Molecular Biology 2015 (Hyogo, Japan) December 1-4, 2015
7. Yuta Watanabe, and Koichiro Ishimori
“Heme is a regulatory molecule for the antioxidant enzyme, peroxiredoxin-1”
The 10th Symposium on Biorelevant Chemistry CSJ (Kanazawa, Japan) September 7-9, 2016

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CHAPTER I

GENERAL INTRODUCTION

Transition metals play an important role in exerting the protein function as a cofactor. Iron is the most essential element in our body among the transition metals, although the weight in standard healthy adults is only 3 ~ 5 g (1). More than 95% of functional (not storage) iron in the human body is in the form of heme (iron-protoporphyrin IX complex) (2). Heme-containing protein (hemoprotein) is essential for the physiological function on the basis of its transferrable redox state and gas binding property. In contrast to the importance of heme, heme that is not the component of hemoprotein (free heme) has the cellular toxicity by its hydrophobicity and high reactivity. Thus, the intracellular heme metabolism is finely regulated in mammal. In Chapter I, the general information for the physiological role and the metabolism of heme is described.

1.1. Physiological Role of Heme.

Heme consists of four pyrrolic rings attached to one another in a cyclic form via methine bridges, and iron atom chelated with nitrogen atom in each pyrrole (Figure 1.1). Hemoproteins are essential for the diverse biological processes such as gas binding and transport, catalytic reactions and electron transfer. Hemoglobin and myoglobin are one of the most ubiquitous hemoprotein

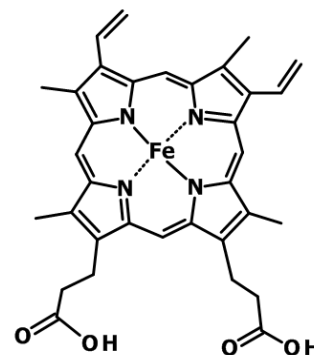


Figure 1.1 The structure of heme.

for transport and storage of oxygen, respectively. The transferrable redox state of iron makes it extremely useful for driving intricate reactions in biology such as the redox reaction and electron transfer (3–5). For example, the redox cycle of heme iron in cytochrome *c* plays a role in electron transfer to its partner protein, cytochrome *c* oxidase, for ATP synthesis (6). Moreover, recent studies have been shown that heme works as the regulatory molecule (7–9), which can modulate many functions such as transcription (10–12), translation (13, 14), protein localization (15), protein degradation (11) and microRNA metabolism (16). The

widespread roles of heme have been established as the invariable positions on the biological reaction.

In contrast to the diverse necessity of heme, however, excess heme has the cellular toxicity that disrupts biomolecules by reaction with reactive oxygen species (ROS) (17–19). The hydrophobic property of heme tends to be embedded to membrane, and the membrane is damaged by the peroxidase activity of heme coupled with hydrogen peroxide (H_2O_2) as the substrate (20), although the cellular H_2O_2 level is normally controlled by the enzymes such as catalase and peroxidase (Figure 1.2) (21). Furthermore, free heme is an abundant source of redox-active iron that can participate in the Fenton's reaction to produce toxic hydroxyl radicals ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-$) (22). The reaction was proceeded not only by iron, but also by heme (23). ROS damage to lipid membrane, proteins and nucleic acids, indicating that the disruption of biomolecule cause oxidative stress (24, 25). It is essential for preventing heme from the undesired degradation or oxidation. Therefore, the heme metabolism must be tightly controlled to provide enough to meet cellular requirements while avoiding excessive levels that are toxic (26).

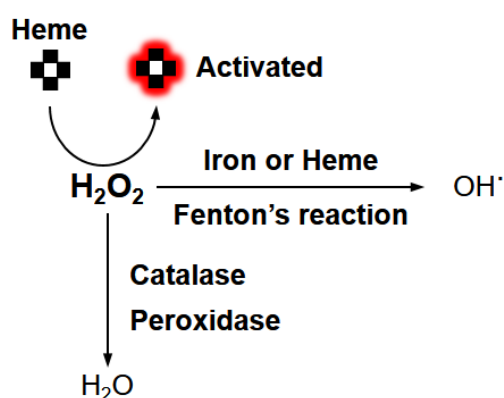


Figure 1.2 Activation and detoxification of hydrogen peroxide.

H_2O_2 was the source of $\text{OH}^\cdot$ production through the Fenton's reaction, or reactive heme derivative. The biological level of H_2O_2 was regulated by catalase or peroxidase, which was rapidly converted to water.

1.2. Intracellular Heme Metabolism.

The regulation of intracellular heme metabolism is involved in the diverse proteins, which can be divided into iron acquisition, heme synthesis, heme export, and heme degradation (Figure 1.3). Prior to description for the regulation of heme metabolism, the outline of the intracellular heme metabolism have been introduced. Iron is delivered in plasma via an iron transport protein, called transferrin (Tf), and binding to transferrin receptor (TfR) lead to the receptor-mediated endocytosis (27). Iron is released to endosome, and then exported to cytosol through the divalent metal transporter-1 (DMT1). Newly assimilated cytosolic iron is transported either to mitochondria for heme synthesis, to ferritin for storage or to outside cell via ferroportin (Figure 1.3) (1, 3). Heme biosynthesis has been elucidated over the past several decades (28, 29). Heme is synthesized through the 8-step enzymatic reactions from glycine and succinyl-CoA as starting materials. The terminal step of heme biosynthesis is insertion of Fe^{2+} by ferrochelatase (FC) (30), which catalyzes the insertion of iron atom into protoporphyrin IX (PPIX), thus forming heme (Figure 1.3). FC is located in inner membrane mitochondria, indicating that heme would be utilized by transportation for other organelles (31). Heme is first released toward the cytosol via mitochondrial transporter FLVCR1b (32–34). The exported heme is incorporated to apoprotein that needs heme. Excess heme was degraded by heme oxygenase (HO) to iron, which is then reused or stored in ferritin (35).

As described previously, heme has the diverse role as the prosthetic group of heme-binding proteins. These heme-binding proteins are localized in nucleus, endoplasmic reticulum as well as cytosol (Figure 1.3). In nucleus, the target DNA-binding activity of some transcriptional factor is regulated by heme. Heme degradation by HO is occurred in the endoplasmic reticulum. However, the toxicity of free heme makes it difficult to spontaneously transport heme to other organelles. In other words, cytosolic heme has the conflicted property,

prosthetic group of protein and cellular toxicity. Thus, the protection of free heme in cytosol must be needed.

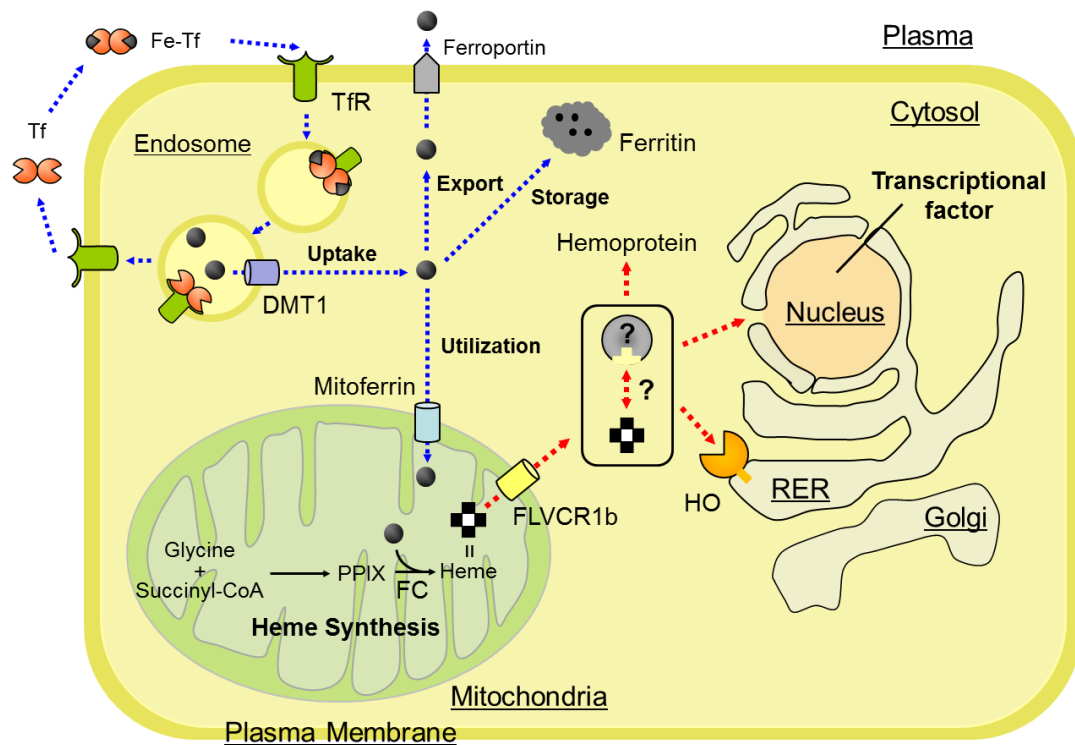


Figure 1.3 Outline of the intracellular heme metabolism

The final step of heme biosynthesis occurs in mitochondria. The nascent heme moiety is exported via putative heme transporter, FLVCR1b. Heme-binding proteins are localized in various organelles, although heme has the inherent peroxidase activity. Free heme can easily disrupt the lipid bilayer of cell plasma membranes. Thus, HO degrades excess heme to prevent the oxidative stress. Abbreviations: Tf, transferrin; TfR, transferrin receptor; DMT1, divalent metal transporter; RER, rough endoplasmic reticulum; Golgi, Golgi body; HO, heme oxygenase; PPIX, protoporphyrin IX; FC, ferrochelatase; FLVCR1b, feline leukemia virus subgroup C receptor 1b.

1.3. Characterization of PRX1 as Heme-Binding Protein (Chapter II).

To prevent the unexpected peroxidation associated free heme in cytosol, heme-binding proteins would be required to part heme from ROS. There are some candidates for heme-binding protein as listed in Table 1.1 (31), because the dissociation constants of heme ($K_{d, \text{heme}}$) for all proteins are below 1 μM , which corresponds to the upper limit of the cytosolic heme level (36). Although these proteins were proved to heme-binding proteins, no experiment has been carried out to show that the function maintains the cytosolic heme homeostasis.

Table 1.1 Cytosolic heme-binding proteins and its binding affinity for heme.

Proteins	$K_{d, \text{heme}}$ (M) ^a	Methods	Reference
L-FABP ^b	1.2×10^{-7}	Fluorescence	(37)
GST ^c	$10^{-6} \sim 10^{-7}$ ^d	Fluorescence	(38)
p22HBP ^e	2.6×10^{-8}	Radioactivity	(39)
HBP23 ^f	5.5×10^{-8}	Fluorescence	(40)

^aDissociation constants of proteins for heme; ^bliver fatty acid binding protein; ^cglutathione S-transferase; ^dmeasured four subtype of GST in Ref. 38; ^e22 kDa heme-binding protein; ^f23 kDa heme-binding protein

In spite of the importance of the heme-binding proteins in cytosol, the functional characterizations of cytosolic heme-binding proteins for the protection of synthesized heme have not yet been confirmed. Thus, I performed the proteomic search for cytosolic heme-binding proteins using hemin-agarose. The proteomics analysis identified Peroxiredoxin-1 (PRX1), which is a human homolog of HBP23 (Table 1.1). PRX1 is originally known as an antioxidant enzyme, which causes reduction of H_2O_2 to water using the cysteine residue. On the other hand, the expression of HBP23 is induced by the hemin-treated cells (41, 42), indicating that HBP23 would be responded to the cytosolic heme level. HBP23 and PRX1 share 97% sequence homology and two characteristic heme binding motifs, Cys-Pro (CP) motifs, in their amino acid sequence (Figure 1.5). Therefore, PRX1 is

thought to be a convincing candidate for the cytosolic heme-binding protein. However, the heme binding to PRX1 was not confirmed, and the functional significance remained elusive.

```

Homo sapiens PRX1      MSSGNAKIGHAPNFKATAVMPDGQFKDISLSDYKGKYVVFYPLDFTF 50
Rattus norvegicus HBP23 MSSGNAKIGHAPNFKATAVMPDGQFKDISLSDYKGKYVVFYPLDFTF 50
*****

Homo sapiens PRX1      VCPTETIAFSDRAEEFKKLNCPVIGASVDSHFCHLAWVNTPKKQGGLGPM 100
Rattus norvegicus HBP23 VCPTETIAFSDRAEEFKKLNCPVIGASVDSHFCHLAWVNTPKKQGGLGPM 100
*****

Homo sapiens PRX1      NIPLVSDPKRTIAQDYGVKKADEGISFRGLFIIDDKGILRQITVNDLPVG 150
Rattus norvegicus HBP23 NIPLVSDPKRTIAQDYGVKKADEGISFRGLFIIDDKGILRQITVNDLPVG 150
*****

Homo sapiens PRX1      RSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK 199
Rattus norvegicus HBP23 RSVDETLRLVQAFQFTDKHGEVCPAGWKPGSDTIKPDVQKSKEYFSKQK 199
*****

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Figure 1.4 Amino acid sequence alignment of human PRX1 with rat HBP23.

The alignment was performed using ClustalX (Version 2.1). The CP motifs and other cysteine residues are shown in a black background and in red, respectively.

In Chapter II, to characterize PRX1 as a heme-binding protein, I constructed the expression and purification system of PRX1 in *E. coli*. Purified PRX1 bound to heme with a stoichiometry 1:1 and a dissociation constant of heme was determined to be 0.17 μM , a value within the concentration range of free heme in the cytosol (43). Spectroscopic characterization, including UV-vis and resonance Raman spectroscopy, revealed that the heme-PRX1 complex contained the five-coordinated high-spin heme with the cysteine ligand. A mutational study showed that Cys52, donated by one of the CP motifs, bound heme, leading to the loss of the original enzyme activity. However, the hemin peroxidase activity and H_2O_2 -mediated hemin degradation of heme-PRX1 were significantly reduced compared with free hemin. These properties are beneficial for cells. Taken together, PRX1 scavenges free hemin to prevent unexpected peroxidation of biomolecules at the cost of diminished enzyme activity, suggesting that PRX1 protects the cytosolic free heme to act as the prosthetic group.

The cytosolic heme is available as a prosthetic group or a regulatory molecule under the protection of heme from the toxicity by PRX1. One of the known cytosolic heme-binding protein is iron regulatory protein 2 (IRP2), which is RNA-binding protein to regulate the translation involved in the iron metabolism such as iron uptake or storage. In iron-replete cells, heme binding to IRP2 triggers the degradation of IRP2 itself, resulting in the loss of the RNA-binding activity. The iron-dependent degradation (IDD) domain, which has a CP motif as a heme-binding site, plays an important role in the degradation. However, IRP2 has other CP motifs except the IDD domain, and the CP motifs are conserved to another homolog, IRP1, allowing me to hypothesize that there is a common regulation mechanism of the RNA-binding activity by heme in both IRPs. Therefore, I focused on the regulation mechanism of IRPs by heme.

1.4. Regulation of Iron Metabolism by IRPs/IRE Systems.

IRPs are the primary regulators of the iron metabolism through the modulation of the expression level of the genes that is involved in the iron metabolism such as iron uptake, storage, utilization, and export (Figure 1.3) (44). IRPs bind to the characteristic iron-responsive element (IRE) in the untranslated regions (UTRs) of mRNA. IRPs/IRE system are involved in the regulation of adequate expression levels of iron metabolism proteins (1, 45). Functional IRE motifs have also been identified in mRNAs encoding divalent metal transporter (DMT1) and ferroportin (46). The wide existence of IRE suggests that IRPs/IRE system plays a central role in the cellular iron metabolism. Thus, I focused on the regulation mechanism of IRPs for the IRE binding.

IRPs determine the fate of several mRNA upon binding to their IRE in their UTRs. Here, I describe the role of IRPs in the mRNAs encoding transferrin receptor 1 (TfR1) and ferritin (Ft), which defines prototype examples of the coordination of post-transcriptional regulation by IRPs/IRE interaction (Figure 1.6). TfR1 plays a role in uptake of iron by interaction with iron-bound transferrin (Tf), which is the main transporter of iron in bloodstream, and Ft is capable of storing the excess iron atoms in cell (47). IREs are evolutionary conserved stem-loop structures of 25-30 nucleotides (48). TfR1 mRNA contains multiple IREs within its 3' UTR, while the mRNAs encoding Ft contain a single IRE in their 5'UTRs (49). The IRE binding activity of IRPs is regulated by cellular iron availability. In iron deficiency, IRPs bind with high affinity to target IREs. This results in stabilization of TfR1 mRNA and steric inhibition of the Ft mRNA translation (50, 51). Under these conditions, accumulation of TfR1 promotes the uptake of cellular iron from plasma Tf, while inhibition of Ft biosynthesis prevents storage of iron, allowing its metabolic utilization (Figure 1.6). Conversely, in response to excess cellular iron, IRPs are inactivated, which leads to the TfR1 mRNA degradation and Ft mRNA translation (Figure 1.6). This behavior minimizes further

internalization of iron via TfR1 and promotes the storage of excessive intracellular iron into ferritin. Therefore, the iron content in cell is adjusted by association/dissociation of IRPs to IRE.

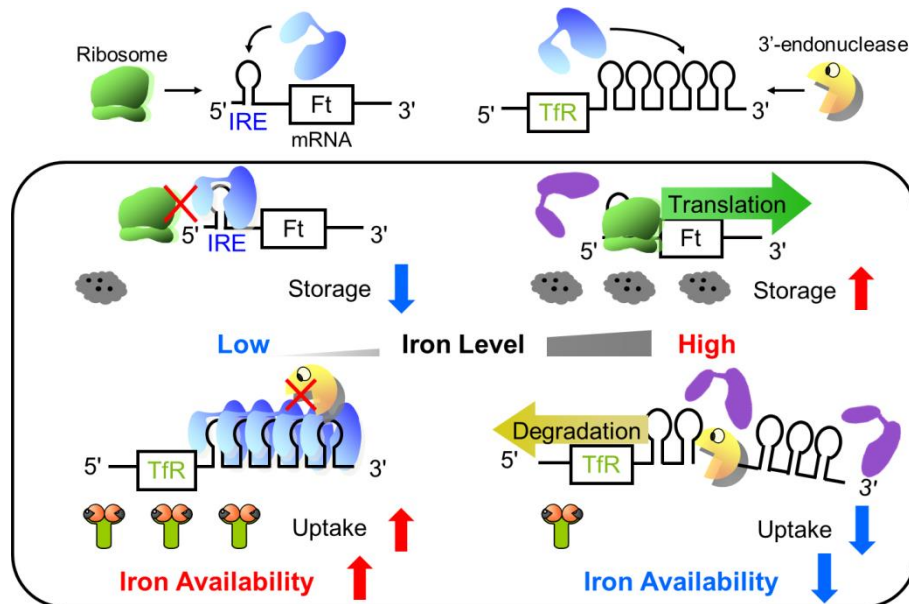


Figure 1.6 Translational regulation via IRPs/IRE system.

In iron-deficient cells, IRPs bind to IRE, present in the 5'-untranslated regions (5'-UTRs) of mRNAs encoding proteins involved in iron storage (Ft). The binding of IRPs to IRE inhibits their translation, whereas IRPs interaction with 3'-UTRs in TfR1 transcript increases its stability. As a consequence, TfR1-mediated iron uptake increases whereas iron storage in Ft decreases, thereby increasing the iron availability in cell. In iron-replete cells, IRPs lose IRE-binding activity, resulting in the opposite effect in iron-deficient cells.

Two homologous proteins, IRP1 and IRP2 share high homology (57% homology) and the regulation mechanism (52). However, the regulatory mechanism for the inactivation of the IRE-binding activity in the iron-replete condition differs between IRPs. IRP1 binds an Fe-S cluster binding causes a conformation change, which is sterically hindered the IRE-binding site of IRP1 (53–56). IRP1 has high level of similarities to aconitase, indicating that IRP1 acts as a multifunctional protein depending on binding Fe-S cluster (57, 58). Thus, Fe-S cluster is thought to be the sole regulatory factor to IRP1 for a long time. Unlike IRP1, IRP2 has a unique IDD domain, to which heme specifically binds. Heme binding to the IDD domain activates molecular oxygen to ROS, which subsequently attacks IRP2 itself to cause oxidative modification (59–62), leading to the proteasomal degradation of IRP2 (63–65). The degradation of IRP2 is concomitant loss of its IRE-binding activity. In contrast, some groups suggested that the oxidative modification was not directly related to the degradation of IRP2 (66, 67).

Recently, some of the previous studies reported that the IRE-binding activity of IRP1 is suppressed by heme (68, 69), although the heme-binding site of IRP1 was not elucidated. It is worthy of note that IRPs have two characteristic heme-binding motifs, CP motifs, in their amino acid sequences, and the both CP motifs are conserved between IRPs. In IRP2, the IDD domain has been already known for the heme binding to the CP motif (62), allowing me to hypothesize that heme regulates the IRE-binding activity not only in IRP2, but also in IRP1. However, because these experiments were performed using cell lysate, the heme-binding environment of IRP1 and the involvement of the IRE-binding activity by heme were not confirmed.

1.5. Effect of Heme on the IRE-Binding Activity of IRP (Chapter III, IV).

An amino acid analysis of IRPs suggests that putative heme-binding CP motifs are present in IRP1. These CP motifs are conserved in IRP2, indicating that the IRE-binding activity of IRPs is commonly regulated by the heme binding. Previous investigation performed in my laboratory showed that IRP1 also bound heme via Cys (70). As expected, the mutational analysis of each CP motif in both IRPs decreased the stoichiometry of heme to IRPs, indicating that the CP motifs were the heme-binding site. Therefore, I investigated the effects of heme binding on the IRE-binding activity of IRPs in both *in vitro* and the cellular condition.

In Chapter III, to elucidate whether heme acts as the regulatory molecule for IRPs, spectroscopic characterization was performed to elucidate the heme-dependent regulation of IRPs. I expressed and purified IRP1 in insect cells. The UV-vis spectrum showed that heme-IRP1 had two coordination environments, which were the Cys and Cys/H₂O coordination. The axial H₂O ligand was retained by hydrogen bonding with distal His as shown in deoxy form of myoglobin. From the crystal structure of IRP1, His207 was the unique His near Cys300, whereas there was no His around Cys118, showing that the coordination environments of Cys118 and Cys300 were Cys and Cys/H₂O, respectively. Fluorescence anisotropy measurement was performed to detect the interaction between IRPs and IRE. Consequently, the IRE-binding activity of IRPs was obviously suppressed in the presence of heme. The results in Chapter III suggest that the cytosolic heme availability affects the IRE-binding activity of IRPs. Considering the cellular condition, however, there are various heme-binding proteins except IRPs, suggesting that the regulation of the IRE-binding activity by heme must be confirmed in cellular condition.

In Chapter IV, to observe the IRE-binding activity of IRPs in cellular condition, I constructed a reporter assay system using *lacZ* as a reporter plasmid, which encodes

β -galactosidase (β -gal). The IRE sequence was inserted into the upstream of the *lacZ* open reading frame (IRE-*lacZ*). These IRP and IRE-*lacZ* plasmids were transfected to mammalian cells, and the catalytic activity of β -gal was monitored. The activity was drastically reduced in IRE-*lacZ* co-expressed with IRP1, indicating that the β -gal expression was inhibited by binding IRP1 to IRE. In contrast, when the transfected cells were cultured in the heme-containing medium, the β -gal activity was recovered. The increase of the β -gal activity was also shown in stimulating the heme biosynthesis by treatment of precursor, although there was no change for treatment of iron. These results indicate that the IRE-binding activity of IRP1 is correlated with the cellular heme content. IRP1 has been reported for binding Fe-S cluster near the IRE-binding site, and Fe-S cluster-free IRP1 was the target for degradation by the ubiquitin-proteasome pathway (71, 72). However, the protein level of IRP1 was not changed by increasing the intracellular heme level, although the expression level of β -gal was also increased. These results indicate that heme acts as the regulatory molecule for IRP1 in cellular condition. Because iron is mainly utilized for heme biosynthesis, the regulation of the IRE-binding activity of IRP by heme would be connected between iron and heme metabolism.

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CHAPTER II

CHARACTERIZATION OF HEME BINDING

ENVIRONMENT AND FUNCTIONAL SIGNIFICANCE OF

HUMAN PEROXIREDOXIN-1 (PRX1)

Abstract

The cytosolic heme-binding protein was important for receiving synthesized heme in mitochondria. Peroxiredoxin-1 (PRX1) was identified from the immunoprecipitation using hemin-agarose, suggesting that PRX1 was a heme-binding protein. PRX1 was the primary peroxidases involved in hydrogen peroxide catabolism. Although PRX1 has a characteristic Cys-Pro heme-binding motif, the significance of heme binding to PRX1 remained to be elucidated. Here, I examined the effect of heme binding to PRX1. PRX1 was expressed in *Escherichia coli* and purified to homogeneity. Spectroscopic titration demonstrated that PRX1 binds heme with a 1:1 stoichiometry and a dissociation constant of 0.17 μM . UV-vis and resonance Raman spectra of heme-PRX1 suggested that Cys52 is the axial ligand of ferric heme. PRX1 peroxidase activity was lost upon heme binding, reflecting the fact that Cys52 is not only the heme-binding site but also the active center of peroxidase activity. Interestingly, heme binding to PRX1 caused a decrease in the toxicity and degradation of heme, significantly suppressing H_2O_2 -dependent heme peroxidase activity and degradation of PRX1-bound heme compared with that of free hemin. By virtue of its cytosolic abundance ($\sim 20 \mu\text{M}$), PRX1 thus functions as a scavenger of cytosolic hemin ($<1 \mu\text{M}$). Collectively, our results indicate that PRX1 has a dual role; Cys-dependent peroxidase activity and cytosolic heme scavenger.

2.1. Introduction

Heme is synthesized in mitochondria and exported to cytosol. Owing to the toxicity of heme, the cytosolic heme-binding protein is essential for utilizing heme safely (Table 1.1). Although there are some candidates for a heme transport protein in cytosol (1, 2), functional significance of heme binding is rarely understood. For the purpose of identification of the cytosolic heme-binding protein, the proteomic analysis using hemin-agarose resin was performed. Lysates from human embryonic kidney 293T are mixed with hemin-agarose resin. Interacting proteins with the hemin-agarose resin were separated on electrophoresis, followed by the digestion and measurement of the matrix-assisted laser deionization time-of-flight (MALDI-TOF) spectra. The obtained mass over charge (m/z) was checked on database. As a result, one of the MS spectra was corresponding to peroxiredoxin-1 (PRX1) (Figure 2.1). PRX1 belongs to the members of peroxiredoxin (Prx: EC 1.11.1.15) family, which are ubiquitous peroxidases found in almost all kingdoms (3–5). On the other hand, a rat homolog of PRX1, 23 kDa heme-binding protein (HBP23) identified as heme-bound form (6). HBP23 and PRX1 shares 97% amino acid identity (Figure 1.4), allowing me to hypothesis that PRX1 acts as the candidate for the cytosolic heme-binding protein. To this purpose, I focused on the heme binding to PRX1.

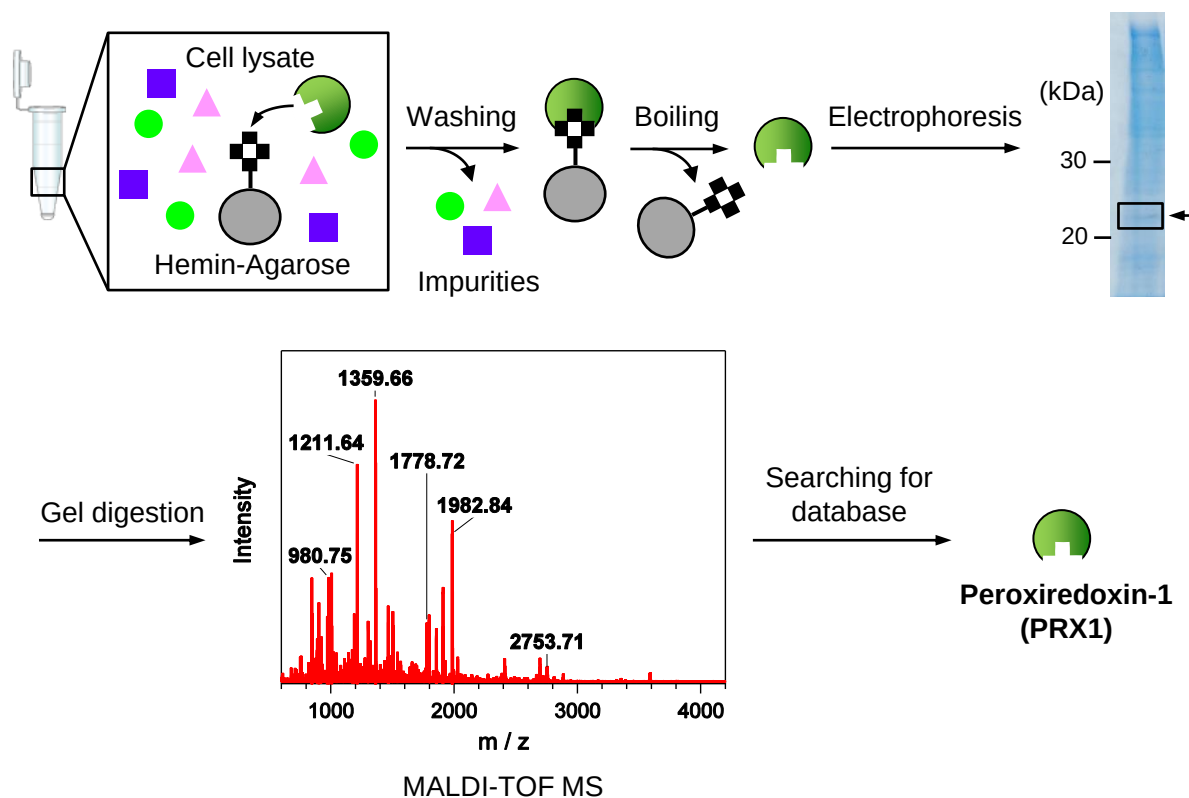


Figure 2.1 Identification of PRX1 as the cytosolic heme-binding protein.

The cell lysates from 293T were mixed with hemin-agarose. After washing the impurities, bound proteins were obtained by boiling, followed by the analysis to electrophoresis. The band corresponding to rectangle in the gel image was digested and measured MALDI-TOF spectra. The ratio of mass for charge (m/z) obtained from spectra was analyzed by the database, matching peroxiredoxin-1 (PRX1).

Prx family is ubiquitous peroxidase, which causes reduction of hydrogen peroxide (H_2O_2), but reaction scheme is definitely different from other heme peroxidases such as horseradish peroxidase (7). The enzymatic reaction of Prx proteins is occurred without requiring cofactors such as metals or prosthetic groups (Figure 2.2). The active center of Prx proteins consists of two Cys residues, and one Cys residue is reactive with H_2O_2 ; thus, members of the Prx family are termed as cysteine-dependent peroxidase. The two cysteine residues in PRX1 are a hallmark of its peroxidase activity, which are an N-terminal peroxidatic Cys (Cys_P-SH) and a C-terminal resolving Cys (Cys_R-SH). Both cysteines are contributed by the CP motifs. Cys_P-SH is oxidized by H_2O_2 to cysteine sulfenic acid (Cys_P-SOH), and then forms an intermolecular disulfide bond in a head and tail manner with

Cys_R-SH in the adjacent monomer (Figure 2.2). Under physiological conditions, the disulfide linkage is reduced by NADPH-dependent thioredoxin and thioredoxin reductase to regenerate Cys_P-SH (8, 9). To the best of my knowledge, there are no other proteins in which the cysteine in the active center of enzymes also forms a CP motif, leading me to hypothesize that the role of PRX1 was replaced upon heme binding. However, the involvement of heme binding to PRX1 in the cysteine-dependent peroxidase activity remains to be elucidated.

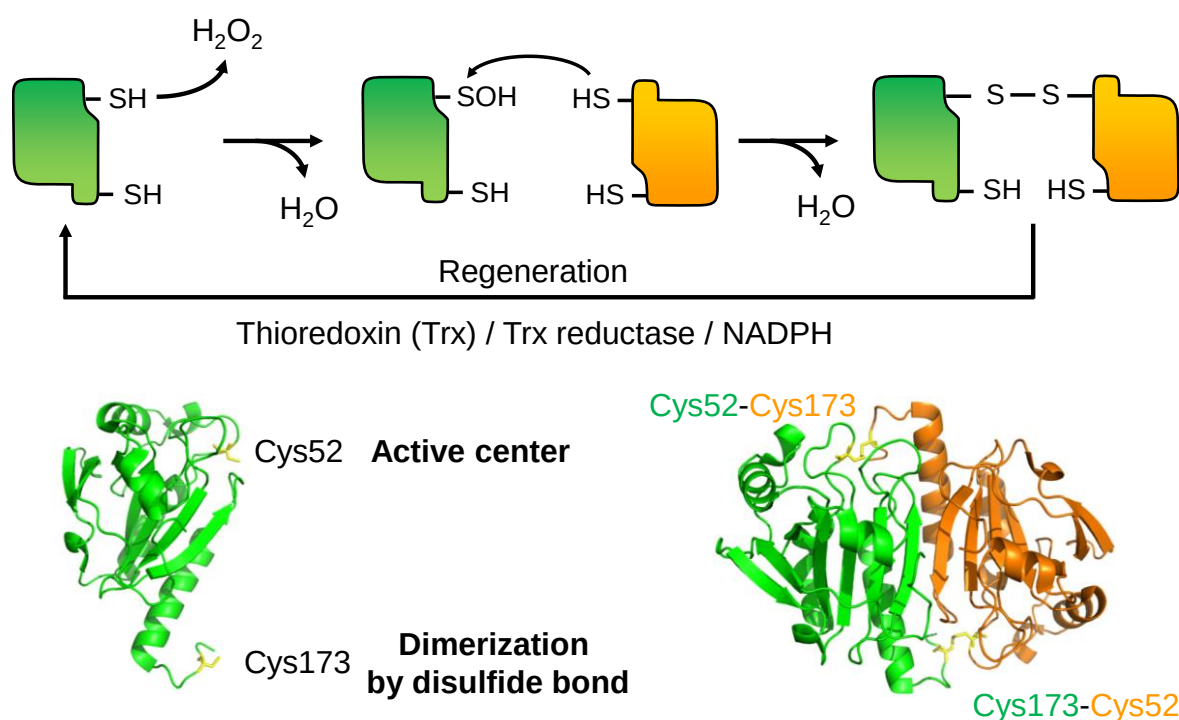


Figure 2.2 Catalytic cycle of Peroxiredoxin-1

Peroxidatic Cys (Cys_P-SH) is reacted with H₂O₂ and heterolytic cleavage of O-O bond in H₂O₂. Cys_P-SOH is easily reacted with another subunit rendering head-to-tail homodimer. This homodimer is regenerated by the NADPH-dependent thioredoxin system.

In Chapter II, to characterize PRX1 as a cytosolic heme-binding protein, heme binding environment was investigated using UV-vis and resonance Raman spectra. Heme binding site was determined by the mutation of Cys residue including the CP motifs. Furthermore, hemin peroxidase activity and H₂O₂-mediated hemin degradation of heme-PRX1 were examined to check the protection of heme from H₂O₂.

2.2. Experimental Procedures

2.2.1. Materials.

All chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), Nacalai Tesque (Kyoto, Japan) and Sigma-Aldrich (St. Louis, MO, USA), and were used without further purification.

2.2.2. Protein Expression and Purification.

A full-length *PRDX1* gene construct, codon optimized for *E. coli* expression, was purchased from Eurofin Genomics (Tokyo, Japan) and amplified by polymerase chain reaction (PCR). The amplified fragment was cloned into the pET-28b vector (Merck Millipore, Darmstadt, Germany) using a Gibson Assembly kit (New England Biolabs, Ipswich, MA, UK). Primers used for the construction of the clone are shown in Table 2.1. The thrombin recognition site (Leu-Val-Pro-Arg-Gly-Ser) in the pET-28b vector was mutated to the HRV 3C protease recognition site (Leu-Glu-Val-Leu-Phe-Gln ↓ Gly-Pro), as described previously (10). The N-terminus of purified PRX1 has extra three amino acids (Gly-Pro-His) from the protease recognition site and NdeI cloning site. After confirming the correct gene sequence by DNA sequencing (Eurofin Genomics), the *PRDX1* expression plasmid was transformed into the *E. coli* BL21(DE3) strain (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and cultured at 37 °C in LB broth supplemented with 50 µg/mL kanamycin. After cultures reached an optical density at 600 nm (OD₆₀₀) of 0.6-0.8, expression of the His-tagged fusion protein was induced with 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG). The cells were further grown at 37 °C for 4 hours and harvested by centrifugation. The cell pellet (~ 3.0 g) was stored at -80 °C until use. The pellet was subsequently thawed on ice and suspended in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1 % Nonidet P-40, and 1 mM dithiothreitol (DTT) at pH 8.0. The suspension was further incubated for 30

minutes at 4 °C after adding 1 mg/mL lysozyme and DNase. The sample was disrupted by the sonication and then centrifuged at $40,000 \times g$ for 30 minutes. The resulting supernatant was loaded onto a HisTrap HP column (GE Healthcare, Uppsala, Sweden) pre-equilibrated with 50 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole (pH 8.0). The resin was extensively washed with 50 mM Tris-HCl, 500 mM NaCl and 50 mM imidazole (pH 8.0), and then bound protein was eluted with 50 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole (pH 8.0). Eluted PRX1 protein was concentrated to ~ 2 mL using an Amicon Ultra (Merck Millipore). The His₆-tag was removed by adding 1 mM DTT and Turbo 3C protease (Accelagen, San Diego, CA, USA) to the solution and incubating for ~ 16 hours at 4 °C. After cleavage, the reaction mixture was again applied to a HisTrap column and the flow-through fraction was collected. Tag-cleaved PRX1 was then applied to a HiLoad 16/600 Superdex 200 preparatory grade gel-filtration column (GE Healthcare) pre-equilibrated with 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). PRX1 contains 199 amino acid residues and has a calculated molecular mass of 22,110 Da. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldose (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) were used as molecular mass protein standard markers for estimation of PRX1 molecular mass. The yield of purified PRX1 was 2-3 mg from 1 L of LB culture. Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5 % polyacrylamide gels. Purified protein in 50 mM HEPES-NaOH, 100 mM NaCl and 1 mM DTT (pH 7.4) was frozen in liquid nitrogen and stored at -80 °C. DTT was removed using a PD-10 MiniTrap column (GE Healthcare) prior to use. The protein concentrations were estimated from the absorbance at 280 nm with a molar extinction coefficient (ϵ_{280}) of $18,450 \text{ M}^{-1} \text{ cm}^{-1}$ using ProtParam (<http://web.expasy.org/protparam/>).

Cysteine residue mutations (C52S, C71A, C83A and C173A) were introduced by PCR using a PrimeSTAR mutagenesis basal kit from Takara Bio (Otsu, Japan). The primers employed for mutagenesis are shown in Table 2.1. All mutations were verified by DNA sequencing.

Table 2.1 Oligonucleotide used for construction of expression vectors.

The underlined bases signify the Gibson Assembly signal sequence (for cloning) and introduced mutations (for mutation). S: sense-strand, AS: anti-sense-strand

Constructs	Strand	Primers (5'→ 3')	Application
pET28b-PRX1	S	<u>CCAGGGGGCCCCATATG</u> TCGAGTGGCAACGCGAAAA	Cloning
	AS	GGAGCTCGAATTCTCATTCTGTTTGGAAAAGTAC	
C52S	S	TTTGTG <u>AGT</u> CCGACGGAAATCATTGCC	Mutation
	AS	CGTCGG <u>ACT</u> CACAAAGGTAAAATCGAG	
C71A	S	CTGAAT <u>GCCCA</u> AGTGATTGGCGCAAGC	Mutation
	AS	CACTTG <u>GGC</u> ATTTCAGTTTCTTGAAGTC	
C83A	S	CACTTT <u>GCC</u> CACTTGCGGTGGGTCAATAC	Mutation
	AS	CAAGTG <u>GGC</u> AAAGTGGGAATCAACGCT	
C173A	S	GAAGTG <u>GCT</u> CCAGCTGGTTGGAAACCA	Mutation
	AS	AGCTGG <u>AGC</u> CACTTCGCCATGTTTGTC	

2.2.3. Absorption Spectroscopy.

All absorption spectra were obtained using a V-660 UV-vis absorption spectrophotometer (JASCO, Japan). Hemin binding studies were conducted by difference absorption spectroscopy. Hemin was dissolved in 0.1 M NaOH, and its concentration was determined on the basis of absorbance at 385 nm using a molar extinction coefficient (ϵ_{385}) of $58.44 \text{ mM}^{-1} \text{ cm}^{-1}$. Aliquots of the hemin solution (1 mM) were added to both the sample cuvette containing 10 μM apo-PRX1 and the reference cuvette at 25 °C. Spectra were recorded 3 minutes after the addition of hemin. The absorbance at 370 or 371 nm was plotted as a function of heme concentration, and the dissociation constant ($K_{d, \text{heme}}$) was calculated using the quadratic binding equation,

$$\Delta\text{Absorbance} = \frac{1}{2}(\epsilon_{\text{binding}} - \epsilon_{\text{free}}) \left\{ [P] + [H] + K_{d, \text{heme}} - \sqrt{([P] + [H] + K_{d, \text{heme}})^2 - 4[P][H]} \right\} \quad (2.1)$$

where $\Delta\text{Absorbance}$ is the absorption difference at a given concentration. $\epsilon_{\text{binding}}$ and ϵ_{free} are the extinction coefficients of heme-PRX1 complex and hemin, respectively. $[P]$ and $[H]$ are the concentrations of the PRX1 and hemin, respectively. The molar extinction coefficient at Soret band of heme-bound PRX1 was determined using the pyridine hemochrome method (11).

Absorption spectrum for the heme-PRX1 complex was measured, and then adding pyridine and NaOH at a final concentration of 12.5% and 0.1 M, respectively, to obtain Fe^{3+} -hemichrome. The reaction mixture was reduced to Fe^{2+} -hemochrome by sodium dithionite. The amount of heme bound to PRX1 was calculated by following the absorbance at 557 nm between Fe^{3+} -hemichrome and Fe^{2+} -hemochrome using an extinction coefficient of $28.15 \text{ mM}^{-1} \text{ cm}^{-1}$. Protein concentrations were determined using a Pierce 660 nm protein assay reagent (Thermo Scientific, Waltham, MA, USA) according to a manufacturer's instruction using BSA as a standard.

2.2.4. Dissociation Rate Constant of PRX1.

Heme transfer measurements were made in a 0.5-mL reaction mixture containing 2 μ M heme-PRX1 and 20 μ M apo-myoglobin in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4) at 25 °C. Apo-myoglobin was prepared by extracting heme from equine skeletal muscle myoglobin using the acid/methylethylketone method (12). The Soret peak of myoglobin (408 nm) was traced using a JASCO V-660 UV-vis absorption spectrophotometer. The dissociation rate (k_{off}) of heme was calculated by fitting the data to a single-exponential (equation 2.2) or double-exponential (equation 2.3) equation using Igor Pro (WaveMetrics, Portland, OR, USA) as follows:

$$A_t = A_0 + A_1 \exp(-k_{\text{off},1}t) \quad (2.2)$$

$$A_t = A_0 + A_1 \exp(-k_{\text{off},1}t) + A_2 \exp(-k_{\text{off},2}t) \quad (2.3)$$

where A_0 is the initial absorbance, A_1 and A_2 are the proportional constants and k_{off} is the dissociation rate constants (s^{-1}).

2.2.5. Resonance Raman Spectroscopy.

Resonance Raman spectra were recorded with a single monochromator (SPEX500M, Jobin Yvon) equipped with a liquid nitrogen-cooled CCD detector (Spec-10:400B/LN; Roper Scientific, Princeton, NJ, USA). Samples were excited at a wavelength of 413.1 nm delivered by a krypton ion laser (BeamLok 2060; Spectra Physics, Santa Clara, CA, USA). The laser power at the sample point was adjusted to ~ 5 mW for the ferric and ferrous forms. A lower laser power (0.1 mW) was used for the CO-bound form to prevent photodissociation. Raman shifts were calibrated using indene, CCl_4 , acetone and an aqueous solution of ferrocyanide. The accuracy of the peak positions of well-defined Raman bands was $\pm 1 \text{ cm}^{-1}$. Samples for resonance Raman experiments were prepared at a concentration of approximately 30 μ M in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4).

2.2.6. Detection of Cysteine-Dependent Peroxidase Activity.

The activity of PRX1 was determined by measuring the amount of dimerization after the reaction with H_2O_2 using non-reducing SDS-PAGE. The reaction was initiated by mixing H_2O_2 (30 μM) with PRX1 (10 μM) at 25 °C, and then stopped 5 minutes after initiating the reaction by adding catalase to remove excess H_2O_2 . Subsequently, 5 $\times$ SDS loading buffer containing 60 mM Tris-HCl, 25% (v/v) glycerol, 2% (w/v) SDS and 0.1% (w/v) bromophenol blue (pH 6.8) was added, followed by incubation for 10 minutes at room temperature. Sample was analyzed on 12.5 % polyacrylamide gels with Coomassie Brilliant Blue staining. H_2O_2 and catalase concentrations were determined spectrophotometrically using an absorption coefficient of 43.6 $\text{M}^{-1} \text{cm}^{-1}$ (at 240 nm) and of 324 $\text{mM}^{-1} \text{cm}^{-1}$ (at 405 nm), respectively. The band intensities were quantified using NIH ImageJ software.

2.2.7. CD Spectroscopy.

CD spectra were recorded with a JASCO J-1500 CD spectrometer using 10-mm path-length cuvettes. Each spectrum represents the integration of three consecutive scans from 190 to 260 nm at 0.2-nm intervals. The spectrum bandwidth was kept at 1 nm, and the scan speed was 20 nm/min. PRX1 protein was diluted to a final concentration of 10 μM in 50 mM sodium phosphate/100 mM NaCl (pH 7.4). Hemin was titrated and incubated for 10 minutes at 4 °C prior to measurement. Ellipticity was expressed as mean residue molar ellipticity ($\text{deg cm}^2 \text{dmol}^{-1}$) calculated using the JASCO software. The ratio of α -helix (f_{H}) content was estimated from the molar ellipticity at 222 nm ($[\theta]_{222}$) using equation 4 (13):

$$f_{\text{H}} = -([\theta]_{222} + 2340)/30300 \quad (2.4)$$

2.2.8. Size-Exclusion Chromatography for Determination of Oligomeric State.

Size-exclusion chromatography was performed using an ENrich SEC 650 10/300 column (Bio-Rad, Hercules, CA, USA) at 4 °C using an ÄKTA 10S instrument (GE Healthcare). The column was calibrated using the same molecular markers as used for protein purification. The eluate was monitored at 280 nm.

2.2.9. Heme Peroxidase Activity Assay.

Heme peroxidase activity was determined spectrophotometrically by measuring co-oxidation of the substrate by H_2O_2 (14). The assay was performed in 0.5 mL of reaction mixture containing with 360 μM H_2O_2 , 1.25 mM 4-aminoantipyrine (4-AAP), 86 mM phenol, and 1.5 μM hemin or heme-PRX1 at 25 °C. The reaction was initiated by adding H_2O_2 , and antipyrilquinoneimine absorbance at 512 nm was monitored using a JASCO V-660 UV-Vis spectrophotometer.

2.2.10. H_2O_2 -Mediated Hemin Degradation.

The hemin-degradation reaction was monitored by UV-vis spectroscopy. Following addition of 30 μM H_2O_2 to 10 μM hemin or heme-PRX1 in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4), the spectrum was recorded at 1-minute intervals for 30 minutes. Soret band peaks at 386 nm and 370 nm correspond to free hemin and PRX1-bound hemin, respectively. The data were normalized by subtracting the zero time point value from subsequent time points.

2.3. Results

2.3.1. Expression and Purification of PRX1.

Human PRX1 was expressed in *Escherichia coli* strain BL21(DE3) and purified using Ni²⁺-affinity and size-exclusion chromatography. The purified PRX1 protein had an apparent molecular mass of 22 kDa and was estimated to be ~95% pure by SDS-PAGE (Figure 2.3A). Three major peaks on the size-exclusion chromatogram, with elution times of 54.8, 76.7 and 88.0 mL, corresponded to a decamer, dimer and monomer, respectively, based on molecular masses estimated from the migration of the bands against standard proteins (Figure 2.3B) (4). Molecular mass of the fraction eluted at 45.3 mL was much larger than 669 kDa, indicating that the fraction is a soluble aggregate (Figure 2.3B). The monomeric form of PRX1 was used in subsequent analysis, because it was a major component of the purified protein, and the dimeric form was inactive to H₂O₂ (Figure 2.3C). The decameric form was highly active, but the amount was too small, and the importance of the decameric form remained to be controversial (15).

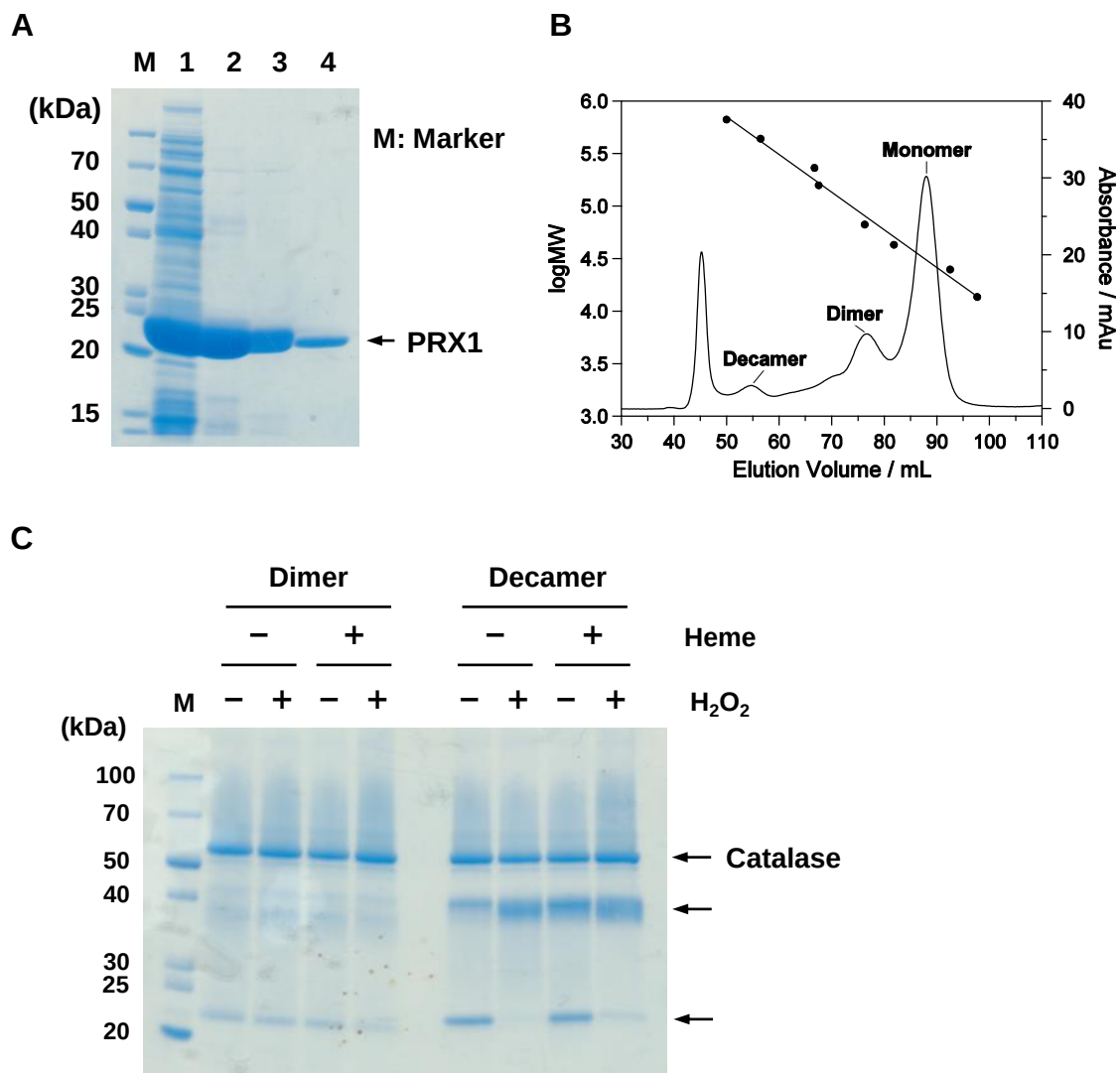


Figure 2.3 Purification Profile of PRX1 and dimerization assay of oligomeric PRX1.

(A) SDS-PAGE gel of PRX1 stained with CBB Stain One including molecular mass marker (Lane M), whole-cell protein extracts (Lane 1), purified His-tagged PRX1 (Lane 2), purified His-tag cleaved PRX1 (Lane 3) and purified PRX1 after gel-filtration chromatography (Lane 4). (B) Profile of PRX1 on a gel-filtration column (HiLoad 10/600 Superdex 200 pg) pre-equilibrated with 50 mM HEPES-NaOH and 100 mM NaCl (pH 7.4). The elution volumes of standard proteins as follows: thyroglobulin, 50.0 mL; ferritin, 56.4 mL; catalase, 66.7 mL; aldose, 67.5 mL; albumin, 76.3 mL; ovalbumin, 81.8 mL; chymotrypsinogen A, 92.5 mL; and RNase A, 97.6 mL. (C) Apo- or holo-dimeric or decameric PRX1 (10 μ M) was treated with 30 μ M H₂O₂ for 5 minutes at 25 °C in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The reaction was stopped by adding 1 μ M catalase to quench excess H₂O₂, after which PRX1 was resolved by non-reducing SDS-PAGE and stained with Coomassie Brilliant Blue. The bands at ~60 kDa correspond to catalase.

2.3.2. Heme-Binding Properties of PRX1.

Although HBP23 is known as a heme-binding protein (6), the UV-vis spectrum of purified PRX1 had no absorption in the visible region, indicating that it was devoid of heme (Figure 2.4A). To confirm the heme-binding ability of PRX1, I performed spectroscopy-based heme-titration experiments. Difference absorption spectra obtained by subtracting the spectrum for free heme from that of PRX1-bound heme at different concentrations are shown in Figure 2.4B. A plot of the difference absorbance versus heme concentration at 371 nm suggested that PRX1 binds to heme with a 1:1 stoichiometry (Figure 2.4B, inset). Because the titration curve was not completely saturated even in the presence of 3 equivalents of heme, the binding stoichiometry of heme to PRX1 was confirmed using the pyridine hemochrome method, which also yielded a value of 1:1 (Figure 2.4C). The $K_{d, \text{heme}}$ of PRX1 for heme calculated from equation 2.1 was $0.17 \pm 0.03 \mu\text{M}$, which is slightly larger than that for rat HBP23 (55 nM) (6), and significantly larger than myoglobin (16). The difference spectrum showed a prominent peak at 413 nm. Because the plot of absorbance difference at 413 nm was monotonously increased, the emergence of this peak suggests non-specific heme binding. However, deconvolution of the Soret band of the purified heme-PRX1 after removal of excess of heme by gel filtration showed no peaks at 413 nm, indicating that the amount of the non-specific heme binding is negligible for this experiment. The millimolar extinction coefficient of heme-PRX1 at 370 nm was determined to be $84 \text{ mM}^{-1} \text{ cm}^{-1}$ by the pyridine hemochrome method (Figure 2.4C).

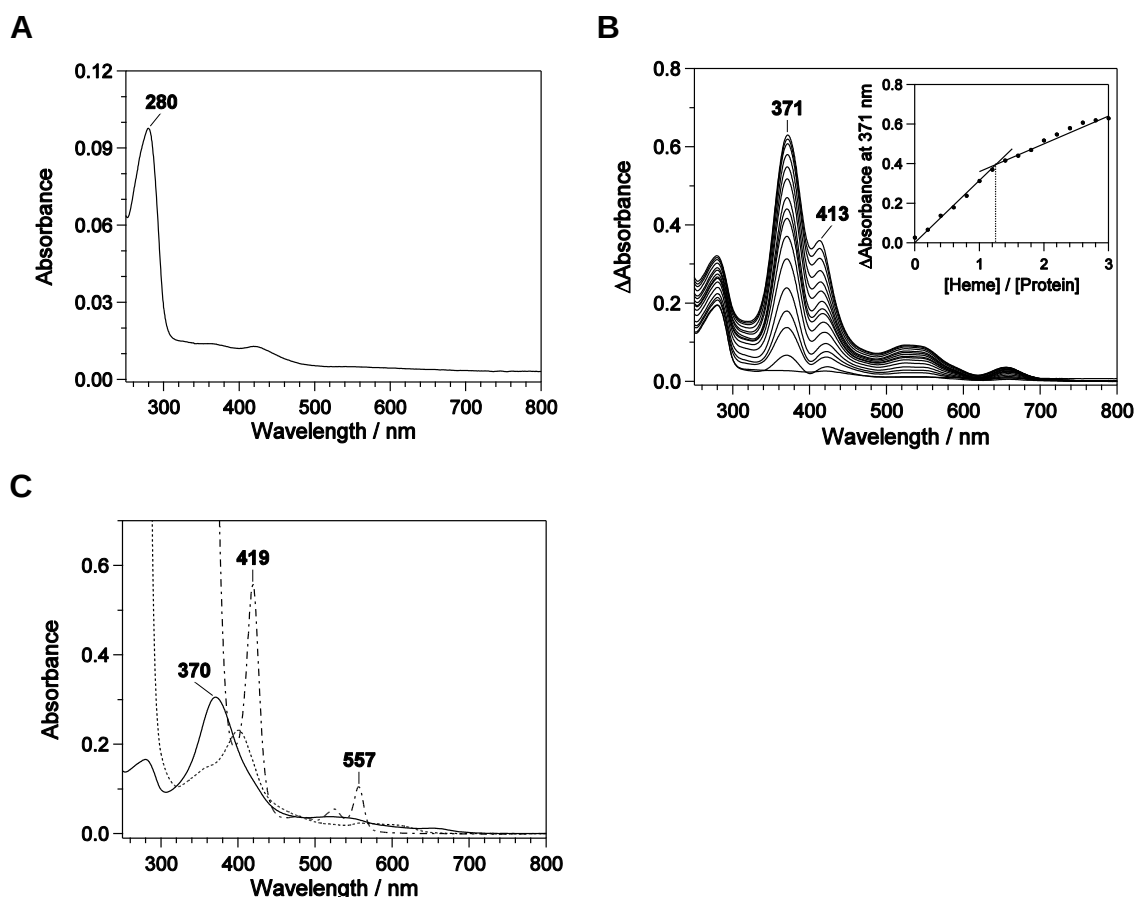


Figure 2.4 Heme titration and pyridine hemochrome method.

(A) Optical absorption spectra of PRX1 as purified. (B) Absorption difference spectra of heme binding to PRX1. Absorption difference spectra of heme binding to PRX1 following stepwise addition of heme (2 – 30 μM) to PRX1 (10 μM) versus buffer blank in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). inset: Absorbance difference at 371 nm as a function of heme concentrations (C) Pyridine hemochrome assay of PRX1. Absorption spectra on the heme-PRX1 complex (solid line), Fe^{3+} -hemichrome (dotted line) and Fe^{2+} -hemichrome (dashed-dotted line). The amount of heme that is bound to PRX1 was calculated by following the absorbance change at 557 nm between oxidized and reduced hemochrome using an extinction coefficient of $28.15 \text{ mM}^{-1} \text{ cm}^{-1}$ (11). The assay was repeated for three times, and the average amount of heme was calculated to be 36.7 μM . Protein concentrations were determined to be 30.7 μM by a Pierce 660 nm protein assay reagent using BSA as a standard.

2.3.3. Absorption Spectra of the Heme-PRX1 Complex.

The heme-binding environment was next investigated using UV-vis absorption spectroscopy. PRX1 was reconstituted with a 1.2-fold excess of heme, and then unbound heme was removed using a gel-filtration column. Absorption spectra of heme-reconstituted PRX1 are shown in Figure 2.5. The Soret absorption maximum of ferric PRX1 was 370 nm, and the visible maxima were 521 and 653 nm. The far blue-shifted Soret peaks at ~370 nm is known as a signature for a five-coordinate high-spin heme with an axial thiol ligand (17, 18) (Table 2.2), indicating that PRX1 binds heme through Cys. Upon reduction of heme by sodium dithionite, the broad Soret band was appeared at 389 nm with a shoulder at approximately 420 nm, indicative of abnormal coordination behavior. The spectrum of the ferrous heme-binding form was different from that of reduced free heme, whereas similar spectra were previously reported for the MBP (maltose-binding protein)-conjugated, iron-dependent degradation domain in IRP2 (iron regulatory protein 2) (19) and heme oxygenase H25Y mutant, in which proximal His is replaced with Tyr (20). For the ferrous H25Y mutant, it was concluded that the bond between heme and axial ligand is disrupted or a weak ligand such as a water molecule is bound upon reduction of heme. Thus, the coordination environment of the ferrous heme of heme-PRX1 would be no proximal ligand, or coordination of a water molecule or protonated Cys to heme. The Soret peak of the carbon monoxide (CO) adduct was observed at 420 nm, with Q-bands at 539 and 569 nm, which is characteristic of the His-Fe-CO coordination (20), indicating that Cys was replaced with His, as observed in cystathionine- β -synthase (CBS) (21).

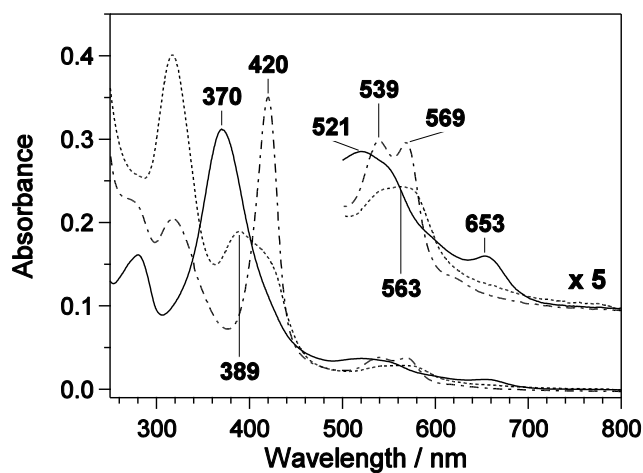


Figure 2.5 Absorption spectra of PRX1.

Absorption spectra shown in ferric (solid line), ferrous (dotted line) and ferrous-CO (dashed-dotted line) measured in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4).

Table 2.2 Absorption maxima of the heme-PRX1 complex compared with those of other heme proteins.

Protein	Ligand	Soret (nm)	Visible (nm)	Reference
PRX1	Cys	370	521, 653	This study
Bach1 (Type 2)	Cys	371	521, 541, 650	(17)
Irr ^a	Cys	372	ND ^e	(18)
P450 _{cam} (+cam) ^b	Cys	391	ND	(22)
CBS ^c	Cys/His	428	ND	(23)
CooA	Cys/Pro ^d	424	541, 566	(24)

^aIron response regulator protein; ^b*d*-camphor-bound P450_{cam}; ^ccystathionine- β -synthase;

^dN-terminal proline binds to heme; ^enot determined.

2.3.4. Dissociation Rate Constants of PRX1.

Because the dissociation rate constant (k_{off}) value is related to the axial ligand of heme (Table 2.3), I confirmed the Cys coordination of PRX1 to the ferric heme by measuring the k_{off} of heme from heme-PRX1. To this end, I mixed heme-PRX1 with a 10-fold excess of apo-myoglobin and then monitored changes in absorption spectra (Figure 2.6A). The Soret band was shifted from 371 nm to 408 nm immediately after the addition of apo-myoglobin, indicating the formation of holo-myoglobin. The increase in absorbance at 408 nm was plotted against time (Figure 2.6B, 1) and fit to both single-exponential (equation 2.2) and double-exponential (equation 2.3) functions. The double-exponential fit, which produced a less random residual contribution than the single-exponential fit (Figure 2.6B, 2 and 3), yielded dissociation rate constants for PRX1 of $k_{\text{off},1} = 4.5 \times 10^{-4} \text{ s}^{-1}$ (56%) and $k_{\text{off},2} = 4.0 \times 10^{-3} \text{ s}^{-1}$ (44%) (Table 2.3), indicating the presence of two binding sites with different affinities, despite the fact that 1 equivalent of heme bound to PRX1, as discussed below (Figure 2.4A). The k_{off} value for PRX1 was closer to that of heme-regulated inhibitor (HRI, also known as eIF2 α kinase), whose axial ligand is Cys (25), than that of His-coordinated myoglobin or Tyr-coordinated BSA (Table 2.3) (16). Therefore, the behavior of the k_{off} value is consistent with Cys coordination to heme.

Table 2.3 Heme dissociation rates for PRX1 and other heme-binding proteins.

Protein	Ligand	$k_{\text{off},1} \text{ (s}^{-1}\text{)}^a$	$k_{\text{off},2} \text{ (s}^{-1}\text{)}^b$	Reference
PRX1	Cys	4.5×10^{-4} (56%)	4.0×10^{-3} (44%)	This study
Myoglobin	His	8.4×10^{-7}	ND ^e	(16)
HRI ^c	Cys	1.5×10^{-3}	ND	(25)
BSA ^d	Tyr	1.1×10^{-2}	ND	(16)

^aRate constants calculated assuming a single-exponential equation (Eq. 2); ^brate constants calculated assuming a double-exponential equation (Eq. 3); ^cheme-regulated eIF2 α kinase;

^dbovine serum albumin; ^enot determined.

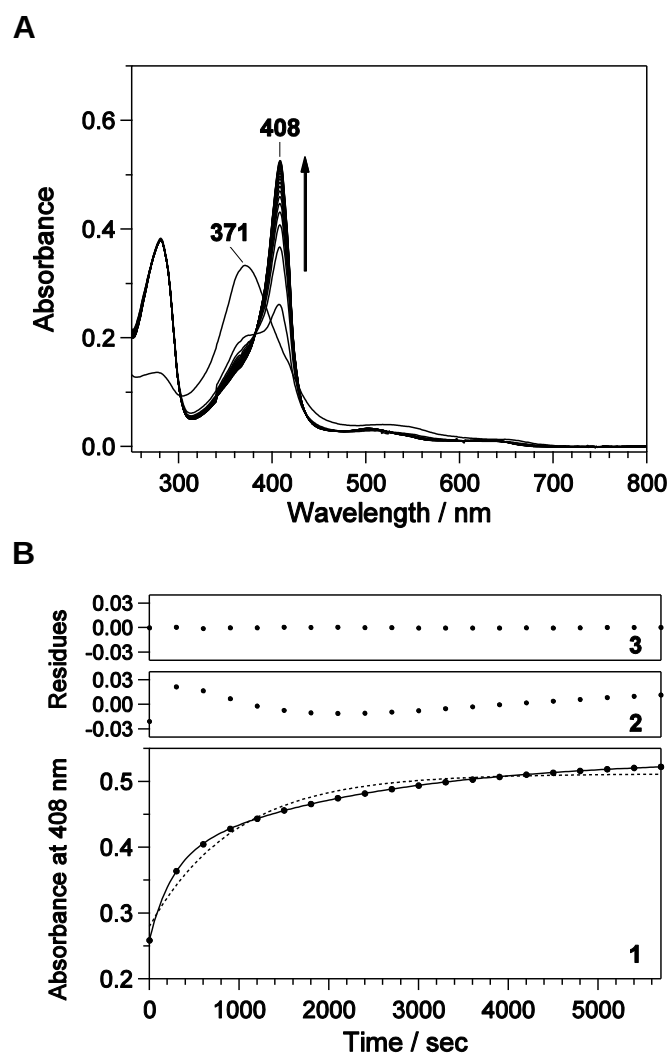


Figure 2.6 Dissociation rate constants of PRX1 using apo-myoglobin.

(A) Displacement of heme from heme-PRX1 (2 μ M) to apo-myoglobin (20 μ M) in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). Spectra were measured at 5-minutes intervals over a period of 100 minutes. (B) Time course of the displacement of hemin from heme-PRX1 to apo-myoglobin, measured as the change in absorption at 408 nm over time (1). Dissociation rate constants were calculated by both single-exponential (dotted line) and double-exponential (solid line) equations. Residuals of single-exponential (2) and double-exponential (3) fittings are shown in the upper panels.

2.3.5. Resonance Raman Spectra of Heme-PRX1.

To investigate the manner in which Cys is coordinated PRX1, I measured resonance Raman spectra. In the ferric form of the heme-PRX1 complex, the spin- and coordination-state marker band, ν_3 and ν_2 were observed at 1489 and 1569 cm^{-1} , respectively (Figure 2.7A), which are characteristic of five-coordinate high-spin heme (26). This is in good agreement with the results obtained by absorption spectra (Figure 2.5). In addition, the small intensity ratio of ν_4 to ν_3 ($I_{\nu_3} / I_{\nu_4} \approx 0.3$) suggests the presence of a weak axial ligand such as anionic oxygen or a sulfur atom (20, 27–29). These observations support Cys coordination to heme as the axial ligand.

Upon reduction, the ν_3 band appeared at 1470 and 1501 cm^{-1} , which represent five-coordinate high-spin and four-coordinate intermediate-spin hemes, respectively (Figure 2.7A) (10, 30–32). The presence of a four-coordinate heme indicates that Cys loosely bound to ferric heme was released upon reduction. Resonance Raman spectra of the ferrous-CO heme complex of PRX1 are illustrated in Figure 2.7B. Both 495 and 1961 cm^{-1} bands were left-shifted to 484 and 1868 cm^{-1} , respectively, upon $^{13}\text{C}^{18}\text{O}$ substitution. Accordingly, I assigned the 495 and 1961 cm^{-1} bands to the Fe-CO stretching mode ($\nu_{\text{Fe-CO}}$) and CO stretching mode ($\nu_{\text{C-O}}$), respectively. The plot of $\nu_{\text{Fe-CO}}$ versus $\nu_{\text{C-O}}$ for PRX1 falls on the line for proteins possessing a neutral histidine (Figure 2.7C), in agreement with results from UV-vis spectra (Figure 2.5). These results also indicate a weak coordination of Cys to the ferric heme.

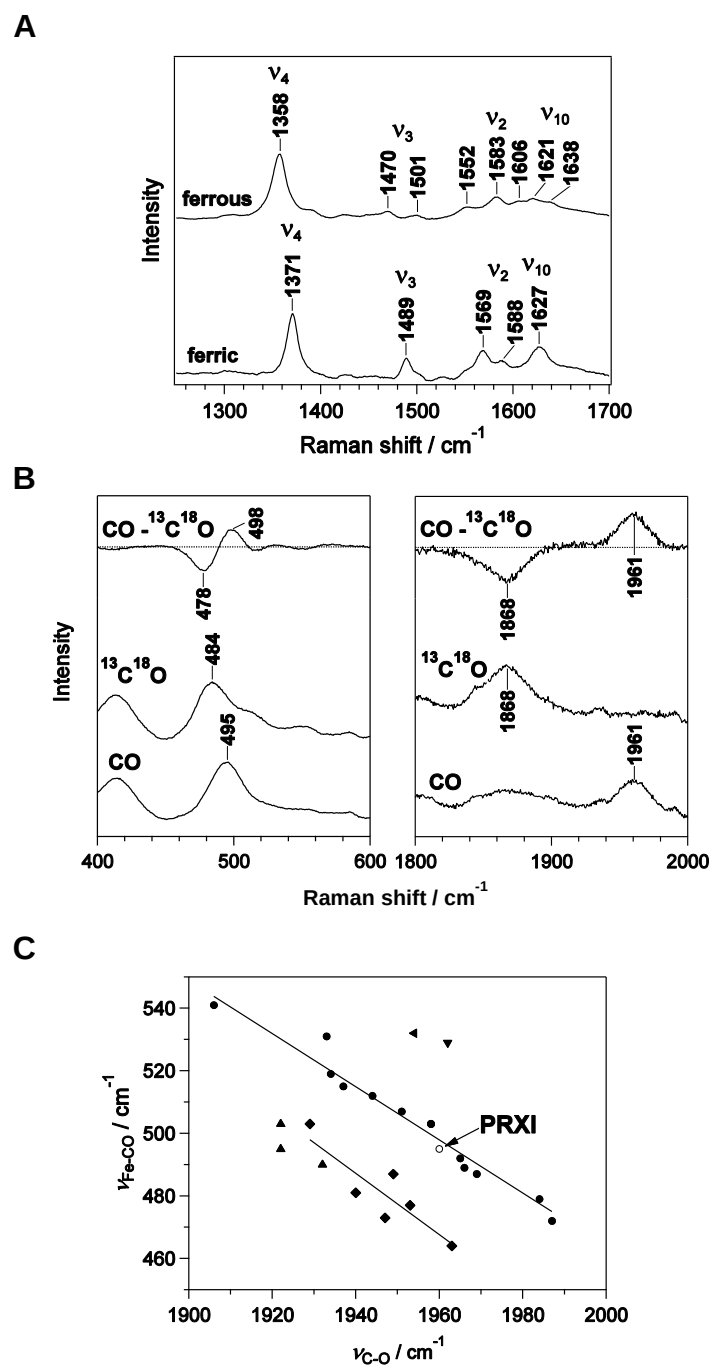


Figure 2.7 Resonance Raman spectroscopy.

(A) Resonance Raman spectra of PRX1 in the high-frequency region excited at 413.1 nm in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). (B) Resonance Raman spectra of the ferrous-CO complex of PRX1 in low-frequency (left) and high-frequency (right) regions with excitation at 413.1 nm. (C) Correlation plot of $\nu_{\text{Fe-CO}}$ versus $\nu_{\text{C-O}}$. The two solid lines correspond to proteins with proximal imidazoles (●), proximal imidazolates (▲), thiolate (◆), and five-coordinate hemoproteins (▼). The data point for PRX1 is presented as an open circle.

2.3.6. Determination of the Heme-Binding Site.

To specify the heme-binding residue, I performed site-directed mutagenesis of Cys. To this end, I replaced each of the four cysteine residues in PRX1 (Cys52, Cys71, Cys83 and Cys173) with Ser (Cys52) or Ala (Cys71, Cys83 and Cys173). Because the Ala mutant of Cys52 showed a strong tendency to aggregate, Cys52 was replaced only with Ser. Heme-titration experiments for all mutants were performed (Figure 2.8). The $K_{d, \text{heme}}$ values for PRX1 mutants C71A, C83A and C173A were 0.033, 0.050 and 0.14 μM , respectively, which are the same or slightly higher than that for wild-type PRX1. In contrast, the $K_{d, \text{heme}}$ for the C52S mutant could not be calculated owing to the drastic decrease in the absorption difference at 370 nm. These results clearly demonstrate that the heme-binding site is Cys52, which is identical to the active center of the cysteine-dependent peroxidase activity.

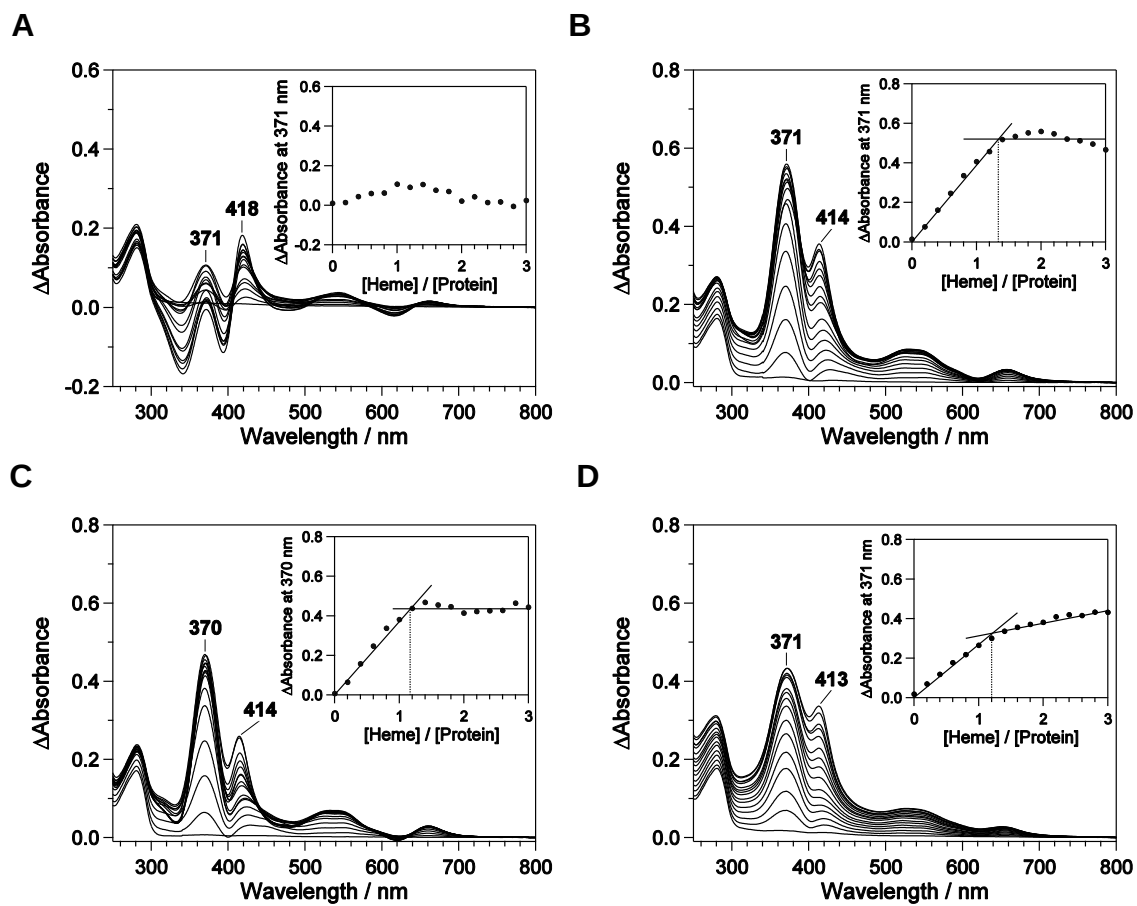


Figure 2.8 Effects of PRX1 mutations (C52S, C71A, C83A and C173A) on heme binding.

Heme titration for C52S (A), C71A (B), C83A (C) and C173A (D) mutants. Absorption difference spectra of heme binding to PRX1 following stepwise addition of heme (2-30 μM) to PRX1 mutants (10 μM) versus buffer blank in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). inset: Absorbance difference at 370 or 371 nm as a function of heme concentrations.

2.3.7. Effects of Cysteine-Dependent Peroxidase Activity on Heme Binding.

I next investigated the effects of heme binding on the cysteine-dependent peroxidase activity of PRX1. The reaction of PRX1 with H_2O_2 results in formation of an intermolecular disulfide bond with an antiparallel conformation (33), as verified by non-reducing SDS-PAGE (34). A gel image of PRX1 after incubation with or without H_2O_2 is shown in Figure 2.9A. In the absence of H_2O_2 treatment (lane 1), PRX1 showed a band at ~ 22 kDa (i), whereas H_2O_2 treatment resulted in the appearance of a new band at ~ 40 kDa (lane 2), corresponding to disulfide-bonded PRX1 (ii). This band shift suggests that PRX1 forms an intermolecular disulfide bond upon reaction with H_2O_2 . When the same assay was performed for heme-reconstituted PRX1, the 40 kDa-band did not appear (lane 3 and 4), indicating that heme-PRX1 remained without a covalent bond, even after treatment with H_2O_2 . To discuss the PRX1 dimerization by H_2O_2 quantitatively, the gel image was analyzed using NIH ImageJ software (Figure 2.9B). The band intensity in lane 4 was lower than that in other lanes owing to degradation of PRX1 by ROS generating from heme and H_2O_2 . Thus, I compared the ratio of intensities between (i) and (ii). In lane 2, the bands of (i) and (ii) had same intensities, although the band of (ii) was significantly decrease compared to (i) in lane 4. These analyses indicated that the dimerization was obviously prevented by reconstituting with heme. The cysteine-dependent peroxidase activity of monomeric PRX1 seemed to be lower than that of reported (Figure 2.9). This might be because some amount ($< 40\%$) of the active center of monomeric PRX1 was hyperoxidized during gel filtration (Figure 2.10). These results show that heme binding to PRX1 inhibits dimer formation through the S-S bridge, implying a loss of cysteine-dependent peroxidase activity.

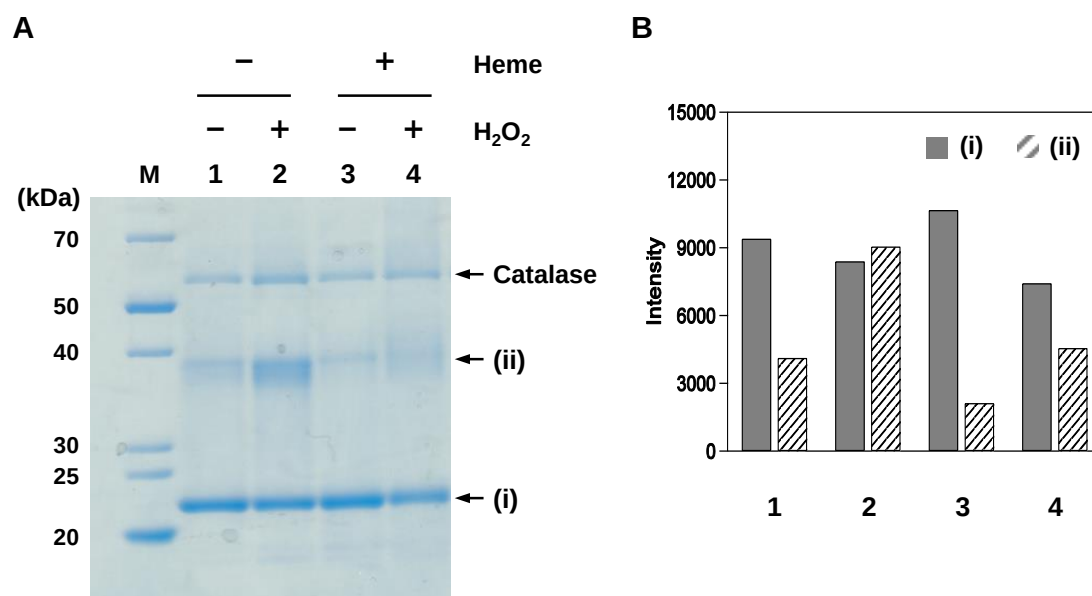


Figure 2.9 Dimerization assay.

(A) Apo- and holo-monomeric PRX1 (10 μ M) was treated with 30 μ M H₂O₂ for 5 minutes at 25 °C in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The reaction was stopped by adding 0.1 μ M catalase to quench excess H₂O₂, after which PRX1 was resolved by non-reducing SDS-PAGE and stained with Coomassie Brilliant Blue. The band at ~60 kDa corresponds to catalase. (B) The band intensities were quantified using NIH ImageJ software. The number below graphic indicated the lane as shown in (A).

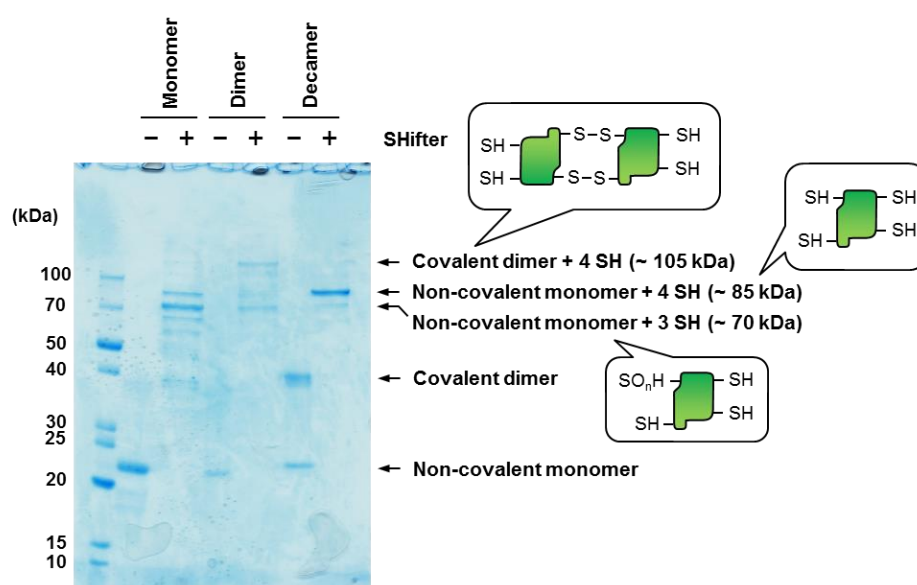


Figure 2.10 Thiol group modification of PRX1 on non-reducing SDS-PAGE.

Quantification of the number of free thiol groups in monomeric, dimeric and decameric PRX1 was performed using the Protein Redox State Monitoring Kit (Dojindo, Kumamoto, Japan). Free thiol group was modified by the maleimide-conjugated peptide (SHifter), resulting in a 15 kDa-shift per modification. The molecular-mass was resolved by non-reduced SDS-PAGE gel.

2.3.8. Functional Characterization of Heme-PRX1.

The above results prompted me to examine the functional role of heme binding to PRX1. It is well known that free heme is toxic to cells owing to heme-mediated oxidation. To elucidate the significance of heme binding to Cys52 in PRX1, I first investigated H₂O₂-dependent heme peroxidase activity. Heme peroxidase activity was monitored under steady state conditions using 4-aminoantipyrine (4-AAP) as a reducing substrate and phenol as an electron donor. The absorbance at 512 nm from the product, antipyrilquinoneimine, was plotted against time (Figure 2.11A) (14). The absorbance developed linearly for approximately 15 minutes, as reported previously (14). Complexation with heme reduced the heme peroxidase activity of PRX1 by up to 55% as shown by the limited increase in the absorbance at 512 nm. When excess ratio (more than 3 times) of PRX1 existed, the hemin peroxidase activity was significantly suppressed (Figure 2.11B). Interestingly, the same effect was observed in the C52S mutant (Figure 2.11C). For this mutant, heme non-specifically binds to the protein as discussed above. These data suggest that heme peroxidase activity is effectively inhibited in heme-reconstituted PRX1, but it is not necessary for heme to bind Cys52.

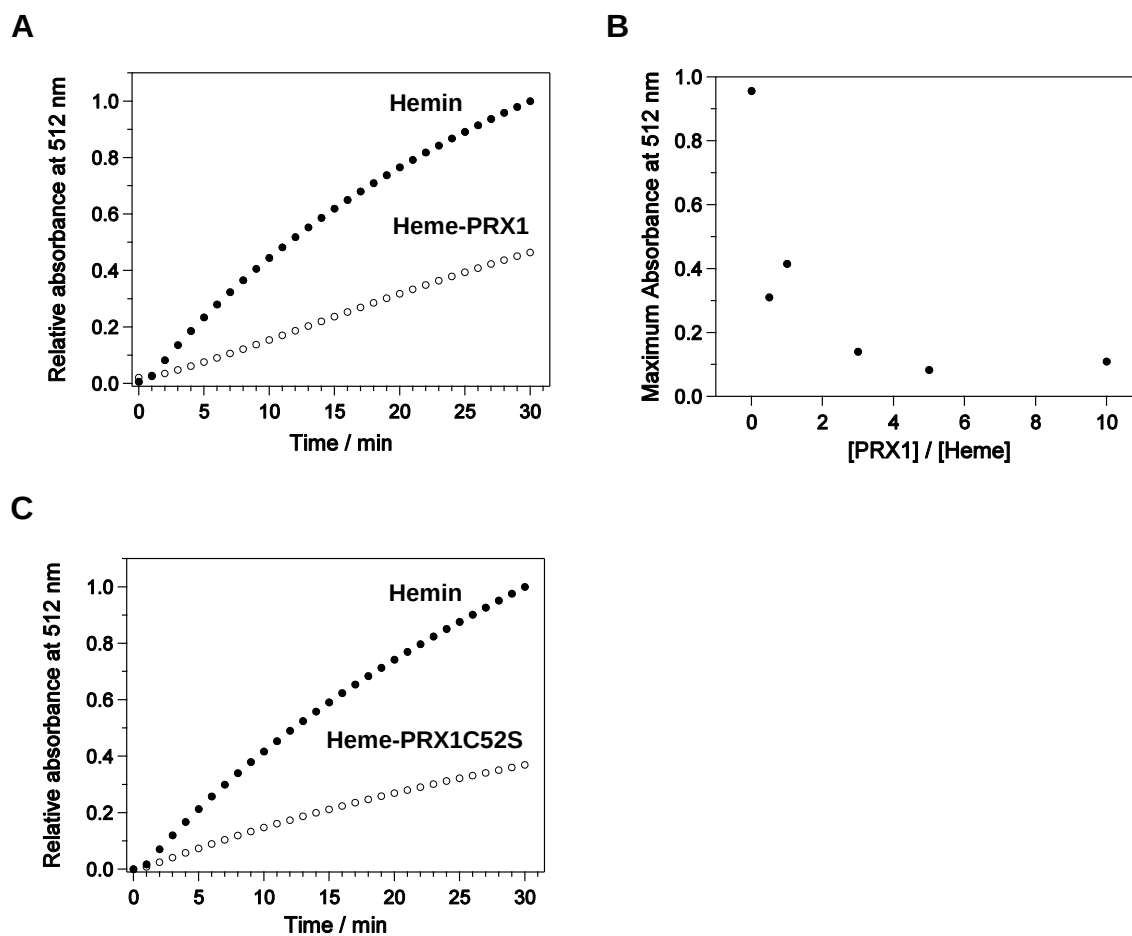


Figure 2.11 Hemin peroxidase activity assay.

(A, C) Hemin peroxidase activity of hemin (●) and heme-PRX1 (○). Reaction mixes containing 1.5 μ M of hemin alone or heme-PRX1WT (A) or C52S (C) were supplemented with 4-AAP (1.25 mM) and phenol (85 mM) followed by addition of H_2O_2 (360 μ M). The formation of oxidized AAP/phenol products was followed at 512 nm for 30 minutes at 1-minute intervals. (B) Hemin peroxidase activity in various concentrations of PRX1. The absorbance at 512 nm after reacting 30 minutes was plotted as a function of PRX1 concentration.

Although free heme is toxic to biological macromolecules, heme itself must be protected against degradation by oxidation during intracellular trafficking. Thus, I next compared the sensitivity of heme to H_2O_2 -mediated degradation (Figure 2.12A). The addition of H_2O_2 to free hemin resulted in a rapid decrease in the Soret band at 386 nm, as reported previously (14), indicating that hemin degradation occurred. In contrast, when hemin was complexed with PRX1, the decrease in absorbance at 370 nm was much slower than that for free hemin. Because heme-PRX1 did not exert the cysteine-dependent peroxidase activity (Figure 2.9), the observed slow degradation of heme bound to PRX1 is not owing to consumption of H_2O_2 as a cysteine-dependent reaction. To further clarify the interpretation, I performed the H_2O_2 -mediated hemin degradation assay using the excess amount of H_2O_2 (500 μM). $\Delta\text{Absorbance}$ of free hemin rapidly reached plateau within 1 second, compared to about 250 seconds for that of heme-PRX1 (Figure 2.12B). Thus, I supposed that the accessibility of H_2O_2 to heme is reduced upon binding to PRX1. These results indicate that PRX1 protects heme from degradation by H_2O_2 . However, the decrease in the Soret band of the heme-C52S mutant by addition of H_2O_2 was much smaller than that of free hemin (Figure 2.12C). Although the C52S mutant lost the specific heme-binding site (Figure 2.8), the presence of small absorbance at 418 nm in the difference spectrum of heme titration (Figure 2.8A) suggests non-specific heme binding. Thus, the non-specific heme binding also prevents heme from degradation by H_2O_2 .

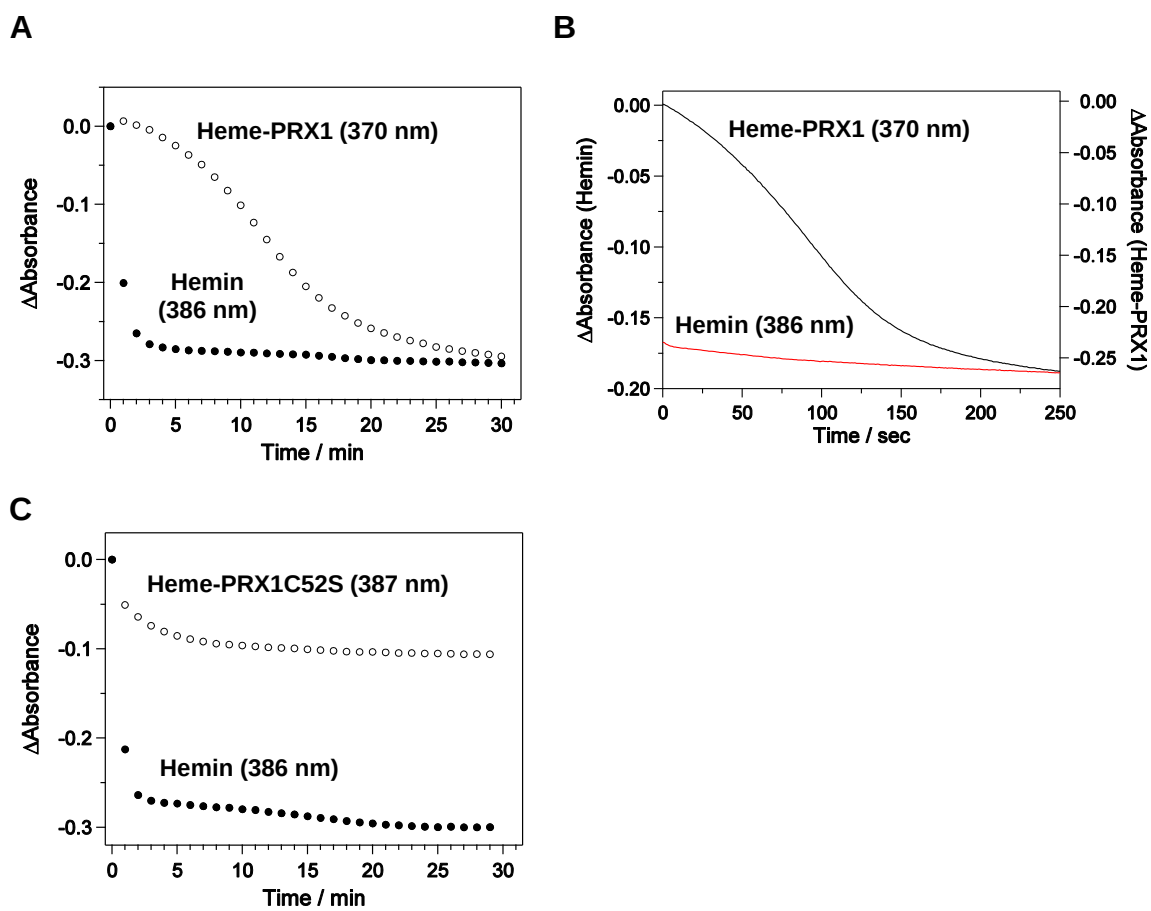


Figure 2.12 H_2O_2 -mediated heme degradation.

H_2O_2 -mediated degradation of heme (●) and heme-PRX1 (○) WT (A) or C52S (C) using H_2O_2 at a concentration of 30 μM (A, C) and 500 μM (B). H_2O_2 was added to heme alone or heme-PRX1 (10 μM), after which decreases in the absorbance of the Soret peak of heme (386 nm) or heme-PRX1 (370 nm) were followed for 30 minutes at 1-minute intervals (A) or for 250 seconds at 1-second intervals (B). Intensities at the indicated time points were subtracted from the respective zero values and plotted.

2.3.9. Effect of Heme Binding on the Secondary Structure of PRX1.

Because heme binding modulates the function of PRX1 (Figures 2.8 and 2.9), I investigated the influence of heme binding on the structure of PRX1. To determine whether heme binding affects the secondary structure of PRX1, I measured far-UV circular dichroism (CD) spectra. CD spectra of PRX1 showed a negative peak at about 220 nm (Figure 2.13A). Using equation 2.4 and the molar ellipticity at 222 nm, I estimated the α -helix content to be 9%, which is slightly lower than the estimate from the crystal structure of rat HBP23 (29%) (33). This difference is due to ambiguity of the estimation of the α -helix content using equation 2.4. The CD spectra of PRX1 with 1, 2 and 4 equivalents of heme were practically indistinguishable from that of apo-PRX1, indicating that the fact that addition of heme did not produce any measurable effects on CD spectra.

Although no change in the CD spectra occurred upon heme binding, there is the possibility of tertiary structural change. Thus, I examined the oligomerization status of PRX1 by gel-filtration chromatography, as described in Material and Methods (Figure 2.13B). For apo-PRX1, a major band appeared at 15.5 mL, corresponding to a monomer. The chromatogram of heme-reconstituted PRX1 showed an increase in the intensity of bands at 13.4, and 14.2 mL corresponding to dimeric, and tetrameric states, respectively, and a concomitant decrease in the band for the monomeric state with a slight low-elution-volume shift (15.3 mL). These results indicate that heme binding induces oligomerization of PRX1.

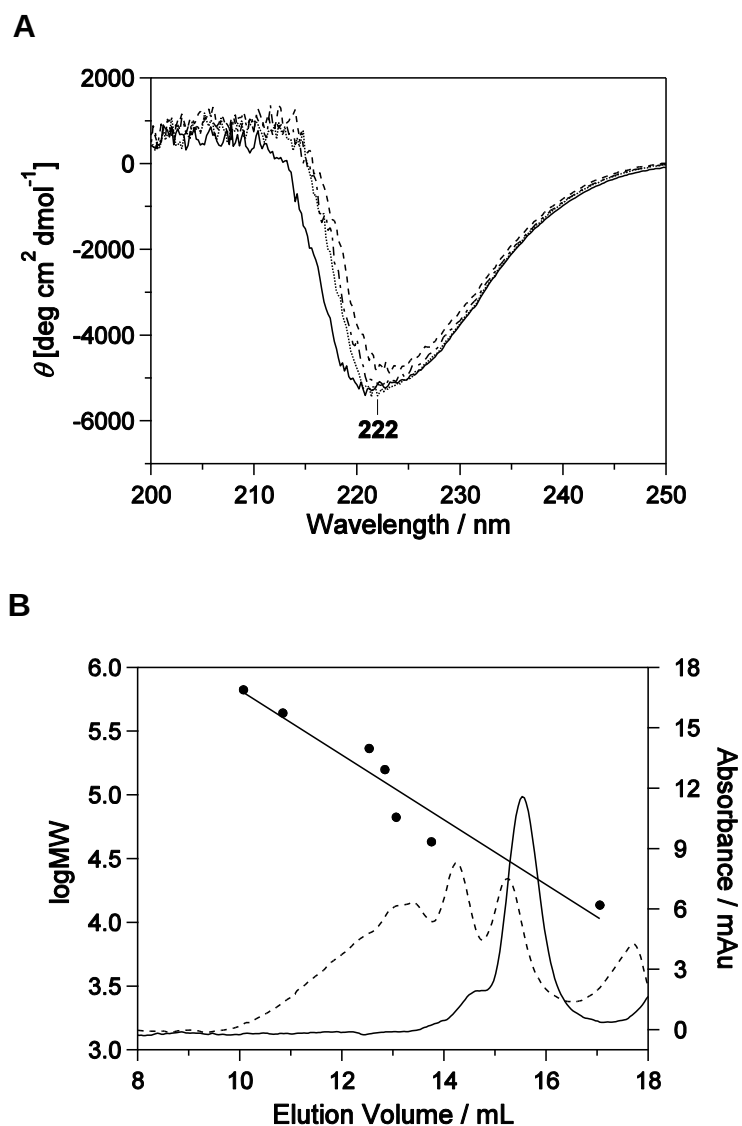


Figure 2.13 Effects of the oligomerization state of PRX1.

(A) CD spectra of PRX1 in the absence (solid line) and presence of 1 (dotted-line), 2 (dashed-dotted line) and 4 (dashed line) equivalent(s) of heme in 50 mM sodium phosphate/100 mM NaCl (pH 7.4). Samples were pre-incubated for 10 minutes at 4 °C prior to measurement. (B) Size-exclusion chromatography of 10 μ M apo- (solid line) and holo- (dotted line) PRX1 in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4) using an ENrich SEC 650 10/300 column (Bio-Rad) with a flow rate of 1 mL/min. The elution volumes of standard proteins were as follows: thyroglobulin, 10.1 mL; ferritin, 10.8 mL; catalase, 12.5 mL; aldose, 12.8 mL; albumin, 13.1 mL; ovalbumin, 13.8 mL; and RNase A, 17.0 mL.

2.4. Discussion

2.4.1. Heme Coordination Environment of PRX1.

Synthesized heme in cytosol is potentially cytotoxic and must be managed by the cell, a protective function that has been suggested to fall on cytosolic heme-binding proteins (1, 2, 35, 36). Although HBP23 is frequently mentioned as such a candidate, knowledge of its heme-binding site and the functional significance of its heme binding have been limited, motivating our characterization of the human homolog, PRX1. Heme titration experiments showed that PRX1 binds 1 equivalent of heme (Figure 2.4B) and the Soret band for ferric heme-PRX1 strongly suggested Cys-coordinated environment (Figure 2.5). Indeed, a mutational study demonstrated that Cys52 is responsible for heme binding (Figure 2.8). The k_{off} value for PRX1 was similar to that of Cys-coordinated heme-binding proteins, such as HRI (Figure 2.6 and Table 2.3), further supporting the Cys coordination of PRX1. The ν_3 band in resonance Raman spectra of ferric heme-PRX1 was characteristic of a five-coordinate high-spin state, and the lower intensity of ν_3 relative to ν_4 indicated coordination of a neutral ligand, such as the thiol (not the thiolate) of Cys or the hydroxyl group of Tyr (27–29, 31). The presence of a four-coordinate heme in the ferrous form and a five-coordinate CO-heme also support the neutral and weak Cys coordination to ferric heme (30). Indeed, the $K_{\text{d, heme}}$ of PRX1 was 0.17 μM (Figure 2.4B), a value within the range of cytosolic heme concentrations (37). Such a moderate heme affinity of PRX1 suggests that PRX1 can serve as a heme transporter to cytosolic apoproteins.

The UV-vis absorption spectrum of heme-PRX1 displayed a Soret band at 370 nm, which is different from that reported previously for HBP23 ($\lambda_{\text{max}} \approx 408$ nm) (6). Gel-filtration chromatography showed that PRX1 exists in monomeric, dimeric, and decameric states (Figure 2.3B). When decameric PRX1 was reconstituted with heme, the Soret band appeared at 415 nm (Figure 2.14), implying that the previously reported Soret maximum (6) is derived from the protein in a mixture of several oligomeric forms. Furthermore, the decameric form of the C52S mutant also provides the Soret absorbance at 416 nm (Figure 2.14) as well as wild-type, although the heme-binding site was mutated. This finding indicates that Cys52 is not involved in heme binding in decameric form. Cys52 might be hidden from solvent, so that heme cannot access to it. Thus, the oligomerization status would influence the heme-binding environment of PRX1, and the heme-binding environment shown in this thesis is different from the previous report.

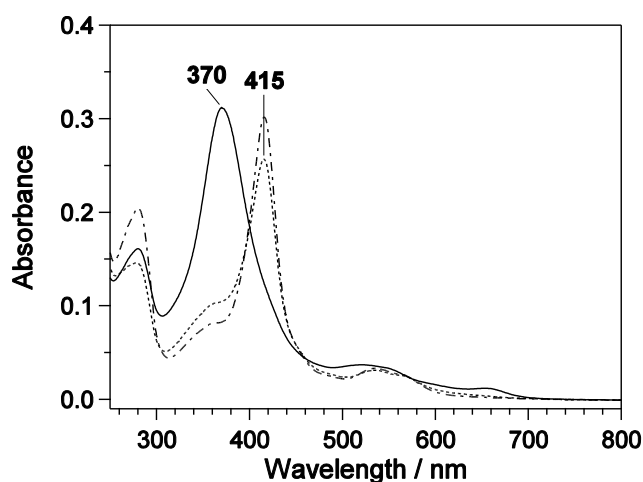


Figure 2.14 Oligomerization effect on the heme binding property.

UV-vis absorption spectra of the heme-monomeric (solid line) and -decameric (dotted line) PRX1WT, and heme-decameric PRX1C52S (dashed-dotted line) in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4).

The oligomerization effect of PRX1 was shown for the dissociation rate constants. PRX1 exhibits biphasic dissociation kinetics, with $k_{\text{off},1}$ and $k_{\text{off},2}$ values of 4.5×10^{-4} and $4.0 \times 10^{-3} \text{ s}^{-1}$, respectively (Table 2.3). One possible interpretation of this kinetic feature is that the heme dissociation rate depends on the oligomerization state. The tendency for heme binding to PRX1 to induce oligomerization was shown by gel-filtration chromatography (Figure 2.13B). Thus, heme binding to PRX1 induces oligomerization of PRX1, which increases the six-coordinate heme ($\lambda_{\text{max}} = 415 \text{ nm}$) and yields a k_{off} different from that of the five-coordinate heme.

Despite the presence of two CP motifs in PRX1, only Cys52 is a favorable heme-binding site. A closer look at the crystal structure of human PRX4 reveals that this preference is largely derived from structural features around Cys52 as described below (corresponding to Cys_P-SH). Sulfenylation of the Cys_P-SH (Cys_P-SOH) by H_2O_2 induces unwinding of the α -helix containing Cys_P-SOH, which positions the sulfenylated cysteine close to Cys_R-SH to allow formation of a disulfide bond (38). Importantly, Cys_P-SH forms two hydrogen bonds with the guanidinium group of Arg128 and the backbone amide group of Pro45 (Figure 2.12). These hydrogen bonds cause a downward shift in the pK_a of the side chain in Cys52 from 8.5, the value for the free cysteine, to approximately 6 (39). Such an environment would make Cys highly active even at a neutral pH. Thus, Cys52 is suitable for heme binding. A similar type of enhancement of ligand coordination by hydrogen bonding to the axial ligand has also been reported for CBS. In this case, the axial ligand of heme is Cys52 (human CBS numbering), and the adjacent Arg266 forms a hydrogen bond with Cys52. Although Arg266 is not directly coordinated to the iron, replacement of Arg266 with Lys leads to a longer and weaker heme-Cys52 bond (40). Enhancement of the nucleophilic nature of the axial tyrosine residue by hydrogen bonding to the neighboring His is also seen in HasA (41, 42). On the other hand, another CP motif, Cys173 (corresponding to Cys_R-SH), existed in the flexible loop. The C-terminus of rat HBP23 was disordered in the crystal structure (33), indicating that

there are no structural features around Cys173. Thus, the nucleophilic environment of Cys52 is favorable for heme binding.

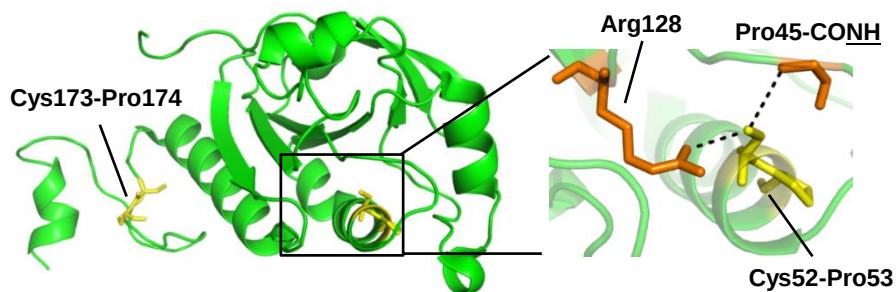


Figure 2.15 Structure of human PRX4 (PDB ID: 2PN8).

CP motifs and hydrogen-bond partners are highlighted in yellow and orange, respectively. Hydrogen bonds are depicted as dotted lines.

2.4.2. Toxicity Suppression Mechanism of Free Heme by PRX1.

Heme-bound PRX1 loses cysteine-dependent peroxidase activity (Figure 2.9), because the heme occupies the active center. This property would seem to be undesirable for PRX1 to function as an antioxidant enzyme. However, PRX1 is highly abundant in cells, with an estimated concentration of 20 μM (43, 44). In contrast, the intracellular levels of free heme must be lower than 1 μM (35), because heme oxygenase-1, with a $K_{d, \text{heme}}$ of 0.84 μM , is capable of degrading excess heme under heme-replete conditions (45, 46). Furthermore, PRX1 is extremely reactive with H_2O_2 , with a rate constant of $\sim 10^7\text{-}10^8 \text{ M}^{-1}\text{s}^{-1}$, which is comparable to that of catalase and selenium-containing glutathione peroxidase (47). It is therefore conceivable that, given its molar excess, PRX1 has sufficient capacity for heme binding to serve an antioxidant role.

The heme-binding residue Cys52 is evolutionary conserved in all members of the Prx family. Although the Pro residue next to Cys is often replaced with other residues (Thr in most cases) in lower, non-mammalian organisms, the hydrogen bond partner (Arg128 in PRX1) is widely conserved. Importantly, heme is an indispensable molecule for all organisms owing to its regulatory functions. This fact allows me to speculate that scavenging heme is a common role for PRX1. Moreover, PRX1 binds heme not only to prevent it from interacting with H_2O_2 , but also to suppress H_2O_2 -dependent heme peroxidase activity (Figure 2.11A) and H_2O_2 -mediated heme degradation (Figure 2.12). This implies that PRX1 acts as “heme shelter” to protect both biomolecules and heme itself from undesired oxidation (Figure 2.16). Therefore, I propose that PRX1 has dual role, in the first case acting as a heme shelter and in the second protecting against oxidative stress through its cysteine-dependent peroxidase activity (Figure 2.13).

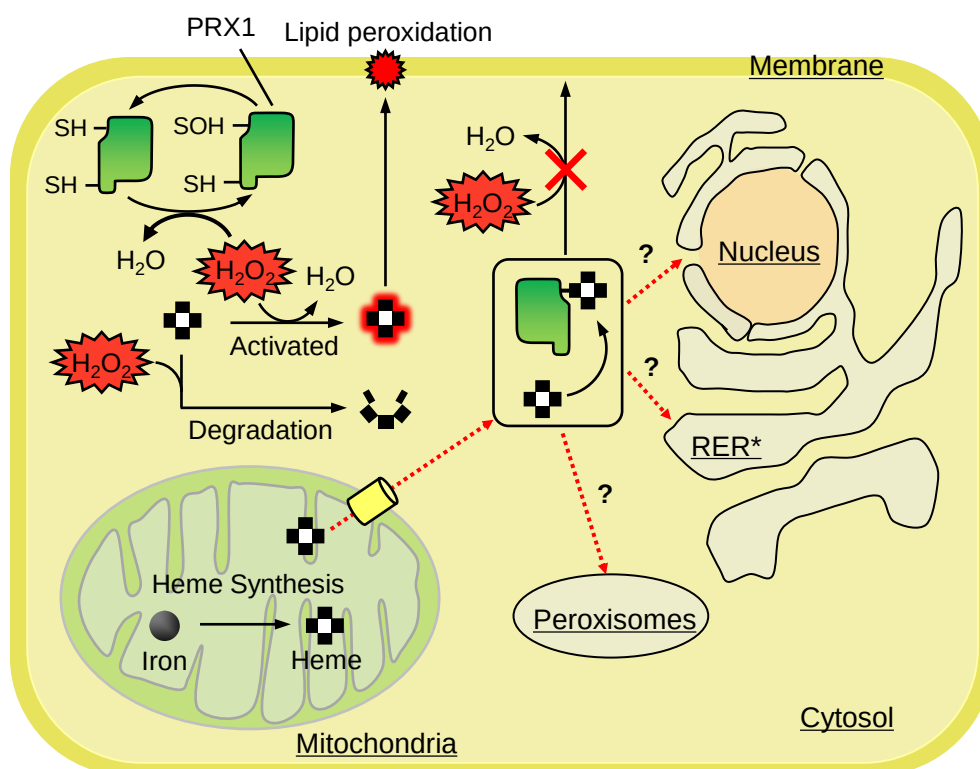


Figure 2.16 Schematic representation of the dual function of PRX1 as a cysteine-dependent peroxidase and a heme-binding protein.

*RER: rough endoplasmic reticulum.

Although I propose that PRX1 functions as shelter for heme in the cytosol, intracellular heme dynamics are poorly understood. AhpC from *Streptococcus agalactiae*, a heme-binding protein conserved to PRX1, protects heme from degradation and was thus proposed to play a role in facilitating intracellular heme transport. However, in contrast to heme-PRX1, heme-bound AhpC shows a significant level of heme peroxidase activity (48), indicating that the role of Prx family members might differ among organisms (e.g., between eukaryotes and prokaryotes). Although it is possible that PRX1 safely handles cytoplasmic heme movement (Figure 2.16), I was unable to identify any proteins that interact with PRX1 in pull-down experiments (data not shown). Therefore, it is still unclear whether PRX1 simply holds heme as a shelter, or also functions as a heme carrier protein. Thus, further study is needed to elucidate the detailed mechanism of intracellular heme transport.

In summary, I present here the first characterization of the human cytosolic heme-binding protein, PRX1, in which Cys52 is bound to a five-coordinate high-spin heme. Additionally, I propose that PRX1 sequesters heme from H_2O_2 , preventing oxidative reactions at the expense of PRX1 cysteine-dependent peroxidase activity. Because PRX1 is highly abundant in cytosol, it might be capable of exerting cysteine-dependent peroxidase activity even if some portion of the PRX1 protein population protects cytosolic free heme. The results in this chapter strongly suggest that PRX1 acts as the cytosolic heme-binding protein to protect synthesized heme from the unexpected reaction with H_2O_2 .

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CHAPTER III

**HEME-DEPENDENT REGULATION OF THE TARGET
RNA BINDING ACTIVITY FOR IRON REGULATORY
PROTEINS (IRPs)**

Abstract

In this chapter, I focused on iron regulatory proteins (IRPs), which is the primary regulation factor for iron metabolism. IRPs bind to specific iron-responsive element (IRE) in their target mRNA. The IRPs-IRE system carries out in a cellular iron-dependent manner, suggesting that sensing of the cellular iron is essential factor for regulation. Because the characteristic heme-binding CP motifs were conserved in each IRP homolog, IRP1 and IRP2, I speculated that heme affects the IRE-binding activity of IRPs. Previous heme titration and mutational study in my laboratory showed that the heme binding to the CP motif in IRPs was confirmed. Indeed, UV-vis spectra for IRP1 showed the Soret peak at 372 nm and 418 nm, showing that the heme coordination environment would be the mixture of 5-coordinate Cys and 6-coordinate Cys/H₂O hemes. The H₂O ligand in the sixth axial ligand was retained by the distal His residue as shown in deoxymyoglobin. From the crystal structure of IRP1, His207 was located near Cys300, whereas there was no histidine residue around Cys118. Therefore, Cys118 is 5-coordinate Cys, and Cys300 is 6-coordinate Cys/H₂O. Next, to reveal the heme binding effect on IRE-binding activity of IRPs, interaction analysis using fluorescence anisotropy was performed. For the titration of IRP1 to fluorescence-conjugated IRE, the interaction was significantly suppressed in the presence of heme, indicating that heme binding perturbs the interactions between IRP1 and IRE. The suppression of the IRE-binding activity by heme was also observed in IRP2. These results indicate that IRPs sense the intracellular iron content upon the heme binding.

3.1. Introduction

In previous chapter, I elucidated that PRX1 protected the synthesized heme in cytosol. The protection of heme provides the regulatory function of various heme-binding proteins. Among them, I next focused on IRPs, which is the RNA-binding protein to modulate the iron storage or uptake. IRPs have CP motifs in their amino acid sequences (Figure 3.1). The function of IRPs is modulation of the gene expression of proteins for iron metabolism. IRP2, one of the homologues of IRPs, has a unique iron-dependent degradation (IDD) domain, to which heme specifically binds. Heme binding to Cys201 in the CP motif activates molecular oxygen to ROS, which subsequently attacks IRP2 itself to cause the oxidative modification, leading to the proteasomal degradation of IRP2 (1–3). On the other hand, based on their primary sequences, IRPs have other conserved CP motifs except for the IDD domain (Figure 3.1A). The conserved CP motifs (Cys120 and Cys375) in IRP2 are also heme-binding sites shown in previous heme titration experiments (4), indicating that it is possible for regulation of the IRE-binding activity of both IRPs by heme binding. Moreover, the CP motifs are conserved to IRP1 despite of lacking IDD domain, suggesting that IRP1 can also bind heme. Indeed, one research group has been already suggested that heme suppressed the IRE-binding activity of IRP1 in the cellular condition (5–7).

From the previous investigation in my laboratory, the heme titration experiment showed that heme-binding sites of IRP1 were the CP motifs, Cys118 and Cys300 (4). A closer look at the crystal structure of rabbit IRP1 (94 % amino acid identity with human IRP1) and frog ferritin IRE complex (8) showed that Cys118 is far from the IRE-binding site and exposes the protein surface of IRP1, but Cys300 is closer to IRE-binding site, and the residues around Cys300 participates in the interaction with IRE (Figure 3.1B). These observations allow me to hypothesis that the heme-binding to conserved CP motif regulates IRE-binding activity.

In Chapter III, to elucidate the effect of heme on the RNA-binding activity of IRPs, I

tried to analyze the heme-dependent regulation of IRPs using fluorescence anisotropy.

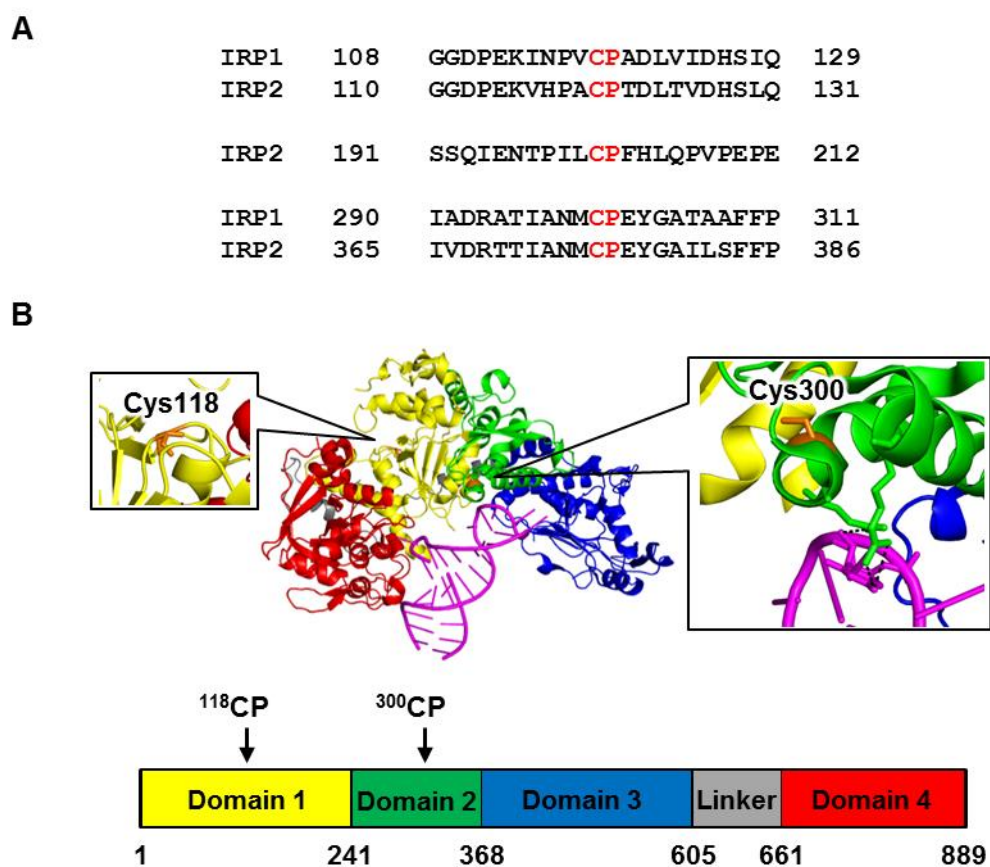


Figure 3.1 Sequential feature of IRPs.

(A) Amino acid sequence alignment of IRPs around CP motifs. (B) Crystal structure of IRP1 (PDB ID: 3SNP) (8), which is colorized each domain following to lower bar. The CP motifs and IRE are highlighted with orange and magenta, respectively. The black dashed line represents the salt bridge.

3.2. Experimental Procedures

3.2.1. Baculovirus Preparation.

The pFastBac HT plasmids encoding *IRP* gene were gifted from Prof. Kazuhiro Iwai, and Dr. Yukiko Takeda (Kyoto University). Recombinant baculovirus were generated by the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA). The plasmid was transformed to Max Efficiency DH10Bac competent cells (Invitrogen) to prepare the baculovirus expression shuttle vector (bacmid). Transformed cells were incubated for 4 hours at 37 °C, and then streaked onto a Luria Broth (LB) agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamycin, 10 µg/mL tetracycline, 40 µg/mL isopropyl β -D-thiogalactopyranoside, and 100 µg/mL 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal) to visualize the insertion of the desired gene by blue colorization derived from degradation of X-gal. At 72 hours after incubation, the white (not degrading X-gal) colony was cultured for ~16 hours at 37 °C in LB medium. The bacmid was purified using a PureLink HiPure Plasmid DNA Purification Kit (Invitrogen). Sf21 cells (Invitrogen) were routinely maintained at 27 °C in Grace's supplemented medium containing 10% fetal bovine serum (FBS), 3.33 g/L lactalbumin, 3.33 g/L yeast extract, 0.35 g/L sodium bicarbonate, 100 IU/mL penicillin G, and 100 µg/mL streptomycin. The cells were cultured on 6-well plates at a density of 3×10^6 cells per well and incubated for 24 hours. Bacmid transfection was carried out using Cellfectin II (Invitrogen) according to the manufacturer's instruction. The amounts of bacmid and Cellfectin II were 1 µg and 8 µL per construct, respectively. After 72 hours, the supernatant containing baculovirus was collected and kept it as the first-generation (P1) baculovirus by covering aluminum foil to avoid light at 4 °C. P1 baculovirus was infected to 3×10^6 cells of Sf21 in a 10-cm plate. After 4-days incubation at 27 °C, the supernatant was collected as the second-generation (P2) baculovirus. The procedure was repeated once more, and then the third-generation (P3) baculovirus were used for expression of proteins.

3.2.2. Protein Expression and Purification.

High Five cells were cultured at 27 °C in Express Five serum-free medium (Invitrogen) supplemented with 16.5 mM L-glutamine, 100 IU/mL penicillin G and 100 µg/mL streptomycin, and infected P3 baculovirus to express His₆-tagged IRPs. At 60 hours after infection, the infected cells were harvested and centrifuged at 1,000 rpm for 3 minutes at 4 °C to remove the medium. The cells were washed with phosphate buffered saline (PBS), and subsequently suspended in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 10 mM 2-mercaptoethanol, 0.2 mg/mL heat-treated ribonuclease A (Roche Diagnosis, Basel, Switzerland) and 1 tablet/50 mL protease inhibitor cocktail tablet (Complete EDTA-free, Roche Diagnosis). After incubation for 10 minutes on ice, the suspended cells were homogenized by Dounce homogenizer (WATSON, Tokyo, Japan). The insoluble fractions were removed by centrifugation at 15,000 rpm for 20 minutes at 4 °C. The supernatant was applied to Ni-NTA agarose resin (QIAGEN, Hilden, Germany) pre-equilibrated with 50 mM Tris-HCl (pH 7.4), and the mixture was incubated for 2 hours at 4 °C. The resin was extensively washed with wash buffer containing 50 mM Tris-HCl (pH 7.4), 20 mM imidazole and 10 mM 2-mercaptoethanol. The bound protein was eluted with 50 mM Tris-HCl (pH 7.4) buffer containing 300 mM imidazole and 10 mM 2-mercaptoethanol. For the purification of IRP1, the eluate was dialyzed against 50 mM Tris-HCl (pH 8.0) containing 10 mM 2-mercaptoethanol, and Turbo TEV Protease (Accelagen, San Diego, CA, USA) to remove imidazole and His₆-tag for 16 hours at 4 °C. After cleavage, the reaction mixture was again applied to Ni-NTA agarose pre-equilibrated with 50 mM Tris-HCl (pH 7.4) containing 10 mM 2-mercaptoethanol. The flow-through was collected and concentrated to ~2 mL using Amicon Ultra (Merck Millipore, Darmstadt, Germany). The sample was applied to a HiLoad 16/600 Superdex 200 preparatory grade gel-filtration column (GE Healthcare) pre-equilibrated with 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The log unit of molecular weight (logMW) was estimated by standard curve of the diffusion coefficients (K_{av}), obtained from the K_{av} of

thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldose (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and RNase A (13.7 kDa) as follows,

$$K_{av} = \frac{V_e - V_{void}}{V - V_{void}} \quad (3.1)$$

where V_e is the elution volume of proteins, V is the bed volume of the gel-filtration column, and V_{void} is the void volume of the column.

The purification procedure for IRP2 was essentially the same as that for IRP1. However, after the eluate was concentrated, buffer was exchanged to 50 mM Tris-HCl, 0.5 mM EDTA, and 1 mM dithiothreitol (DTT) (pH 8.0) using PD-10 MidiTrap column (GE Healthcare) instead of a HiLoad 16/600 Superdex 200 gel-filtration column to avoid partial aggregation during purification. The sample was incubated with Turbo TEV protease for ~12 hours at 4 °C. The reaction mixture was applied to Ni-NTA agarose resin to separate His₆-tag and TEV protease as described for IRP1. The collected flow-through was exchanged with 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The protein concentrations of IRP1 and IRP2 were estimated from the absorbance at 280 nm with extinction coefficients (ϵ_{280}) of 84,690 and 77,240 M⁻¹ cm⁻¹ using ProtParam (<http://web.expasy.org/protparam/>), respectively.

3.2.3. Absorption Spectroscopy.

Absorption spectra were obtained using a V-660 UV-vis spectrophotometer (JASCO, Japan). Hemin was dissolved in *N, N*-dimethylformamide (DMF). The hemin concentration was determined by the absorption spectrum of 500 μL of solution containing 15 % pyridine, 0.1 M NaOH, and approximately 30 mg sodium dithionite, which was mixed with 5 μL of hemin solution, using an extinction coefficient at 418 nm (ϵ_{418}) of $191.5 \text{ mM}^{-1}\text{cm}^{-1}$. The Soret band in the absorption spectra for heme-IRP1 was fitted with Gaussian curves as follows,

$$\text{Absorbance} = A_0 + \sum_{i=1}^n B_n \exp\left[-(\text{wavelength} - \lambda_{\text{max}, n})^2 / C_n^2\right] \quad (3.2)$$

where A_0 is the initial absorbance, n is the number of Gaussian curves, B_n is the heights of the peak absorbance, $\lambda_{\text{max}, n}$ and C_n are the wavelengths and widths of the peaks, respectively.

The dissociation constants of IRP1 for heme (K_i) was calculated by plotting the absorbance difference as a function of heme concentration, and using the quadratic binding equation,

$$\Delta \text{Absorbance} = \frac{1}{2} (\epsilon_{\text{binding}} - \epsilon_{\text{free}}) \left\{ [P] + [H] + K_i - \sqrt{([P] + [H] + K_i)^2 - 4[P][H]} \right\} \quad (3.3)$$

where $\Delta\text{Absorbance}$ is the absorption difference at a given concentration. $\epsilon_{\text{binding}}$ and ϵ_{free} are the extinction coefficients of the heme-IRP1 mutant complex and hemin, respectively. $[P]$ and $[H]$ are the concentrations of IRP1 and hemin, respectively.

3.2.4. Fluorescence Anisotropy Measurement.

Human ferritin IRE, whose 5'-end is covalently bound to 6-FAM (Figure 3.2A), was purchased from Eurofins Genomics as HPLC grade. The secondary structure of IRE is shown in Figure 3.2B. In this chapter, the 6-FAM-labeled IRE is simply depicted as IRE. IRE was dissolved in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). After dissolving in buffer, IRE was annealed by heating to 94 °C for 3 minutes with slow cooling to 25 °C over 6 hours in a

water bath. The prepared IRE was stored at -20 °C. Fluorescence anisotropy was recorded with a fluorometer FP-8500 (JASCO). The configuration for fluorescence anisotropy measurement is illustrated in Figure 3.2C. Fluorescence anisotropy r is defined as follows:

$$r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} \quad (3.4)$$

$$G = \frac{I_{HV}}{I_{HH}} \quad (3.5)$$

where I is the intensity of fluorescence with two letters of subscript expressed the directions of excitation and emission polarizer, respectively. V and H represent vertical and horizontal, respectively. For example, I_{VH} is depicted for the fluorescence intensities by passing through vertical-polarized excitation and horizontal-polarized emission light. The instrumental function, G , is used to calibrate the detector sensitivity between the vertical and horizontal light. The G value was calculated using equation 3.5 by the measurement for buffer containing 1 nM IRE. The concentrations of IRE for titration by IRP1 and IRP2 were 0.3 and 1 nM, respectively. To examine the effect of heme on the IRE-binding, IRPs were mixed with heme for approximately 15 minutes prior to addition of IRE. The excitation and emission wavelengths were 495 and 515 nm, respectively. Each data point represented the integration of five consecutive measurements. A plot of fluorescence anisotropy r versus protein concentrations x was analyzed using the Hill equation as follows:

$$r = a + \frac{b - a}{1 + (K_d / x)^n} \quad (3.6)$$

where a is the minimum anisotropy difference, b is the maximum anisotropy difference, n is Hill coefficient and K_d is the dissociation constant. All instruments were treated with RNase remover (RNase ZAP, Ambion) to remove the contamination of RNase, and all buffers were sterilized by 0.2 µm filter.

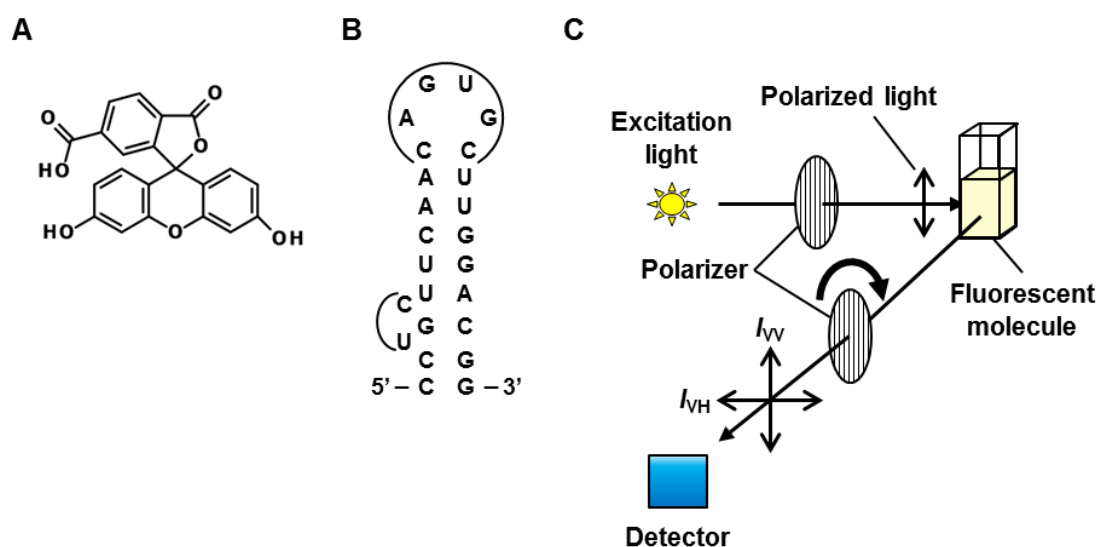


Figure 3.2 Outline of fluorescence anisotropy measurement.

(A) Structure of fluorescent molecule, 6-carboxyfluorescein termed 6-FAM, conjugated to the 5'-end of IRE. (B) Schematic secondary structure of human ferritin IRE. IRE was composed of a hairpin hexaloop (CAGUGC), stems, and internal loop/bulge (CGU). (C) Schematic representation of apparatus of fluorescence anisotropy. Both excited and emission lights pass through the polarizer.

3.3. Results

3.3.1. Expression and Purification of IRP1.

IRP1 was expressed in an insect cell strain, High Five, and purified using Ni^{2+} -affinity and size-exclusion chromatography. The purified IRP1 protein was estimated to be ~95% pure by SDS-PAGE (Figure 3.3A). An apparent molecular mass of 98 kDa for purified IRP1, which contains 889 amino acid residues, was consistent with a calculated molecular mass of 98,399 Da. A major peak on the size-exclusion chromatogram was obtained at the elution volume of 70.2 mL (Figure 3.3B). The K_{av} of IRP1 was calculated by equation 3.1 to be 0.330. The logMW was estimated from the standard curve obtained from the migration of bands against standard proteins (Figure 3.3B). The standard curve was fitted to a linear function, obtaining $K_{av} = 2.23 - 0.370 \cdot \log\text{MW}$. Thus, the K_{av} of 0.330 corresponds to logMW of 5.13, which is closer to molecular mass of monomeric IRP1. The yield of purified IRP1 was 1-2 mg from 400 mL of High Five culture.

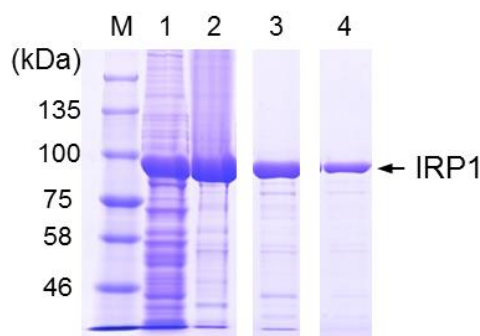
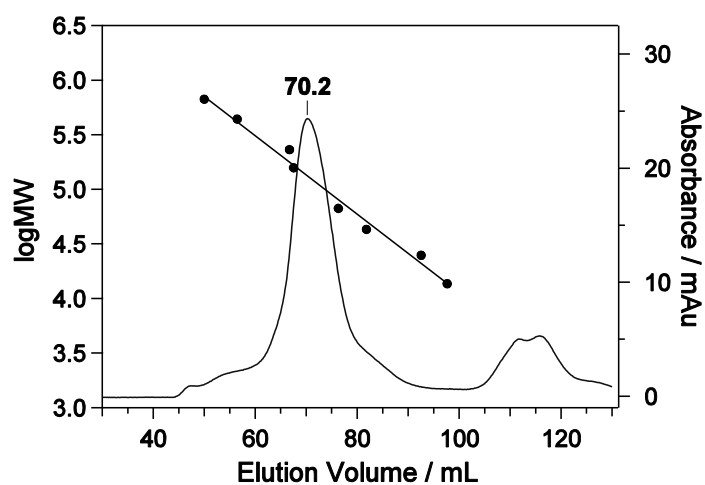
A**B**

Figure 3.3 Purification and size-exclusion chromatography.

(A) SDS-PAGE gel of IRP1 stained with CBB Stain One including molecular mass marker (Lane M), whole-cell extracts (Lane 1), purified His-tagged IRP1 (Lane 2), purified His-tagged cleaved IRP1 (Lane 3), and purified by size-exclusion chromatography (Lane 4). (B) Profile of IRP1 on a gel-filtration column (HiLoad 10/600 Superdex 200 pg) pre-equilibrated with 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). Analytical gel filtration was performed using a Superdex 200 pg column with a flow rate of 1 mL/min. The plot shows elution volume versus the log of molecular mass including the following protein standards; chymotrypsinogen (25 kDa), albumin (66 kDa), aldose (158 kDa), catalase (232 kDa), ferritin (440 kDa), and blue dextran (2,000 kDa) (GE Healthcare).

3.3.2. Absorption Spectra for IRP1.

The heme-binding environment was investigated using UV-vis absorption spectroscopy. Because it was reported that the stoichiometry of the heme binding to IRP1 was 1:2, IRP1 was reconstituted with a 2.4-fold excess of heme, and then unbound heme was removed using a gel-filtration column. Absorption spectrum of heme-reconstituted IRP1 is shown in Figure 3.4A. The Soret absorption maximum of ferric IRP1 was 375 nm with a shoulder at approximately 415 nm, and the Q-band at 637 nm. This spectrum distinctly differs from that for free hemin (Figure 3.4A). Because the Soret band for heme-IRP1 contained two bands, the Soret band was deconvoluted using equation 3.2, resulting in two peaks at 372 and 418 nm (Figure 3.4B). Both peaks of heme-IRP1 were different from the deconvoluted peaks of free hemin (Figure 3.4C), showing that heme specifically coordinates to IRP1. The far blue-shifted Soret peak at ~370 nm is known as a signature for a five-coordinate high-spin heme with an axial thiol ligand (Table 3.1), indicating that IRP1 binds heme through Cys. The absorbance at 418 nm was typical of the six-coordinate Cys-Fe-H₂O structure as shown in *d*-camphor-bound cytochrome P450 (9) rather than Cys/His coordination like Bach1 (10) or bis-His coordination like HrtR (11). Accordingly, heme binds to Cys in IRP1 with the mixture of the five-coordinate and six-coordinate species.

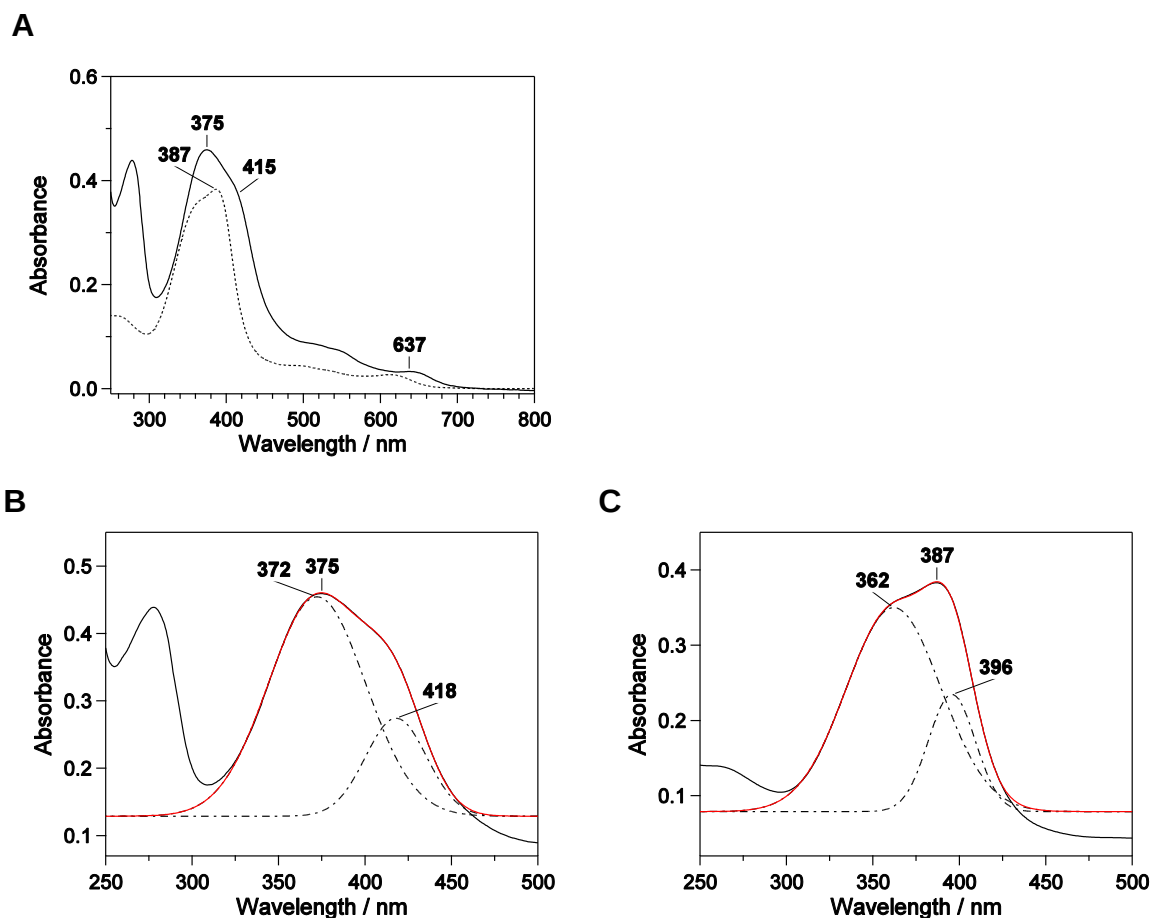


Figure 3.4 Electric absorbance spectra of the heme-IRP1 complex.

(A) UV-vis absorption spectra of ferric heme-IRP1 (solid line) and free hemin (dotted line) in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). (B and C) Enlarged absorption spectra (250-500 nm: black solid line) for the Soret region of IRP1 (B) and free hemin (C) fitted with two Gaussian functions (red), and resolved into two spectra (dashed-dotted line).

Table 3.1 Absorption maxima of the heme-IRP1 complex compared with those of other heme proteins.

Protein	Ligand	Soret (nm)	Visible (nm)	Reference
IRP1	Cys, Cys/H ₂ O	372, 418	637	This study
Bach1 (Type 1)	Cys/His	423	540, 580	(10)
Bach1 (Type 2)	Cys	371	521, 541, 650	(10)
Irr ^a	Cys	372	ND ^e	(12)
P450 _{cam} (+cam) ^b	Cys	391	ND	(13)
P450 _{cam} (H ₂ O)	Cys/H ₂ O	417	536, 569	(9)
HrtR	His/His	413	536, 562	(11)

^aIron response regulator protein; ^b*d*-camphor-bound P450_{cam}; ^ccystathionine-β-synthase;

^dN-terminal proline binds to heme; ^enot determined.

3.3.3. Detection of Complex Formation by Fluorescence Anisotropy.

The binding of IRP1 to IRE was examined by the fluorescence anisotropy measurements. The fluorescence anisotropy of IRE, whose sequence is 5'-CCU GCU UCA ACA GUG CUU GGA CGG, was measured in the presence of a various concentration of IRP1. The anisotropy differences (Δr) obtained by subtracting the anisotropy of free IRE from that of IRP1-bound IRE at each concentration are shown in Figure 3.5. The value of Δr is related to the remaining polarization of the fluorescence shown in equation 3.3, reflecting the speed of Brownian motion of IRE. When the Brownian motion becomes slower owing to the formation of a complex, fluorescence remains polarized, leading to increase in the value of Δr . Thus, an increase in Δr indicates that IRP1 binds to IRE. At 0.3 nM of IRP1, Δr was nearly 0, meaning that IRP1 did not bind to IRE. In contrast, at more than 100 nM of IRP1, Δr was increased to 0.05. The plot of Δr versus IRP1 concentration was best fitted to the Hill equation (equation 3.6), yielding the K_d of 6.9 ± 0.3 nM, which is comparable to those of reported previously (14, 15). To exclude the possibility that Δr was derived from non-specific binding, IRE was also titrated with bovine serum albumin (BSA: Figure 3.5). BSA did not induce any changes of Δr at any concentrations, suggesting that the specific interactions between IRP1 and IRE were detected using fluorescence anisotropy measurements.

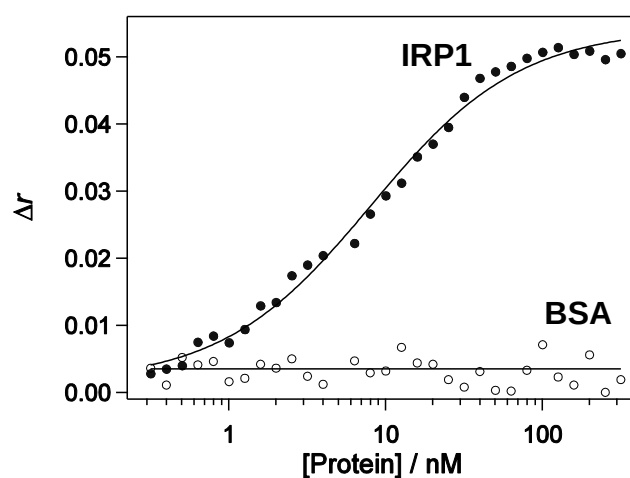


Figure 3.5 Fluorescence anisotropy measurement in the absence of heme.

Fluorescence anisotropy measurement for detection of interaction with IRE. The fluorescence was measured following titration of IRP1 (●) and BSA (○) to 0.3 nM IRE in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The excitation and emission wavelengths were 495 and 515 nm, respectively. The protein concentrations were displayed in logarithm unit.

3.3.4. Heme-Dependent Regulation of Interaction between IRP1 and IRE.

To elucidate the role for the heme binding to IRP1, the effects of heme binding on formation of the IRP1-IRE complex were investigated. In the presence of 4 equivalents of heme, Δr of heme-IRP1 was smaller than that of apo-IRP1 (-Heme) at any concentrations; Δr at 300 nM of heme-IRP1 was 0.025, which is about half as large as that for apo-IRP1 (Figure 3.6). This observation indicates that heme suppresses the formation of the IRP1-IRE complex. At more than 300 nM of IRP1, Δr in the presence of 4 equivalents of heme was not able to be obtained correctly owing to significant increase in the non-specific interaction. Therefore, the curve was not completely saturated. However, if the saturation point was postulated to be 0.05 on the basis of the plot for apo-IRP1, the apparent dissociation constant was estimated to be approximately 400 nM, which is ~60-fold higher than that for apo-IRP1. When 8 equivalents of heme was added, Δr was less than 0.01 over the range of 0.3-300 nM, indicating that the complex formation was almost inhibited (<20%). Therefore, the IRE-binding activity of IRP1 was suppressed in a heme-dependent manner.

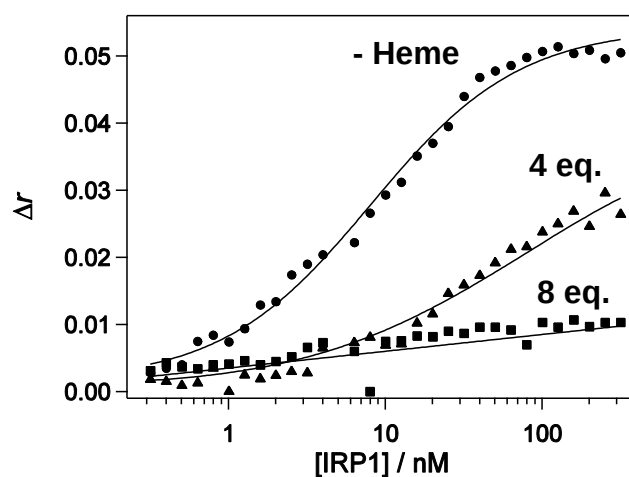


Figure 3.6 Fluorescence anisotropy measurement in the presence of heme.

Fluorescence anisotropy measurement for heme-IRP1 and IRE. The fluorescence was measured following titration of IRP1 (●) and mixing with 4 equivalents (▲) and 8 equivalents (■) of heme to 0.3 nM IRE in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The excitation and emission wavelengths were 495 and 515 nm, respectively. The protein concentrations were displayed in logarithm unit.

3.3.5. Expression and Purification of IRP2.

I found heme-dependent inhibition of the IRE-binding activity of IRP1. The CP motifs in IRP1 was conserved in IRP2, allowing me to examine effects of the heme binding on the activity of IRP2. IRP2 contains 963 amino acid residues and has a calculated molecular mass of 105 kDa. Although IRP2 share 57% amino acid identity with IRP1, IRP2 has unique IDD domain. IRP2 was so unstable that the purification was completed by exchanging buffer to 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4) using desalting PD-10 MiniTrap column (GE Healthcare) instead of a HiLoad 16/600 Superdex 200 gel-filtration column. This change made the time for purification significantly short, because the bed volume of PD-10 column (5 mL) was much smaller than that of the gel-filtration column (120 mL). The protein purity was assessed by SDS-PAGE (Figure 3.7). A band shown at approximately 80 kDa was probably derived from the degradation product of IRP2. The purity of purified IRP2 was approximately 80%, which was lower than that of IRP1 owing to omitting the purification by the gel-filtration chromatography. Because a large portion of the purified protein was composed of a full-length IRP2, I used this protein for fluorescence anisotropy experiments. The yield of purified IRP2 was 1-2 mg from 400 mL High Five culture.

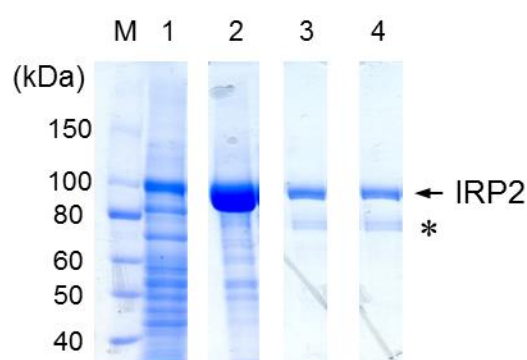


Figure 3.7 Purification of IRP2.

SDS-PAGE gel of IRP2 stained with CBB Stain One including molecular mass marker (Lane M), whole cell extracts (Lane 1), purified His-tagged IRP2 (Lane 2), purified His-tagged cleaved IRP2 (Lane 3), and exchanged the buffer to 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4: Lane 4). Asterisk (*) represents impurity band probably derived from the degradation product of IRP2.

3.3.6. Heme-Dependent Regulation of Interaction between IRP2 and IRE.

The interaction between IRP2 and IRE was analyzed using fluorescence anisotropy measurements. Δr was obtained over the range of 0.055-300 nM of IRP2 in the absence of heme. Δr for the titration of IRP2 for IRE resulted in the saturation around 0.06 at more than 100 nM of IRP2, indicating that IRP2 specifically interacts with IRE (Figure 3.8). Δr plot for apo-IRP2 (-Heme) was successfully fit to the Hill equation (equation 3.6), which provided the K_d of 18.2 ± 1.6 nM. The K_d is slightly lower than that of IRP1 (Figure 3.6), and comparable to those of other RNA-binding proteins (Table 3.2). The effects of heme on the interaction between IRP2 and IRE were investigated. Δr at 100 nM of IRP2 in the presence of 4 equivalents of heme was approximately 0.01, which is as half as that of IRP1 under the same condition (~ 0.02) (Figure 3.6). This result suggests that the complex formation was almost completely suppressed even in the presence of 4 equivalents of heme. Thus, the IRE-binding activity of IRP2 is suppressed at smaller equivalent of heme than that of IRP1. In the presence of 1 equivalent of heme, Δr at more than 50 nM was saturated to approximately 0.02, which is drastically smaller than that of apo-IRP2. The curve was fit to equation 3.6 to yield the K_d of 28.1 ± 2.2 nM, which is close to that of apo-IRP2. This tendency was different from IRP1, indicating that the sensitivity for heme would be varied between IRPs.

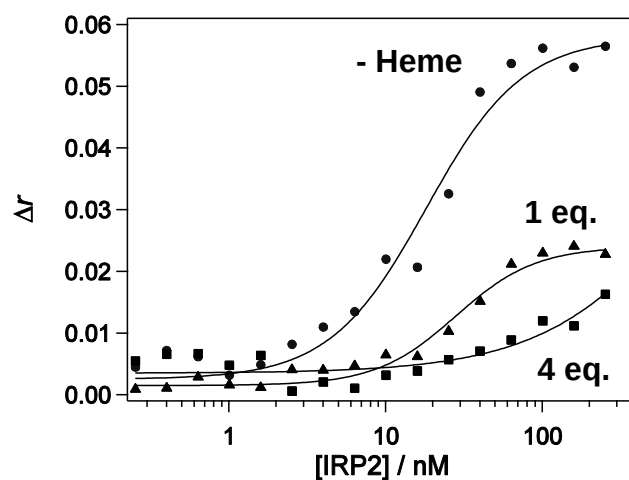


Figure 3.8 Fluorescence anisotropy for IRP2 with IRE.

Fluorescence anisotropy measurement for heme-IRP2 and IRE. The fluorescence was measured following titration of IRP2 (●) and mixing with 1 equivalents (▲) and 4 equivalents (■) of heme to 1 nM IRE in 50 mM HEPES-NaOH/100 mM NaCl (pH 7.4). The excitation and emission wavelength were 495 and 515 nm, respectively. The protein concentrations were displayed in logarithm unit.

Table 3.2 Dissociation Constants of RNA-binding protein measured using fluorescence anisotropy.

Protein	$K_d (\times 10^{-9} \text{ M})$	Reference
IRP1	6.9	This Study
IRP2	18.2	This Study
Vts1	17	(16)
NS5A	10	(17)
Hsp70	28	(18)

3.4. Discussion

3.4.1. Heme Coordination Environment for IRPs.

I expressed and purified IRPs using an insect cell-baculovirus expression system (Figure 3.3). The Soret band of UV-vis spectrum for heme-reconstituted IRP1 exhibited the mixture of 5-coordinate Cys and 6-coordinate Cys/H₂O hemes (Figure 3.4). The coordination of H₂O to heme in IRP1 was shown by deconvolution of the Soret band in the absorption spectrum (Figure 3.4). Such a distal water ligand is known to be stabilized by hydrogen bond as observed in the deoxy form of myoglobin, in which adjacent His64 forms hydrogen bond with the iron-bound water molecule (Figure 3.9A) (19). The water ligand disappeared when His64 was replaced with Leu (20), indicating that histidine residue plays a role in fixing a water molecule in the distal side. In IRP1, I found that His207 was located near Cys300 (Figure 3.9B), suggesting that His207 stabilizes the water ligand of heme bound to Cys300. In contrast, there is no histidine residue around Cys118, which allows me to conclude that the 5-coordinate Cys and 6-coordinate Cys/H₂O hemes can be assigned to Cys118 and Cys300, respectively.

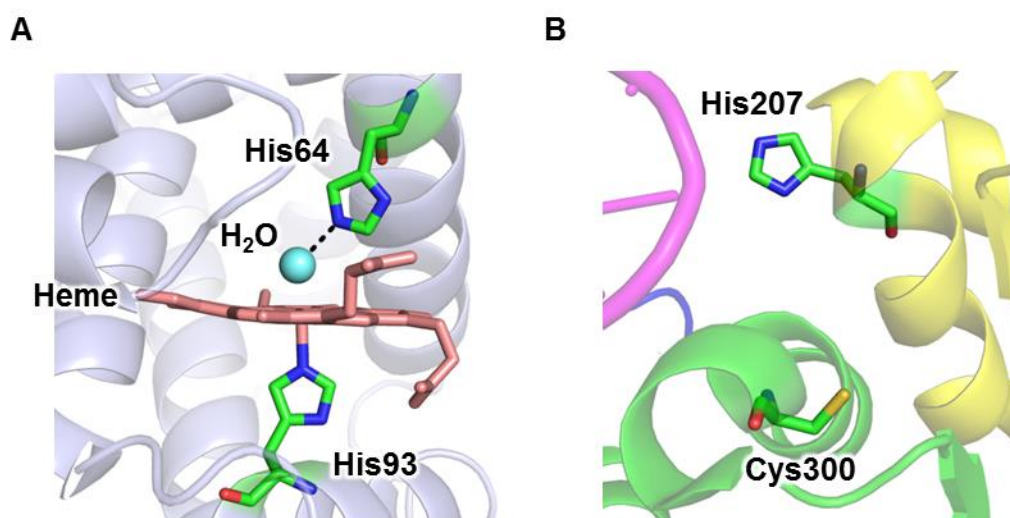


Figure 3.9 The distal pocket of myoglobin and IRP1.

(A) The heme pocket of deoxy myoglobin (PDB ID: 1VXH). The water molecule hydrogen-bond with His64. (B) The crystal structure of IRP1 around Cys300 (PDB ID: 3SNP). The candidate for hydrogen-bonding partner for water ligand with neighbor His207.

3.4.2. Heme Effect on Binding between IRPs and IRE.

IRP specifically bound to IRE observed in the fluorescence anisotropy measurements, and the IRE-binding activity of IRPs was suppressed by heme (Figure 3.6 and 3.8). To consider effects of heme binding to IRP on the IRE-binding activity, the heme binding property described in previous experiment (4) was discussed. The stoichiometry of IRP1 for heme was determined to be 1:2 from the heme titration analysis (4). When Cys118 and Cys300 in the CP motifs were mutated to Ala, the heme binding stoichiometry was decreased to 1 (4). Thus, the heme axial ligands of IRP1 are determined to be Cys118 and Cys300. The heme titration plot for C118A and C300A (4) were analyzed using equation 3.3 (Figure 3.10). The obtained affinities, dissociation constants, of C118A and C300A mutants for heme were 0.15 and 0.05 μM , respectively, which are similar to that of wild-type (0.05 μM) (4). In the case of IRP2, Cys120, Cys201 and Cys375 in the CP motifs are also determined to be the heme binding sites (4). The affinity for heme to IRP2 wild-type was 0.05 μM . These observations indicate that the heme binding affinity for IRPs was comparable with the range of cytosolic heme concentrations (21), so that heme bound to IRPs would act as a regulatory molecule.

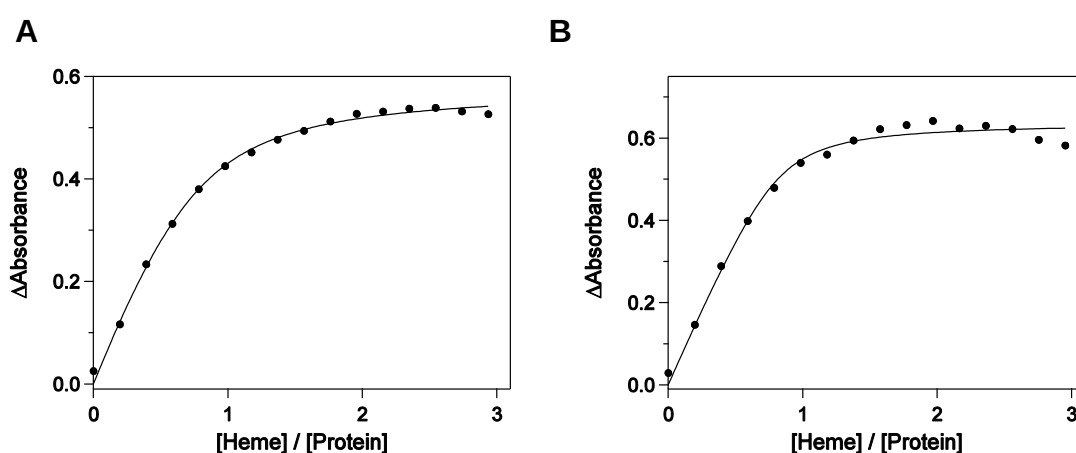
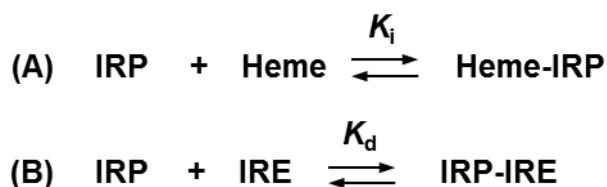


Figure 3.10 Heme titration for IRP1 Cys mutants.

The titration curve for C118A (A) and C300A (B) mutant (4). The curve was fitted to the equation 3.3 to calculate the dissociation constants.

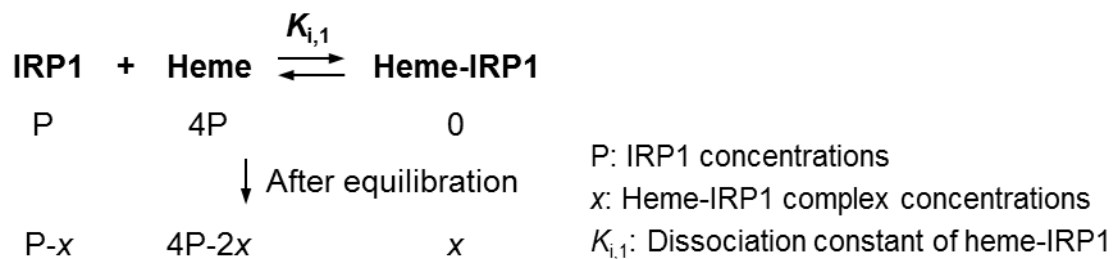
The concentration of IRP-bound heme (Heme-IRP) was calculated. The equilibrium during the measurement was shown in Scheme 3.1. In these measurements, IRP was first mixed with heme (Scheme 3.1A), and subsequently incubated with IRE (Scheme 3.1B).



Scheme 3.1 The equilibrium shown in the fluorescence anisotropy measurement.

Equilibrium for mixing IRP with heme (A) and with IRE (B). K_i : dissociation constant between IRP and heme, K_d : dissociation constant between IRP and IRE.

In the case of adding 4 equivalents of heme, when the concentration of IRP1 was P, the concentrations of heme corresponded to 4P. The concentration of the heme-IRP1 complex is settled as x at the equilibrium. The concentration of free heme is decreased by $2x$ owing to 2 equivalents of heme binding for IRP1 (Scheme 3.2).



Scheme 3.2 The equilibrium between IRP1 and heme.

The dissociation constants of heme-IRP ($K_{i,1}$) is expressed as follows,

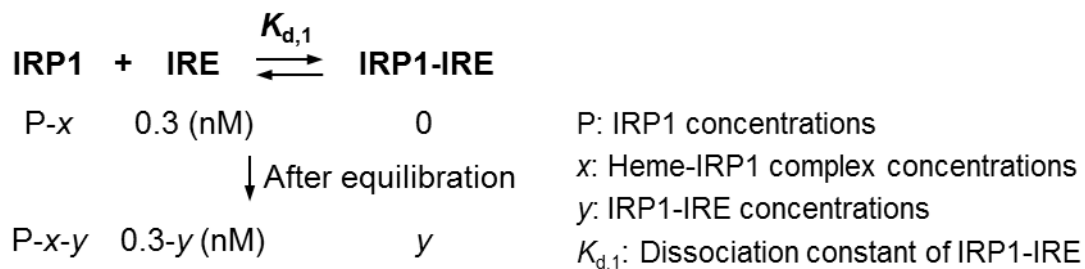
$$K_{i,1} = \frac{(P - x)(4P - 2x)}{x} \quad (3.7)$$

Then, equation 3.7 was transformed by solving the quadratic equation for x ,

$$x = \frac{(6P + K_{i,1}) - \sqrt{(6P + K_{i,1})^2 - 32P^2}}{4} \quad (3.8)$$

From equation 3.8, x is determined in each IRP1 concentration after reaching equilibrium, and the remaining concentration of IRP1 was simultaneously determined as $P-x$. Then, IRE (0.3

nM) was added to the solution containing IRP1 and heme. The reaction mixture reaches equilibrium as shown in Scheme 3.3.



Scheme 3.3 The equilibrium between IRP1 and IRE.

The $K_{d,1}$ is shown as follows,

$$K_{d,1} = \frac{(P - x - y)(0.3 - y)}{y} \quad (3.9)$$

Under the conditions used in these measurements, the value of x is large excess compared to that of y ($x \gg y$), simplifying to

$$K_{d,1} = \frac{(P - x)(0.3 - y)}{y} \quad (3.10)$$

Then, equation 3.10 is transformed by solving the linear equation for y ,

$$y = \frac{0.3(P - x)}{K_{d,1} + (P - x)} \quad (3.11)$$

Using these equations, the concentration of the IRP1-IRE complex was calculated. The value of $K_{i,1}$ and $K_{d,1}$ are 50 nM and 6.9 nM, respectively. The calculated results were expressed as the fraction of IRE concentration and plotted to the protein concentrations overlapped with the measured value for IRP1 (Figure 3.11).

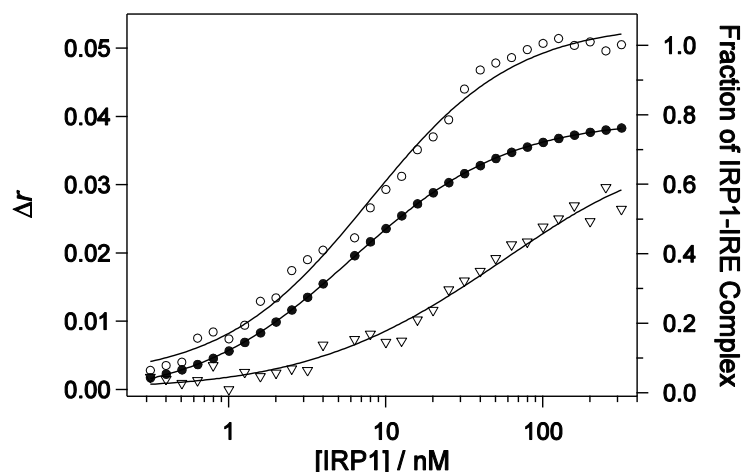
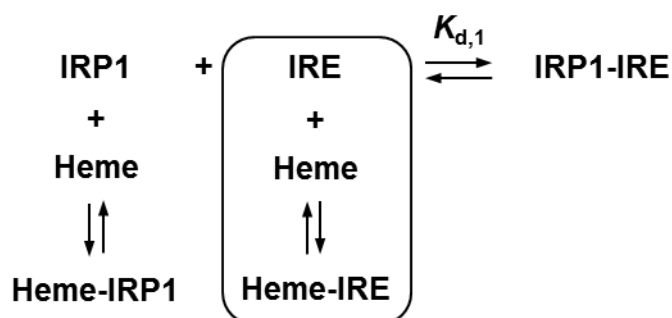


Figure 3.11 The complex formation between IRP1 and IRE.

The fraction of IRP1-IRE complex was calculated for the maximum Δr in apo-IRP1 as 1. The measured value for apo-IRP1 ($\circ$) and in the presence of 4 equivalents of heme (∇). The calculated value in 4 equivalents of heme for the $K_{d,1}$ of 6.9 nM ($\bullet$).

The calculated curve (Figure 3.11, $\bullet$) was drawn under the assumption where heme-bound IRP1 did not bind to IRE. To judge whether the assumption is correct, the calculated curve was compared to the measured curve (Figure 3.11, ∇) in the presence of heme. If heme-IRP1 can bind to IRE, the calculated IRP1-IRE would be identical to the measured curve of apo-IRP1 (Figure 3.11, $\circ$). If heme-IRP1 can NOT bind to IRE, the calculated curve would consistent with the measured curve in the presence of heme (Figure 3.11, ∇). However, the calculated curve (Figure 3.11, $\bullet$) is significantly larger than the measured curve in the presence of heme (Figure 3.11, ∇). Namely, the number of IRP1-IRE complex was smaller than that of calculated value, indicating that the value of K_i or K_d was varied in the presence of heme. Because the value of $K_{i,1}$ is not affected by heme, $K_{d,1}$ would be varied by adding heme. Thus, I hypothesize that the difference between the measurements and calculation was derived from the shift of the $K_{d,1}$ by addition of heme (Scheme 3.4). Indeed, if $K_{d,1}$ was assumed to be 20 nM in IRP1, the calculated curve (Figure 3.12, $\blacklozenge$) is well fitted to the measured curve (Figure 3.12, $\bullet$). Thus, heme affects the value of $K_{d,1}$.



Scheme 3.4 The equilibrium to correct the difference between calculated and measurement value.

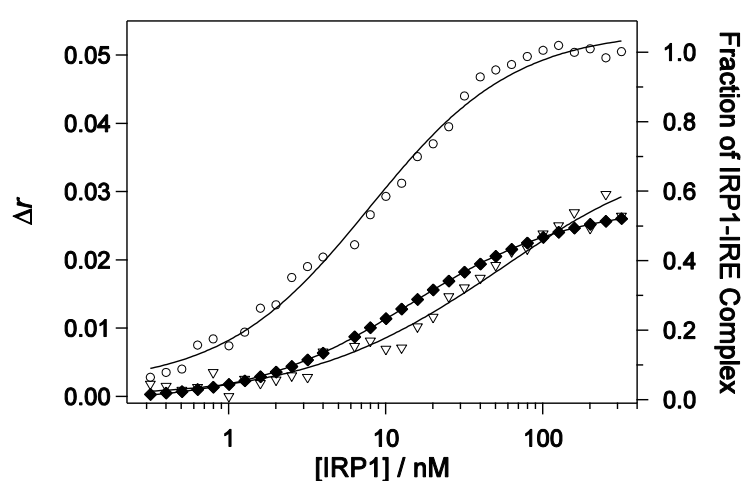


Figure 3.12 The complex formation between IRP1 and IRE.

The fraction of IRP1-IRE complex was calculated for the maximum Δr in apo-IRP1 as 1. The measured value for apo-IRP1 ($\circ$) and in the presence of 4 equivalents of heme (∇). The calculated value in 4 equivalents of heme for the $K_{d,1}$ of 20 nM ($\blacklozenge$).

Nevertheless, the fraction of IRP1-IRE complex was obviously decreased by heme binding in the initial fitting of K_d calculated from the absence of heme (Figure 3.11). The IRE-binding site in IRP1 was not overlapped the binding site of heme from the crystal structure of IRP1-IRE complex. It is difficult to inhibit the IRE-binding activity by the heme binding competitively, suggesting that heme binding to IRP1 induce a conformational change. The conformational change of IRP1 are occurred by conjugation with the Fe-S cluster, which enclose the IRE-binding cleft for the access between domain 3 and 4 (Figure 3.13A). Cys300 and His207 are located for the different domain, and the side chain is faced with the opposite

site each other. Although Cys118 is the axial ligand for the 5-coordinate heme, a water ligand in trans to Cys300 forms the hydrogen bond with His207. Thus, the estimated mechanism for loss of the IRE-binding activity is drawn for Figure 3.13B. Heme coordinates to Cys300 and ligation of water for the sixth ligand, which would attract the side chain of His207 by the hydrogen bond with the water ligand.

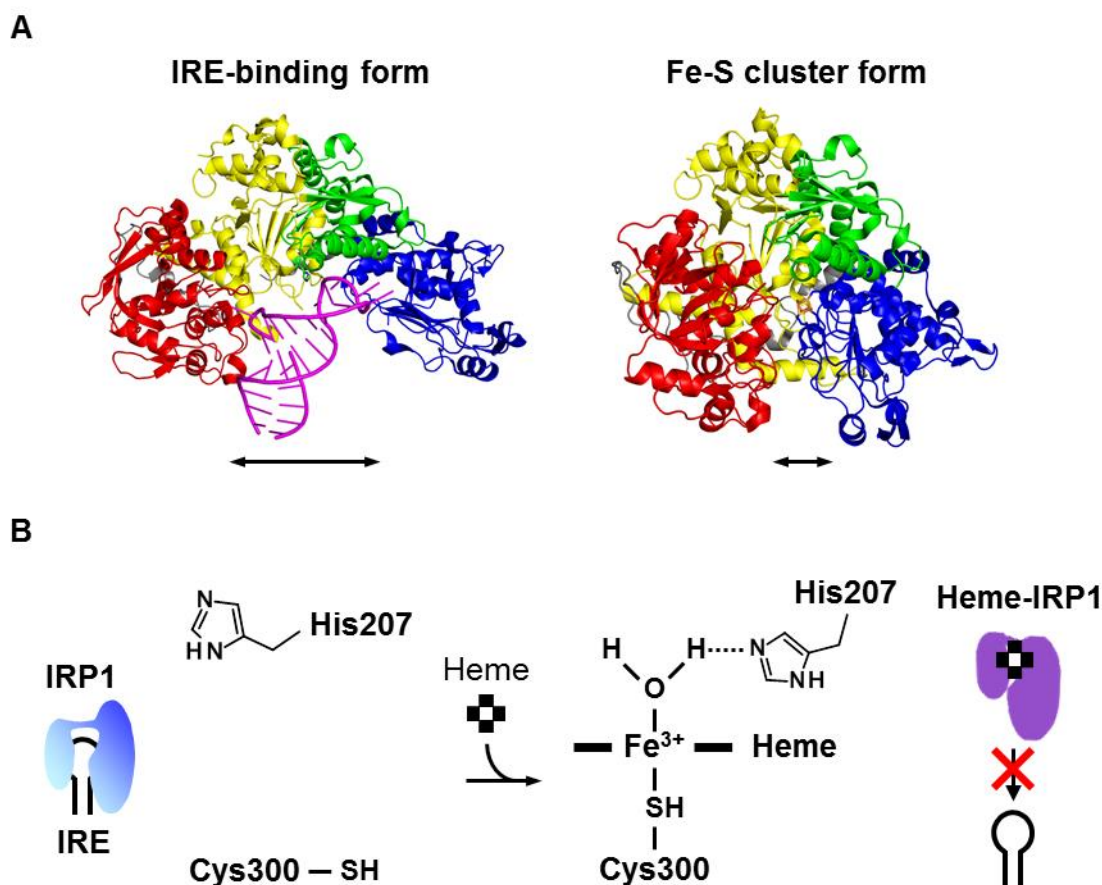
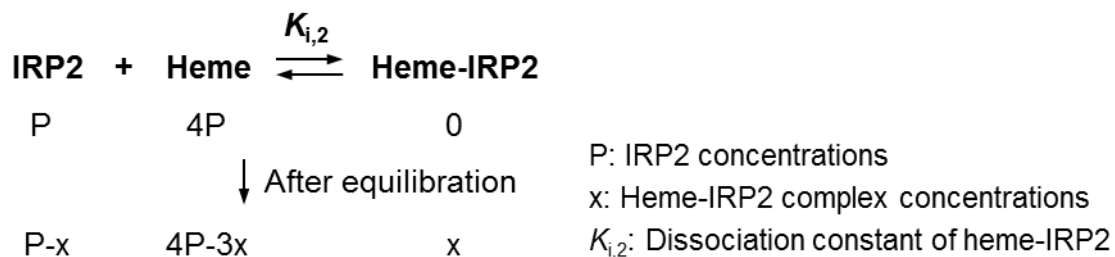


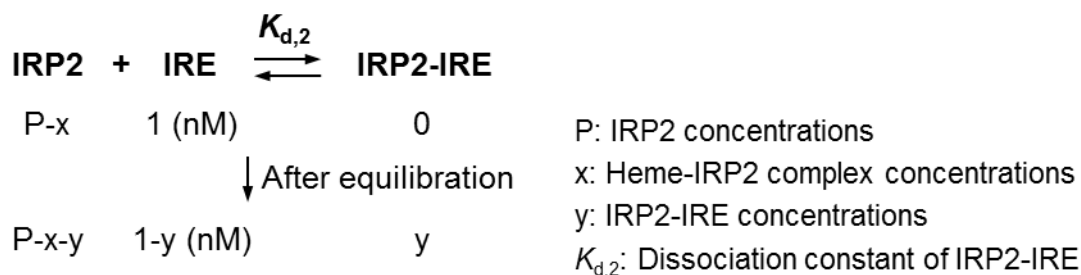
Figure 3.13 Heme effect on the IRE-binding activity.

(A) Comparison of the crystal structure of IRP1 in IRE-binding form (PDB ID: 3SNP) and Fe-S cluster-bound form (PDB ID: 2B3X). The arrows represent the room for cavity of the IRE-binding site. (B) Schematic representation for the conformational change of IRP1 induced by heme. Cys300 and His207 are faced in the different direction. Heme binding and the hydrogen bond between water ligand and His207 induced the conformational change of IRP1.

The procedures were applied for IRP2, obtaining below relationship for IRP2 concentration and the fraction of IRP2-IRE complex (Schemes 3.5 and 3.6). In the case of adding 4 equivalents of heme, when the concentration of IRP2 was P, the concentrations of heme also corresponded to 4P.



Scheme 3.5 The equilibrium between IRP2 and heme.



Scheme 3.6 The equilibrium between IRP2 and IRE.

The equilibrium between IRP2 and heme is shown in Scheme 3.5. The concentration of free heme was decreased by 3x owing to 3 equivalents of the heme binding for IRP2. The value of x is calculated from the dissociation of $K_{i,2}$ (Scheme 3.5) as follows,

$$K_{i,2} = \frac{(P-x)(4P-3x)}{x} \quad (3.12) \quad x = \frac{(7P + K_{i,2}) - \sqrt{(7P + K_{i,2})^2 - 48P^2}}{6} \quad (3.13)$$

The concentration of IRP2-IRE (y) is calculated from the equilibrium between IRP2 and IRE shown in Scheme 3.6. x-y is considered as x owing to $x \gg y$,

$$K_{d,2} = \frac{(P-x-y)(1-y)}{y} \approx \frac{(P-x)(1-y)}{y} \quad (3.14) \quad y = \frac{(P-x)}{K_{d,2} + (P-x)} \quad (3.15)$$

The value of $K_{i,2}$ and $K_{d,2}$ were 50 nM and 18.2 nM, respectively. The calculated results were

plotted to the protein concentrations (Figure 3.14).

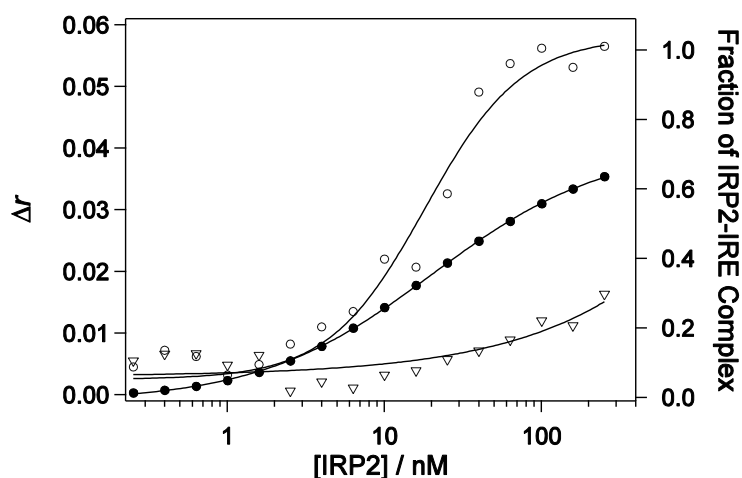
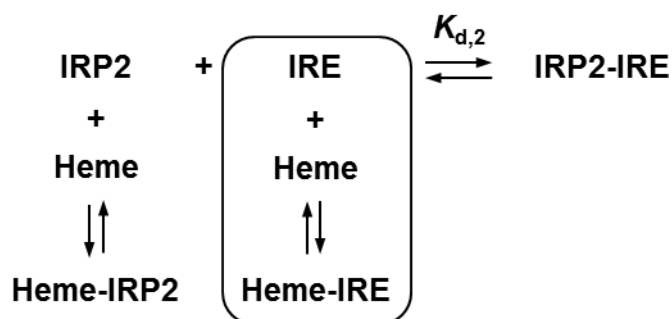


Figure 3.14 The complex formation between IRP2 and IRE.

The fraction of IRP2-IRE complex was calculated for the maximum Δr in apo-IRP2 as 1. The measured value for apo-IRP2 ($\circ$) and in the presence of 4 equivalent of heme (∇). The calculated value in 4 equivalent of heme for the $K_{d,2}$ of 18.2 nM ($\bullet$).

The plot of the calculation curve (Figure 3.14, $\bullet$) was clearly larger than that of the measured curve in the presence of heme (Figure 3.14, ∇) as well as IRP1. Thus, the shift of $K_{d,2}$ by heme binding to IRE was expected (Scheme 3.7). As a consequence, when the $K_{d,2}$ was estimated for 75 nM, the calculation curve (Figure 3.15, $\blacklozenge$) is close to the measured curve (Figure 3.15, ∇). Thus, it is possible to describe that the higher shift to $K_{d,2}$ affects the complex formation between IRP2 and IRE.



Scheme 3.7 The equilibrium to correct the difference between calculated and measurement value.

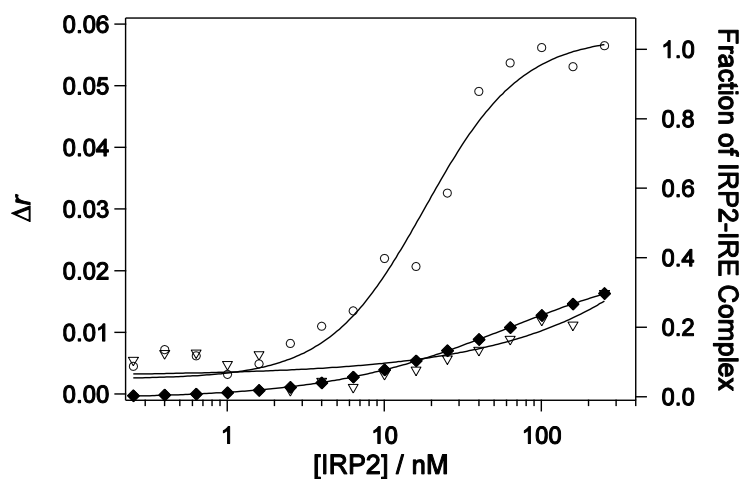


Figure 3.15 The complex formation between IRP2 and IRE.

The fraction of IRP2-IRE complex was calculated for the maximum Δr in apo-IRP2 as 1. The measured value for apo-IRP2 ($\circ$) and in the presence of 4 equivalent of heme (∇). The calculated value in 4 equivalent of heme for the $K_{d,2}$ of 75 nM ($\blacklozenge$).

Both IRPs show the shift of K_d by adding heme, probably caused by the interaction of heme with IRE (Schemes 3.4 and 3.7). The interaction of small compound with IRE was reported in the ferrous ion (Fe^{2+}), by which the secondary structure of IRE was destabilized, leading to dissociation of IRP (14, 15, 22). The same phenomenon was also observed in other divalent metals such as manganese and magnesium (14). In addition, there is evidence that some metals bind to IRE from solution NMR (23, 24), showing that the cationic property of metal would attract anionic RNA. Thus, the electrostatic interactions between heme and IRE lead to the conformational change of IRE, which shifts the larger dissociation constants of IRP and IRE. Nevertheless, the fraction of the IRP2-IRE complex was obviously decreased in the initial fitting using K_d calculated from the results in the absence of heme (Figure 3.14), indicating that heme inhibits the IRE-binding activity of IRPs.

3.4.3. Functional Significance for Regulation by Heme.

Although IRPs are mainly regulated by the expression of genes involved in iron metabolism, the IRE-binding activity of IRPs are determined depending on the heme binding, not iron. This result suggests that heme acts as the iron sensing factor for iron metabolism in IRPs. The specific interaction between IRPs and IRE is crucial for modulation of the cellular iron availability by regulation of gene translation as shown in ferritin mRNA (25–27) or TfR mRNA (28, 29). This effect requires a strongly conserved structured region, suggesting that IRE strongly determines stability of the mRNA. The main purpose of iron utilization is synthesized heme in mitochondria. Especially, erythroid cells are required a lot of iron to produce the hemoglobin in erythrocyte. Based on the genetic analysis in IRE, the first enzyme of the heme biosynthesis, erythroid-specific δ -aminolevulinic acid synthase (eALAS) has IRE, indicating that the translation of eALAS is regulated by IRPs (30, 31). Furthermore, functional IRE was seen in hypoxia inducible factor-2 α to produce erythropoietin, which promotes the erythropoiesis (32). This finding suggests that heme biosynthesis is firmly related to IRPs-IRE regulation system. The heme level must be monitored in case of the lack of heme biosynthesis. It is therefore useful for the regulation of IRE-binding activity by heme. Besides, a wide range of mRNAs containing IRE have been discovered (33). These are not limited to cellular iron metabolism; transcripts of mitochondrial aconitase (citric acid cycle) (34, 35), β -amyloid precursor protein (APP) (36, 37), and α -synuclein (neuronal functions) (38). Although the relationship between these genes and heme is unclear at present, the IRPs-IRE regulation system is related to various pathways.

In summary, to functional characterize the heme binding effects of IRPs on the IRE-binding activity, IRPs were expressed in insect cells and purified. The fluorescence anisotropy measurements clarified that both IRPs lost the IRE-binding activity upon heme binding. The structural change upon heme binding to IRE affects the interaction between IRPs and IRE. I found, for the first time, IRP1 utilizes heme as a regulatory molecule to modulate the IRE-binding activity in *in vitro* experiments.

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CHAPTER IV

HEME EFFECT OF IRPs ON IRE-BINDING ACTIVITY IN CELL USING β -GALACTOSIDASE REPORTER ASSAY

Abstract

The results in Chapter III suggested that the IRE-binding activities of IRPs were regulated by the binding heme, whereas cytosolic heme would not be present in the free form. In addition, IRP1 are known for a Fe-S cluster-bound protein, indicating that the role for two regulatory molecules was overlapped. To reveal the significance of heme in the IRE-binding activity of IRPs, I constructed the cell-based reporter assay using human embryonic kidney 293T cell. The *lacZ* gene, which encodes β -galactosidase (β -gal), was used as a reporter plasmid, and the IRE sequence was inserted into the upstream of the *lacZ* coding region (IRE-*lacZ*). The IRP1 and IRE-*lacZ* plasmids were transfected to 293T cell, and the expression level of β -gal was monitored through the enzymatic activity of β -gal. The transfected cells were cultured in a hemin-treated medium, resulting in the increase of β -gal activity compared with non-treated cells. This result suggests that the IRE-binding activity of IRP1 weaken by exogenously treating heme. To further clarify the relationship between intracellular heme content and the IRE-binding activity, the heme biosynthesis precursor, δ -aminolevulinic acid (ALA) and iron source, ferric ammonium citrate (FAC) were added for the medium. The treatment of ALA and FAC in medium showed the drastic increase in the β -gal activity, although the FAC treatment was no effects on the β -gal activity and cellular heme content. These observations strongly suggest that the affinity of IRP1 for IRE was reduced in increasing the cellular heme content. The IRE-binding activity by heme was, therefore, prevented by the excess supplement of iron for intracellular heme biosynthesis.

4.1. Introduction

In Chapter III, I focused on the heme binding to IRPs, because the CP motifs were conserved in IRPs. The interaction analysis between IRPs and IRE clarified that the IRE-binding activity of IRPs are effectively suppressed in the presence of heme. This result suggested that heme functions as the regulatory molecule for IRPs. However, the interaction profile of the IRPs-IRE complex was not fitted to the calculation estimated using the dissociation constants of the heme-IRPs and IRPs-IRE complex. This difference between measured and calculated curve was thought to be occurred from the weaker IRPs-IRE interaction as discussed in Chapter III. Because the transition metal was reported to affect the structure of IRE through the electrostatic interaction (1–3), the larger affinity of IRPs for IRE was derived from a conformational change of IRE by heme.

As described in Chapter II, cytosolic heme was generally captured by the putative cytosolic protein to avoid the cellular toxicity. This fact suggests that there was no “free heme” under the physiological condition, which made it difficult for the interaction between heme and IRE. A difference between the experimental and physiological condition needs to be filled. Furthermore, early study has reported that IRP1 binds Fe-S cluster to convert the cytosolic aconitase, which catalyzes citrate to isocitrate (4) and the Fe-S cluster binding perturbs the IRE-binding site (5–7). To overcome the problem, a practical method is that the interaction of IRPs-IRE observed in the physiological condition. Reporter assay is the most commonly used to analyze the regulatory function of genes, because the reporter functions as an indicator of transcriptional or translational activity in cells (8). To observe the regulation activity of a region of interest, the region is joined to the reporter gene. The resulting recombinant is used to detect the interaction of the region with the partner protein. The expression level of reporter gene is easily monitored by the optimized method (8). Under this concept, the interactions of IRE with IRPs are monitored in mammalian cells in various heme and/or iron conditions to

investigate the correlation of the IRE-binding activity of IRPs and intracellular heme content.

In Chapter IV, I tried to construct the reporter assay using mammalian cells to confirm the heme effect of IRPs on IRE-binding activity. The *lacZ* gene, which codes β -galactosidase, was used as the reporter gene, and the IRE sequence was inserted into the upstream of the *lacZ* coding region. The expression level of β -galactosidase was monitored in mammalian cells introduced IRPs and IRE-inserted *lacZ*.

4.2. Experimental Procedures

4.2.1. Materials.

All chemicals were purchased from Thermo Scientific (Waltham, MA, USA), Nacalai Tesque (Kyoto, Japan), and Sigma-Aldrich (St. Louis, MO, USA) and were used without further purification.

4.2.2. Plasmids.

The *IRP* gene in pFastBac used in Chapter III was amplified by PCR and cloned into the pcDNA3.1 vector (Thermo Scientific) via the NotI-XbaI restriction sites using KOD -Plus-Neo (TOYOBO, Osaka, Japan). Myc tag (Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu) was inserted between the C-terminal of IRP sequence and stop codon by PCR using a PrimeSTAR mutagenesis basal kit. The pSV β -galactosidase Control Vector (Promega, Madison, WI, USA) coding *lacZ* was used as the reporter plasmid and inserted the human ferritin IRE sequence (IRE-*lacZ*) by PCR. The primers used in this chapter were listed in Table 1. All constructs were confirmed by DNA sequencing.

4.2.3. Reagents.

Hemin (0.13 g) was dissolved in 0.3 M aqueous ammonia, and adjusted pH to 8.0 by adding HCl drop by drop not to precipitate hemin. The solution was diluted to 10 mM by 0.3 M aqueous ammonia whose pH was adjusted to 8.0 in advance. The stock solution was sterilized by filtration, and stored at -80 °C until use. Ferric ammonium citrate (FAC), δ -aminolevulinic acid (ALA), deferoxamine (DFO), and 4,6-dioxoheptanoic acid (succinylacetone: SA) were dissolved to 10 mg/mL, 100 mM, 10 mM, and 200 mM in sterile water, respectively. In ALA and SA, the solutions were sterilized by filtration, and stored at -20 °C until use. DFO and FAC were stored at 4 °C.

Table 4.1 Oligonucleotide used for construction of expression vectors.

The underlined bases signify the recognition sequence of digestion enzymes (for cloning: NotI in sense-strand and XbaI in anti-sense-strand) and introduced bases (for insertion). S: sense-strand, AS: anti-sense-strand

Constructs	Strand	Primers (5'→3')	Application
pcDNA3.1-IRP1	S	ATAG <u>GCGGCCGC</u> ATGAGCAACCCATTTCGCACACCTT	Cloning
	AS	<u>CTCTAGAC</u> TACTTTGGCCATCTTGCGGATCATG	
pcDNA3.1-IRP2	S	TGCGGCCGCATGTCGTACTACCATCACCATCACCA	Cloning
	AS	GCGCT <u>CTAGATT</u> TATGAGAATTTTCGTGCCACAAAG	
Myc tag-IRP1	S-1	<u>GAGCAGAAGCTGATCT</u> AGTCTAGAGGGCCCGTT	Insertion
	AS-1	<u>GATCAGCTTCTGCTCCT</u> TGGCCATCTTGCGGAT	
	S-2	<u>TCAGAGGAGGACCTGT</u> AGTCTAGAGGGCCCGTT	
	AS-2	<u>CAGGTCCTCCTCTGAG</u> ATCAGCTTCTGCTCCTT	
Myc tag-IRP2	S-1	<u>GAGCAGAAGCTGATCT</u> AATCTAGAGGGCCCGTT	Insertion
	AS-1	<u>GATCAGCTTCTGCTCT</u> GAGAATTTTCGTGCCAC	
	S-2	<u>TCAGAGGAGGACCTGT</u> AATCTAGAGGGCCCGTT	
	AS-2	<u>CAGGTCCTCCTCTGAG</u> ATCAGCTTCTGCTCTGA	
IRE- <i>lacZ</i>	S-1	<u>CCTGCTTCAACAGTGT</u> CACATGAGCGAAAAATA	Insertion
	AS-1	<u>CACTGTTGAAGCAGGAG</u> TGTCCCAGCCTGTTTA	
	S-2	GTGCTTGGACGGTCACATGAGCGAAAAATA	
	AS-2	TGACCGTCCAAGCACTGTTGAAGCAGGAGT	

4.2.4. Cell Culture and Transfection.

The human embryonic kidney cell line, 293T (RCB2202) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin G, and 100 µg/ml streptomycin at 37 °C in ambient air with 5 % CO₂. Transient transfections were carried out in Opti-MEM using Lipofectamine 2000 according to the manufacturer's protocol. Typically, 3×10^6 cells were plated per 60-mm dish and incubated for 24 hours prior to transfection with pcDNA-IRP1 and IRE-*lacZ*. When Opti-MEM medium was replaced with DMEM medium after transfection, SA (0.5 mM) or DFO (100 µM) was treated in the case of decreasing iron level. On the following day, cells were treated with the indicated concentration of hemin, FAC, and ALA for the times indicated in manuscripts. At ~ 16 hours after treatment, cells were washed using ice-cold PBS for two times, and harvested with 1 mL PBS to sterile microtube using cell scraper. PBS was removed after centrifugation at $10,000 \times g$ for 5 minutes. Harvested cells were incubated in mammalian cell lysis buffer containing 20 mM HEPES-NaOH, 150 mM NaCl, 0.5 % Triton-X100, and 1 tablet/50 ml Complete EDTA-free (pH 7.4) on ice for 10 minutes, and subsequently lysed by vortex. The lysates were cleared by centrifugation at $15,000 \times g$ for 10 minutes prior to the assay. Protein concentrations in lysates were calculated by Pierce 660 nm Protein Assay Reagent (Thermo Scientific) using BSA as a standard.

4.2.5. Western Blotting.

The lysates (50 μ g) was separated on 10% SDS-PAGE gel with Precision Plus Protein Western C Protein Standards (Bio-Rad) for determination of the molecular mass. Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad) with semi-dry method. Membranes were blocked with 5% skim milk containing PBS and 0.05% Tween-20 for 1 hour at room temperature, and then probed with 1:1000 dilution of peroxidase conjugated anti-myc monoclonal antibody in skim milk for approximately 12 hours at 4 °C. The probed membrane was extensively washed by PBS containing 0.05% Tween-20. The proteins were visualized by EzWest Blue (ATTO, Tokyo, Japan) according to the manufacturer's protocol.

4.2.6. β -galactosidase Reporter Assay.

The activity of β -galactosidase (β -gal) was measured by the enzymatic reaction of the substrate, *o*-nitrophenyl- β -D-galactopyranoside (ONPG) (8). The reaction mixture was prepared by mixing 30 μ L of cell lysates, 66 mM sodium phosphate (pH 7.5), 1 mM MgCl_2 and 45 mM 2-mercaptoethanol. The assay was initiated by adding 66 μ L of 4 mg/mL ONPG to the reaction mixture at 37 °C. When the solutions turned faint yellow, the reaction was stopped by adding 1 M sodium carbonate. After centrifugation, the optical density at 420 nm (OD_{420}) derived from *o*-nitrophenol was measured. The activity was normalized by total protein concentrations of cell lysate.

4.2.7. Quantification of Heme Content.

Heme contents were determined by the fluorometric method (9, 10). The lysates were treated with 0.5 mL of 2 M oxalic acid, and heated at 100 °C for 30 minutes. The mixtures without heating were used as a blank for measurement of endogenous porphyrins. After heating, the mixtures were centrifuged at $15,000 \times g$ for 10 minutes at 4 °C. The fluorescence was recorded in a JASCO FP-8500 fluorometer. The excitation wavelength was 400 nm, and the fluorescence emission was determined at 600 nm. Hemin solutions, containing 0, 50, 100, and 250 pmol of hemin, were prepared as standards for the same way as samples.

4.3. Results

4.3.1. Construction of β -gal Reporter Assay Using 293T Cells.

I constructed a reporter assay system composed of IRPs and the reporter gene *lacZ*, which expresses a glycolytic protein, β -galactosidase (β -gal) (Figure 4.1). I prepared the two plasmids to express the IRPs and β -gal. To identify the interaction between IRPs and IRE, the human ferritin IRE sequences were inserted into the upstream of the *lacZ* gene (IRE-*lacZ*). The interaction between IRPs and IRE was observed through the expression of β -gal. The *IRP* gene was inserted to the pcDNA3.1 vector. To monitor the binding IRP to IRE, the expression level of β -gal was investigated in the mammalian cells that were transfected the two plasmids (Figure 4.1). When IRP binds to IRE, the β -gal protein could not be expressed owing to inhibition of the association of ribosome to mRNA. In contrast, when IRP loses the IRE-binding activity, β -gal was expressed.

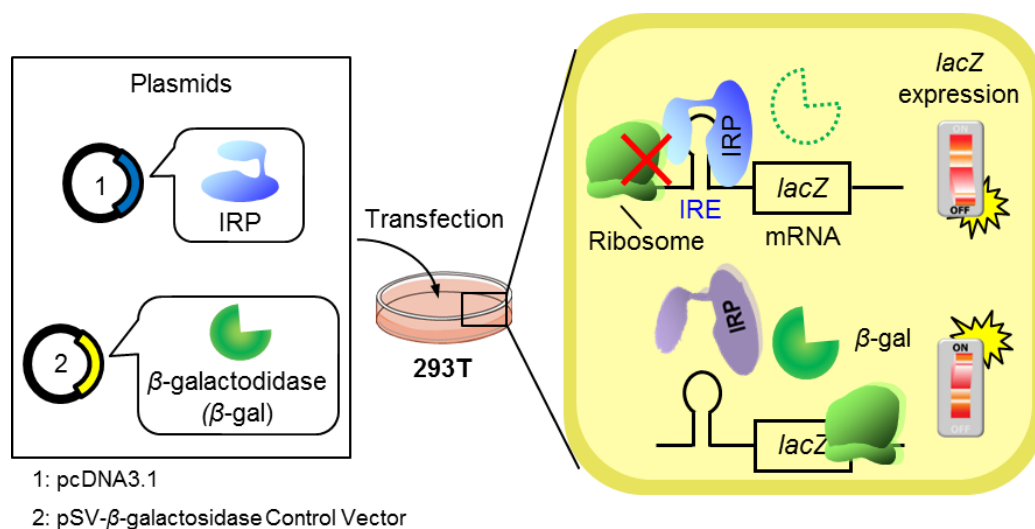


Figure 4.1 Schematic representation of the reporter assay using 293T cells.

Prior to construction of the reporter assay, expression of IRP in 293T cells was first examined. A myc-tag was attached to the C-terminal of IRP to detect the expression of IRP by immunoblotting. IRP1 and/or IRE-*lacZ* plasmids were transfected to 293T cells using a lipofection method. The cytosolic extracts of the cells were separated by SDS-PAGE, and subsequently transferred to the PVDF membrane. The immunoblotting using anti-myc antibody was performed to visualize IRP1 bands. The 293T cells that harbor IRP1 plasmid successfully expressed IRP1 (Figure 4.2). IRP1 was also expressed when co-transfected with IRE-*lacZ* (Figure 4.2). The band was corresponded to the estimated molecular mass of IRP1, indicating that IRP1 remained intact in 293T cells. This result showed that transfection of IRP1 plasmid was successful in the presence of IRE-*lacZ* plasmid together.

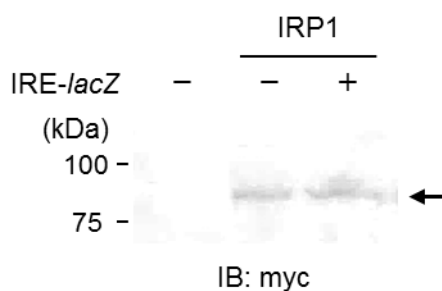


Figure 4.2 Western blotting for myc-IRP1.

Cytosolic extracts from 293T cells with no plasmid, IRP1 or both IRP1 and IRE-*lacZ* plasmids were electrophoresed. IRP1 was detected by immunoblotting (IB) using anti-myc antibody. The black arrow represents that the estimated molecular mass of IRP1.

The suppression of the β -gal activity depending on IRP1 was examined. The expression level of β -gal was quantified via its enzymatic activity, which hydrolyzes the substrate, *o*-nitrophenyl- β -D-galactopyranoside (ONPG), to β -D-galactoside and *o*-nitrophenol (Figure 4.3A). ONPG has no visible color, whereas *o*-nitrophenol exhibits yellow color in solution. The product can, therefore, be spectrophotometrically followed by monitoring the absorbance at 420 nm. To optimize the ratio of plasmids for suppression of the β -gal activity, the amount of IRP1 plasmid was changed in the fixed amount of IRE-*lacZ* plasmid (1 μ g). Transfected cells were lysed, and the enzymatic reaction of β -gal was analyzed by adding ONPG (Figure 4.3B). The activity of the cell without IRP1 plasmid was normalized to 1. When 1 or 2 μ g of IRP1 plasmid was added, the relative activity was 0.8 or 1.1, respectively. The promoter of the expression plasmid of IRP1 and IRE-*lacZ* were cytomegalovirus (CMV) and SV40 early promoter, respectively. In 293T cells, the transcriptional activity of CMV promoter was 4-fold higher than that of the SV40 early promoter (11). However, the expression level of IRP1 in this plasmid content was not enough to suppress the β -gal level. In fact, when 4 μ g of IRP1 plasmid was transfected, the relative β -gal activity was less than 0.4 (Figure 4.3B). Because suppression of the β -gal activity was derived from IRE-binding of IRP1, the interaction between IRP1 and IRE in cellular condition could be monitored. Therefore, the ratio of IRP1 to IRE-*lacZ* plasmids for later experiments was determined to be 4:1.

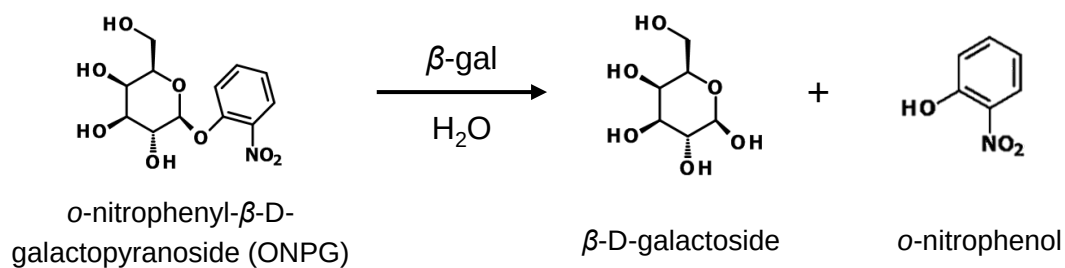
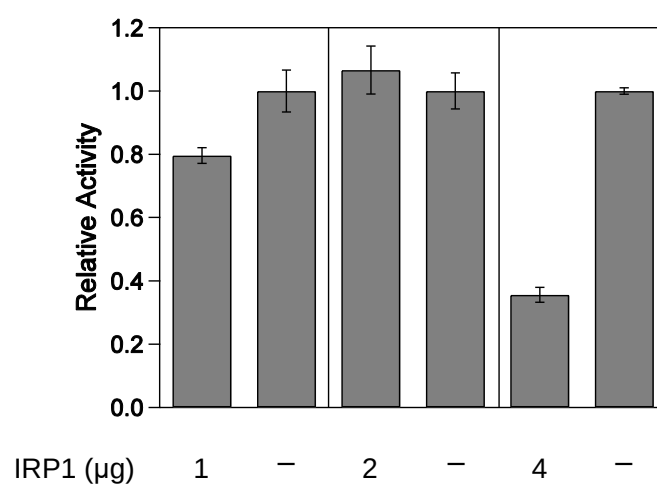
A**B**

Figure 4.3 β -gal activity co-transfected with a various amount of IRP1 plasmid.

(A) The enzymatic reaction of β -gal using the substrate, *o*-nitrophenyl- β -D-galactopyranoside (ONPG). The absorbance of 420 nm product, *o*-nitrophenol, colorization was monitored. (B) Cells were transfected 1 μ g of IRE-*lacZ* and indicated amount of IRP1 plasmid. Minus (-) represents the absence of IRP1 plasmid. The independent measurement was performed in each ratio.

4.3.2. Reporter Assay for the Cells Treating Exogenous Heme.

The IRP1 binding to IRE was confirmed by observing the suppression of the β -gal activity. To observe the heme binding effects on the IRE-binding activity of IRP1 through the expression level of β -gal, the cells transfected IRP1 and IRE-*lacZ* were cultured in a hemin-containing medium (Figure 4.4A). The relative β -gal activity was decreased to less than 0.4 in the presence of IRP1 and IRE-*lacZ*. When 5 μ M of hemin was added, the relative β -gal activity was almost no change (less than 0.5). In contrast, in the presence of 15 μ M of hemin, the activity was recovered to 0.7, suggesting that the IRE-binding activity of IRP1 was suppressed by heme. Then, to confirm that heme was not degraded by endogenous heme-degrading enzyme like heme oxygenase into the cells, the heme content in lysates was quantified. The cytosolic extract was treated with oxalic acid to produce the iron-free porphyrin, protoporphyrin IX, and then fluorescence from protoporphyrin IX was measured. The amount of heme in samples was normalized for the total protein concentration (Figure 4.4B). The heme content in cells not treated heme in medium was almost same (170 ~ 200 pmol/mg protein). The transfected cells of IRP1 and IRE-*lacZ* were cultured in the medium with 5 and 15 μ M hemin, resulting in the increase of the heme content to 20.1 and 80.1%, respectively, compared to the cells without plasmid (Figure 4.4B). To exclude the possibility that the recovery of the β -gal activity was derived from the degradation of IRP1, the expression of IRP1 was monitored using western blotting (Figure 4.4C). The expression level of IRP1 was constant even in the hemin-treated cells, indicating that the β -gal activity was recovered due to suppression of the IRE-binding activity, not degradation of IRP1. This observation suggests that the recovery of the β -gal activity was correlated with the cytosolic heme content.

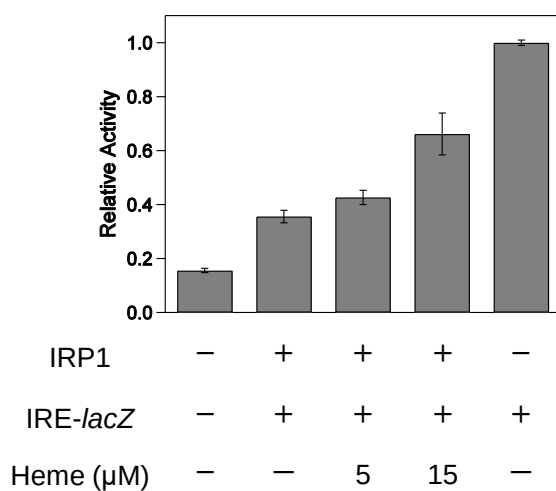
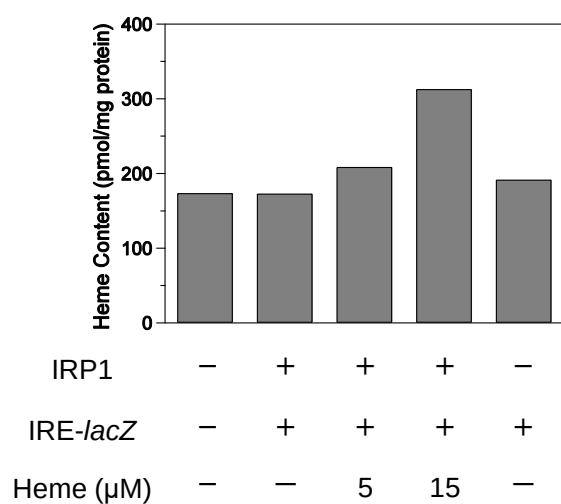
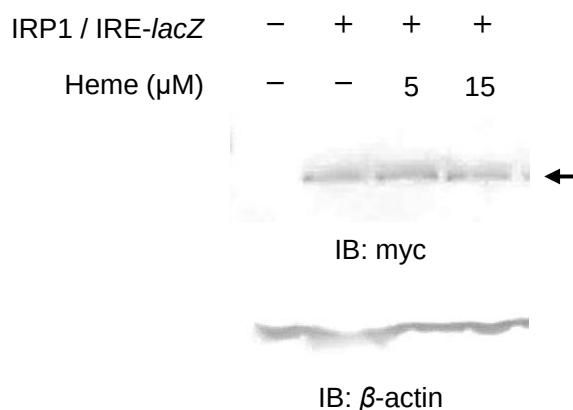
A**B****C**

Figure 4.4 The β -gal assay for the hemin-containing medium.

(A,B) 293T cells were transiently transfected with the pcDNA3.1-IRP1 and the IRE-*lacZ* plasmids, then treated with or without the indicated concentration of hemin for 16 hours. The supernatant of the cell lysate was used for the β -gal reporter assay (A) and quantification of the heme content (B). (C) Cytosolic extracts from 293T cells with no plasmid (-) or both IRP1 and IRE-*lacZ* plasmids (+) were cultured in hemin-treated medium at indicated concentration. IRP1 was detected by immunoblotting (IB) using anti-myc antibody. The black arrow represents that the estimated molecular mass of IRP1. anti- β -actin were detected as a loading control.

4.3.3. Reporter Assay for the Cells in Stimulating Heme Biosynthesis.

The IRE-binding activity of IRP1 was weakened by exogenous heme treatment. Although the cellular heme importer was still controversial (12), the intracellular heme content was successfully increased in a dose-dependent manner (Figure 4.4B). In contrast to the exogenous treatment, heme is also stimulated by treatment of the heme precursor, ALA via heme biosynthesis pathway (13), suggesting that the effect of intracellular heme biosynthesis on the β -gal activity was clarified (Figure 4.5). ALA and iron was treated on the previous day of the assay, and the cells were cultured for about 16 hours. The relative β -gal activity of transfected cells in the ALA, Fe-treated medium was similar for the only IRE-*lacZ* transfected cells corresponding to nearly 1 (Figure 4.5A). In this experimental condition, the intracellular heme content of ALA, Fe-treatment cells was also drastically increased to more than 500 pmol/mg protein (Figure 4.5B). This observation suggests that the β -gal activity was proportional to the intracellular heme content. Namely, IRP1 was loss of the IRE-binding activity in response to increasing the heme content.

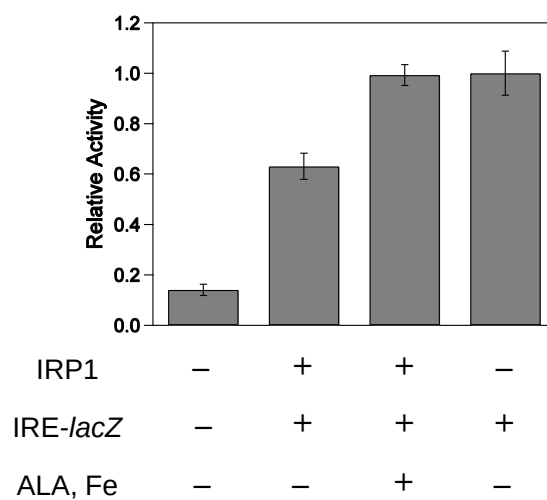
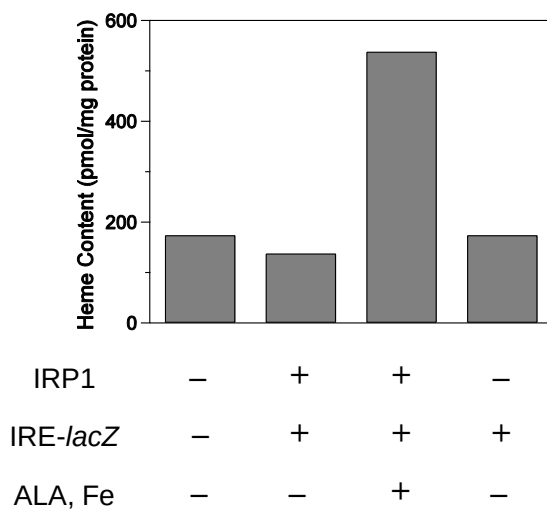
A**B**

Figure 4.5 The β -gal assay for the treatment of ALA and Fe.

293T cells were transiently transfected with the pcDNA3.1-IRP1 and the IRE-*lacZ* plasmids, then treated with or without the indicated concentration of ALA (1.2 mM) and FAC (100 μ g/mL) for 16 hours. The supernatant of the cell lysate was used for the β -gal reporter assay (A) and quantification of the heme content (B).

4.3.4. Reporter Assay for Cells Treating Iron.

Because the early cellular experiment suggested that the IRE-binding activity was affected in an iron-dependent manner (14), the effect of iron on the IRE-binding activity of IRP1 was investigated. Immediately after transfection of the plasmids, the cells were cultured in the DFO-containing medium to reduce cellular iron (DFO-pretreated in Figure 4.6A). The medium was replaced with the FAC-containing medium on the following day, and the cells were incubated another 16 hours. The relative β -gal activity of transfected cells in DFO-pretreated medium was of approximately 0.4 (Figure 4.6A), which was same as that in non-treated medium (Figure 4.4A). This result indicates that the iron deprivation does not affect the IRE-binding activity of IRP1 in this reporter system. Furthermore, when 50 and 200 $\mu\text{g/ml}$ of FAC was treated to the medium, the relative β -gal activity showed no change compared to the DFO-treated medium (Figure 4.6A). At this time, the absolute heme level of DFO-treated cells (~ 80 pmol/mg protein) was lower than that of non-treated cells (~ 150 pmol/mg protein) due to the deficient of iron level by treating DFO (Figure 4.6B). In contrast, the heme level was not increased in the presence of iron, indicating that heme biosynthesis was independent of the exogenous treatment of iron in this reporter system (Figure 4.6B). Because the heme biosynthesis was not stimulated by exogenous iron treatment as suggested previously (10), the β -gal activity was not recovered in FAC-treatment medium. Therefore, taken together my current results, I successfully demonstrated that the IRE-binding activity of IRP1 was responded to the intracellular heme level.

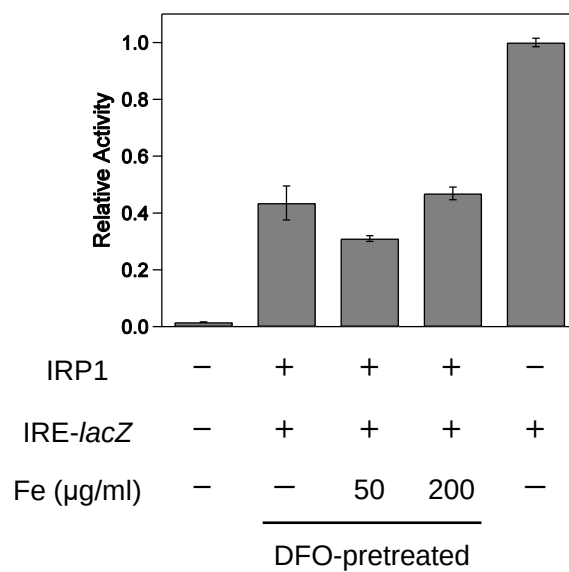
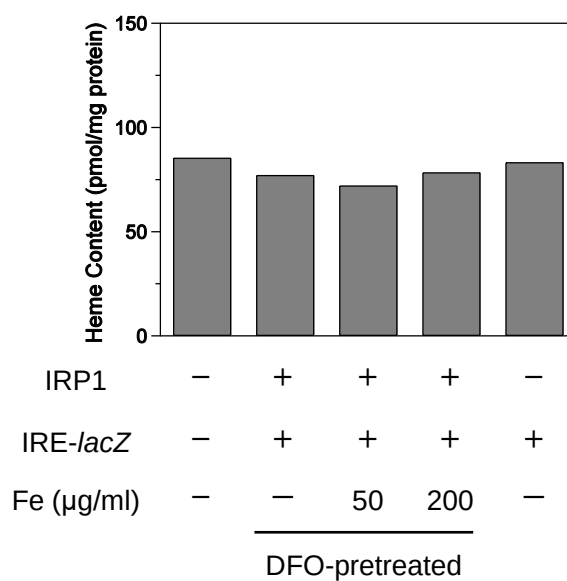
A**B**

Figure 4.6 The β -gal assay for the iron-treatment medium.

293T cells were transiently transfected with the pcDNA3.1-IRP1 and the IRE-*lacZ* plasmids, then treated with or without the indicated concentration of FAC for 16 hours. The supernatant of the cell lysate was used for the β -gal reporter assay (A) and quantification of the heme content (B).

4.3.5. Expression of IRP2 in 293T Cells.

As well as IRP1, IRP2 and/or IRE-*lacZ* plasmids were transfected to 293T, and the expression of IRP2 was checked by western blotting. However, the expression of IRP2 under the same condition as IRP1 was not confirmed (Figure 4.7, ①-③). Because IRP2 was easily degraded in the iron-replete condition, the expression check was performed using transfected cells cultured by various iron/heme level (Figure 4.7, ④-⑪). Unfortunately, the expression of IRP2 was not confirmed at any conditions. Because IRP2 was sensitive to the proteasomal degradation triggered by the heme-mediated oxidation (10, 15), IRP2 was degraded under this experimental condition. Thus, it would be useful for decrease in the heme level using heme-depleted serum (16, 17), or mutagenesis of the site involved in oxidation of IRP2 (10).

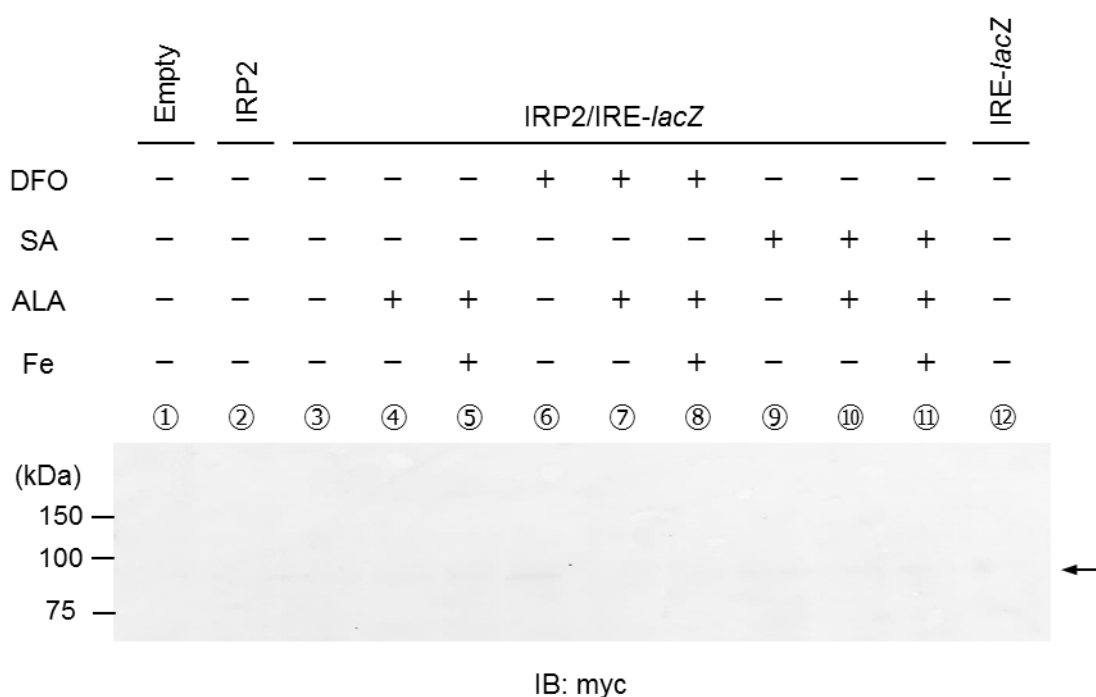


Figure 4.7 Western blotting for myc-IRP2.

293T cells were transfected the plasmids shown above the graphic, and incubated the reagents for 16 hours at the following concentrations: DFO, 100 μ M; SA, 0.5 mM; ALA, 1.2 mM; and FAC, 100 μ g/mL. Myc-IRP2 was analyzed by immunoblotting using anti-myc antibody. The black arrow represents the estimated molecular mass of IRP2.

4.4. Discussion

4.4.1. IRE-Binding Activity of IRP1 Response to Cytosolic Heme Level.

In this reporter system, the β -gal activity was recovered by increasing both exogenous and endogenous heme level (Figure 4.4 and 4.5). In both experiments, the heme content was increased. Especially, there are two possible explanations by exogenous heme treatment,

- I. Cells directly utilize the absorbed heme as a regulatory molecule.
- II. Incorporated heme was once degraded by heme-degrading enzyme (heme oxygenase), and produced iron. The iron was subsequently utilized for heme biosynthesis, leading to increase the heme level.

In explanation II, the iron level affects the heme biosynthesis, because generation of iron was mediated. Thus, if explanation II was true, the same result was observed for the treatment of exogenous iron, because iron generates as the intermediate. The iron treatment has clearly no effects on both activity and heme content (Figure 4.6), indicating that exogenous heme directly functions as a regulatory molecule. The treatment of the heme precursor, ALA, significantly affects the intracellular heme level independent of adding iron. In contrast, when iron was removed from medium using DFO, heme biosynthesis was not stimulated. This is because cells could obtain iron from the medium. Therefore, the ALA treatment strongly supports the involvement of heme in the activity. The cellular heme content was comparable to that of previous investigation using ALA (9), indicating that the reporter system works well. Moreover, an important aspect of heme as a regulatory molecule is that heme exists in the equilibrium between free and protein-bound state (18). Taken together, these observations suggest that the IRE-binding activity of IRP1 was regulated by the intracellular heme level, independent of the supply pathway (exogenous or endogenous).

4.4.2. Involvement of Heme in the Regulation of IRP1.

There are some reports on the effect of heme on the IRP-IRE system. Initially, the expression level of ferritin protein promoted in hemin-treated cells (19–21). For IRP1, Fe-S cluster, not heme, has been known to work as a unique regulatory molecule for the IRE-binding activity. However, in this thesis, I found that IRP1 utilizes heme as a regulatory molecule. The question is how IRP1 distinguishes between Fe-S cluster and heme. Recent studies have shown that the mutation of the Fe-S cluster binding site in IRP1, which renders the Fe-S cluster free form, triggers for destabilization and degradation by the ubiquitin-proteasome pathway by culturing with 100 μ M hemin (22, 23). Furthermore, an E3 ubiquitin ligase (FBXL5) was identified, which recognizes mainly IRP2, but it also recognizes Fe-S cluster-free IRP1 (24, 25). These observations enable me to hypothesis that heme-bound IRP1 becomes the target for degradation, although Fe-S cluster-bound IRP1 was converted to aconitase. However, IRP1 was still expressed even in the treatment of at most 15 μ M hemin (Figure 4.4C), in spite of that the expression level of the β -gal was recovered (Figure 4.4A). Accordingly, heme affects the IRE-binding activity of IRP1, which is consistent as described in Chapter III. Taken together, IRP1 would obtain the following roles upon heme binding: one is the suppression of IRE-binding activity, and the other is the target for degradation by ubiquitin-proteasome pathway (Figure 4.8).

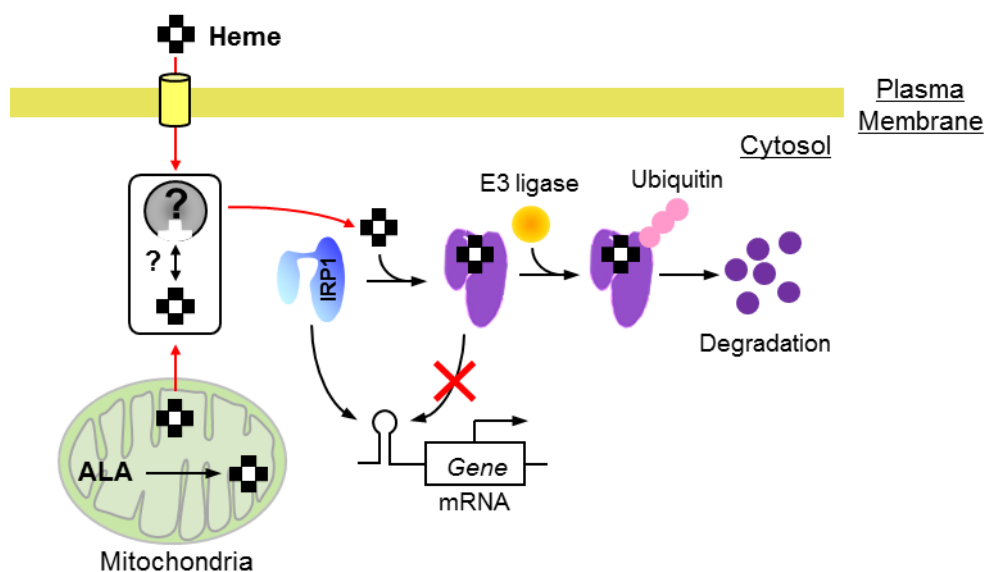


Figure 4.8 Schematic representation of the response for IRP1 by increasing cytosolic heme.

In summary, to characterize the heme-mediated regulation of IRE-binding activity in cellular condition, the reporter assay was constructed by observing the expression level of IRE-inserted *lacZ* gene. I successfully examine the IRE-binding activity of IRP1 by monitoring the enzymatic activity of the product, β -gal. Using this reporter system, it was confirmed that the IRE-binding activity of IRP1 was suppressed, depending on the increase in cytosolic heme level. The response was independent of the pathway for both endogenous and exogenous, indicating that heme acts as the cytosolic iron sensing factor for IRP1.

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CHAPTER V

CONCLUSIONS

In the present thesis, I aimed to elucidate the mechanism for heme metabolism by heme-dependent regulation of protein. Free heme has high reactivity with ROS, resulting in disruption of biomolecule (1–3). Thus, the protection of free heme in cytosol must be needed. Some cytosolic heme-binding proteins are thought to be the candidates for separating the synthesized heme from cytosolic ROS. However, the functional characterization of these proteins has never been performed. Because the proteomics analysis using hemin-agarose identified peroxiredoxin-1 (PRX1) from cytosolic extracts of mammalian cells, I focused on PRX1, among cytosolic heme-binding proteins in Chapter II. For utilization of cytosolic heme as a regulatory molecule, iron regulatory proteins (IRPs) are the candidate for regulation of its binding region, iron-responsive element (IRE). IRPs have conserved CP motifs in amino acid sequences, suggesting that the IRE-binding activity of IRPs is regulated by heme. In Chapter III, the interaction analysis elucidated that heme effectively suppressed the IRE-binding activity of IRPs. Furthermore, heme works as the regulatory molecule for IRE-binding activity confirmed in the cellular condition in Chapter IV. In Chapter V, the results and discussion in this thesis, and future perspectives are highlighted.

PRX1 Provides the “Scavenger” for Cytosolic Free Heme. (Chapter II)

UV-vis and resonance Raman spectroscopies elucidated that PRX1 was a heme-binding protein via Cys52 as an axial ligand, which was identical to the active center of the antioxidant activity. The affinity of PRX1 for heme was calculated to be 0.17 μM . Heme-bound PRX1 significantly diminished its cysteine-dependent peroxidase activity, by which hydrogen peroxide is reduced to water. Instead, the hemin peroxidase activity and H_2O_2 -mediated hemin degradation were reduced by binding to PRX1 compared with free hemin, indicating that PRX1 prevents the cytosolic free heme from reacting with ROS. Therefore, cytosolic free heme scavenges by PRX1 for protection of the hemin degradation.

Heme as the Regulatory Molecule for IRPs (Chapter III)

For iron metabolism, IRPs regulates the translation of mRNAs that have the consensus IRE sequence. The iron sensing factor would be necessary for modulation of the IRE-binding activity. In high iron concentration, IRPs lose its IRE-binding activity. IRPs have conserved CP motifs in their amino acid sequences. Thus, I investigated the heme effects on the IRE-binding activity in IRPs. The heme coordination environment for IRP1 was estimated to the mixing state of Cys and Cys/H₂O from UV-vis spectroscopy. Moreover, fluorescence anisotropy clarified that the IRE-binding activities of both IRPs were significantly diminished in the presence of heme. Calculation of the number of IRPs-IRE complexes from the dissociation constant was not fitted to the measured results owing to the interaction of heme with IRE, resulting in the upper shift for the affinity of IRPs-IRE. Even deducting this effect, the IRE-binding activity of IRPs is suppressed upon heme binding.

Effect of Heme Binding on IRP1 in Mammalian Cell. (Chapter IV)

According to the results in Chapter III, heme-bound IRPs lost the IRE-binding activity. Next, I prompted to observe heme effects on the interaction between IRP1 and IRE in cellular condition. To this end, I constructed the β -gal reporter assay. The IRE sequence was inserted to the upstream of the *lacZ* reporter gene, and the catalytic activity of β -gal was monitored to quantify the expression level. The expression level of β -gal was drastically recovered by treatment of heme or ALA to the medium compared with non-treated medium, whereas the treatment of iron in medium was no effect on the expression level of β -gal. These findings suggest that the IRE-binding activity of IRP1 was regulated in a heme-dependent manner, not an iron-dependent manner. Collectively, heme was potentially acts as the cellular iron sensing factor for iron metabolism.

Heme-Mediated Regulation of the Intracellular Heme Metabolism.

From the results in my thesis, the regulation of the intracellular heme metabolism is proposed via the regulation of the protein function upon heme binding (Figure 5.1). The point to be emphasized is that the cytosolic heme level is the indicator of the protein regulation. The proteins studied in this thesis have the affinity for heme within the estimated cytosolic heme level (0.1-1 μM) (4), indicating that the protein regulation by heme was routinely carried out to maintain the cytosolic heme homeostasis. IRP1 (and probably IRP2) sense the cytosolic heme to regulate the expression level of genes involved in iron metabolism through the modulation of the IRE-binding activity (Figure 5.1). In shortage of cytosolic heme level, IRP1 is active for suppression of the gene expression such as ferritin, leading to increase the iron availability for heme biosynthesis. In contrast, when the cytosolic heme satisfies the requirement of biological activity, cells do not synthesize heme any more. Cytosolic heme suppresses the heme biosynthesis by inhibition of ALAS translocation (5, 6), and the IRE-binding activity of IRP1 for derepression of ferritin expression (Figure 5.1). Then, the excess iron that is generated in cytosol is scavenged by ferritin, resulting in the feedback for the heme biosynthesis level. Therefore, the cycle from the heme biosynthesis to IRP1-IRE regulation plays an essential role in maintenance of appropriate heme level. In case of generation of free heme by oxidative stress, HO-1 is expressed to degrade heme to iron. The iron was stored in ferritin, whose translation was activated by degradation of IRP1 in case of excess heme level (7, 8).

The cytosolic free heme was scavenged by PRX1 to prohibit from the oxidative stress or degradation (Figure 5.1). PRX1 has enough abundance to handle the elevation of heme level (9, 10), indicating that PRX1 could be responded to the wider range than the estimated cytosolic heme level. The scavenged heme must be transported to apoprotein as prosthetic group. Although it is unknown how cytosolic heme is mobilized to other organelle (11), some

bacterial heme transport proteins were identified such as *Pseudomonas aeruginosa* PhuS (12, 13), *Shigella dysenteriae* ShuS (14), *Yersinia enterocolitica* HemS (15), and *Vibrio Cholerae* HutX (16). These bacterial proteins participate in the intracellular heme storage and facilitate heme transfer to apoprotein via protein-protein interaction. These findings suggest that there is the possibility for receiving heme from heme-bound PRX1 by other heme transport protein to deliver to other organelle.

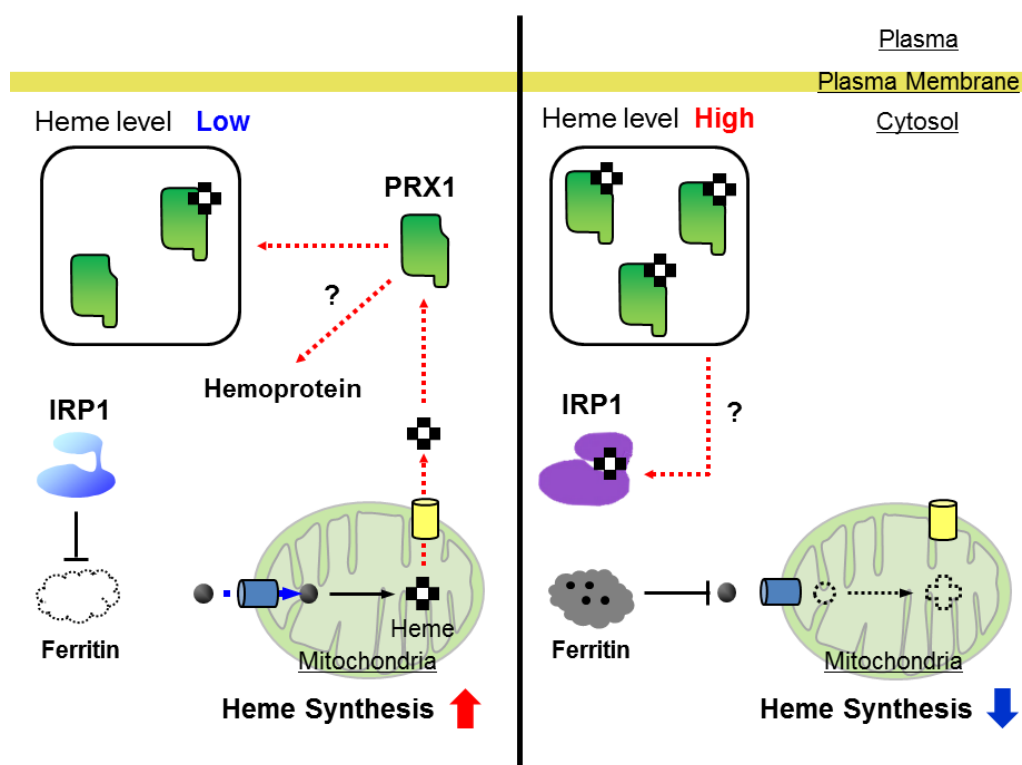


Figure 5.1 Schematic representation of the intracellular heme metabolism.

PRX1 was protected heme from its toxicity by scavenging. It is not clear that PRX1 transport heme to cytosolic hemoprotein or organelle, suggesting that other putative heme transporter was needed to correctly deliver the synthesized heme. IRPs sense the cytosolic heme level to modulate the IRE-binding activity for adjusting the iron availability via iron storage protein, ferritin.

I repeatedly emphasized that the content of free heme must be strictly regulated in cell owing to prevention of the toxic effect. Recently, it has been reported that the development of some diseases is caused by the toxic effect of free heme. Especially, Alzheimer's disease (AD), which is one of the symptoms for the senile dementia, is shed light on the relationship with free heme (17). AD has become a severe problem for the elderly with the increase of general life expectancy, suggesting that the mechanism of AD development has to be elucidated. It has been described that the production and oligomerization of amyloid β ($A\beta$) contributes the neuronal dysfunctions, leading to AD (18). However, Atamna *et al.* reported that $A\beta$ forms a stable complex with heme, and the complex exhibits severe peroxidase activity higher than free heme (19). Heme- $A\beta$ can oxidize neurotransmitters like serotonin and 3,4-dihydroxyphenylalanine (DOPA) in the presence of H_2O_2 . Hence, the abnormal neurotransmission and oxidative stress observed in AD may arise from this complex. Although heme binding to $A\beta$ is prevented from its aggregation in contrast to the pathology observed in AD patient, the diminished bioavailability of heme and the harmful peroxidase activity of the heme- $A\beta$ complex have a dangerous effect on the metabolic consequence (20). Nowadays, the relationship between the heme coordination environment and the peroxidase activity was investigated in detail (21–24). These facts strongly emphasize the significance of regulation of free heme, and elucidation of the trafficking mechanism of cytosolic free heme would contribute the prevention of the AD development or the indicator for the diagnosis of AD.

In conclusion, I prompted to reveal the regulation mechanism of the protein function in intracellular heme metabolism using heme as the regulatory molecule. Although cytosolic heme is essential for the prosthetic group to exert the various biological reaction, free heme causes the disruption of biomolecule. Thus, I focused on the protection of cytosolic heme. PRX1 scavenges the cytosolic free heme to avoid the undesired degradation by reacting with ROS. Moreover, I elucidated that IRPs utilize the cytosolic heme as the regulatory molecule for its IRE-binding activity to modulate the iron availability in cell. The heme effect on the IRE-binding activity is confirmed in the cellular condition for IRP1. These studies suggest that the cytosolic heme play a role in the sensor for the regulation the protein activity to adjust the intracellular heme metabolism.

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