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Review

Heme–thiolate proteins

Tsuneo Omura

Kyushu University, Fukuoka, Japan

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Abstract

Cytochrome P450 was the first hemoprotein found to have a thiolate anion as the axial ligand of the heme. Several other heme–thiolate proteins, including nitric oxide synthase, were later found in animals, plants, and microorganisms. Both cytochrome P450 and nitric oxide synthase, two major members of the heme–thiolate protein family, catalyze monooxygenase reactions, but the physiological functions of other heme–thiolate proteins are apparently highly diverse. Chloroperoxidase of a mold, *Caldaryomyces fumago*, catalyzes a haloperoxidase reaction. CooA of a bacterium, *Rhodospirillum rubrum*, and heme-regulated eIF2α kinase of animals function as the sensors for carbon monoxide and nitric oxide, respectively, to elicit biological responses to these gases. The role of heme in the enzymatic activity of cystathionine β-synthase is still unknown. It is likely that more heme–thiolate proteins with diversified functions will be found in various organisms in the future.

Keywords: Heme–thiolate proteins; Thiolate anion; Cytochrome P450

"Heme–thiolate proteins" are a group of hemoproteins whose prosthetic group is protoheme with a thiolate anion of a cysteine residue as the axial ligand. They constitute one of the two major hemoprotein groups found in living organisms; the other is the "heme–imidazole protein" group that includes hemoglobin. When carbon monoxide binds to the reduced forms, heme–thiolate proteins usually give a characteristic optical absorption spectrum with a prominent Soret peak at around 450 nm, whereas the corresponding absorption spectrum of heme–imidazole proteins, whose axial ligand is an imidazole group of a histidine residue, show a Soret peak at around 420 nm.

Cytochrome P450 was the first hemoprotein found to have a 450-nm absorption peak in the carbon monoxide-binding spectrum. It was initially regarded as a peculiar solitary hemoprotein in comparison with many other known hemoproteins that show a 420-nm absorption peak when bound with carbon monoxide. However, we now know that cytochrome P450 is a member of the growing family of heme–thiolate proteins, in which nitric oxide synthase is another important member. The major physiological function of heme–thiolate proteins is apparently the activation of molecular oxygen for various oxygenase reactions, but recent findings indicate a role of some heme–thiolate proteins in sensing CO or NO to elicit cellular responses to these gaseous effectors. This review article for the special issue "Oxygenases" of this journal provides a brief summary of the history and the present outlook of the research on heme–thiolate proteins.

Discovery of heme–thiolate proteins

When the carbon monoxide-binding pigment of liver microsomes discovered in 1958 [1] was found to be a hemoprotein and named "cytochrome P-450" (P450) in 1962 [2,3], the ligand structure of the protoheme prosthetic group of P450 attracted much attention because of the remarkable red shift of the Soret peak of its carbon monoxide compound compared with other hemoproteins known at that time. The same microsomal component was also detected by ESR measurements of liver microsomes and named "microsomal Fex" in 1962 [4]. The 450-nm optical absorption peak of the carbon monoxide compound of

E-mail address: omurat@mxs.mesh.ne.jp

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P450 shifted to 420 nm (P420 form) when microsomes were solubilized with detergents [2,3]. The role of sulfhydryl groups in this peculiar optical property of P450 was suggested by the conversion of P450 (low-spin Fex) to P420 (high-spin Fex) by treating microsomes with sulfhydryl reagents [5] and its reversal by GSH [6], and the first solid evidence for the thiolate anion as the axial ligand of the heme of P450 was provided by a model compound experiment reported in 1974 [7]. As many different forms of P450 were purified and characterized, comparison of the amino acid sequences of various P450s indicated a conserved cysteine residue near to the carboxy terminus of the P450 proteins as the amino acid residue donating the thiolate anion ligand to the heme [8]. The highly conserved amino acid sequence around this particular cysteine residue, which is called ''heme-binding region'', is FXXGXRX**C**XG. The ligand structure of the heme of P450 was later confirmed by X-ray crystallographic analysis of a soluble bacterial P450, P450cam [9].

The second member of heme–thiolate protein family is a soluble extracellular chloroperoxidase of a mold, *Caldaryomyces fumago*, which was found to show a P450-like 443-nm optical absorption peak when bound with carbon monoxide in 1973 [10]. The heme ligand of this enzyme was confirmed to be a thiolate anion in 1976 [11], but the P450-type heme-binding region was not found in its primary sequence [12]. The third member is cystathionine β-synthase, which was originally found in the cytosolic fraction from pig liver by its P450-like absorption spectrum and named H-450 in 1976 [13]. The proximal ligand of H-450 was identified to be a thiolate anion in 1984 [14], and its amino acid sequence elucidated in 1990 by the cloning of its cDNA indicated that H-450 is entirely different from P450 [15]. Two years later, the amino acid sequence of rat cystathionine β-synthase was elucidated by cDNA cloning, and it was identical with that of rat H-450 [16].

Nitric oxide synthase (NOS) became the fourth member of the heme–thiolate protein family in 1992 when its carbon monoxide-binding spectrum was found to show a P450-type 443-nm peak [17,18]. The heme-containing domain of NOS showed no sequence homology with P450, although its reductase domain showed significant similarity to microsomal NADPH-P450 reductase [19].

In recent years, two interesting members were added to the heme–thiolate protein family. One is ''CooA'' of a bacterium *Rhodospirillum rubrum* [20], and the other is ''heme-regulated eIF2α kinase'' of animals [21]. CooA is a transcriptional regulator, which senses CO and activates the expression of a group of enzymes that participate in the oxidation of CO. Heme-regulated eIF2α kinase is activated by NO and regulates the protein synthesis in reticulocytes by phosphorylating eIF2α. The heme prosthetic group of both CooA and eIF2α kinase has a thiolate anion as the axial ligand in the oxidized form, but the thiolate anion is displaced when the heme is reduced and binds with CO or NO. Therefore, the carbon monoxide compounds of these hemoproteins do not show a 450-nm optical absorption peak that is a common signature for other heme–thiolate proteins. Table 1 shows the members of heme–thiolate protein family reported to date.

Oxygenase and peroxidase activities of heme–thiolate proteins

The oxygenase activity of P450 was first found with a microsomal P450, P450c21, of adrenal cortex in 1963 [22]. It was a monooxygenase reaction, which required molecular oxygen and NADPH, introducing an oxygen atom into the steroid molecule. It was soon found that microsomal and mitochondrial P450s catalyze various monooxygenase reactions utilizing a wide variety of endogenous and xenobiotic organic compounds as the substrates [23]. Hydrophobic compounds are preferred substrates for most P450s, although some P450s catalyze oxygenation of certain hydrophilic compounds like ethanol and acetone. The P450 molecule has a hydrophobic substrate-binding pocket, which is large enough to accommodate a bulky hydrophobic molecule like steroids, and the heme is situated at the bottom of the pocket. The P450-catalyzed oxygenation reaction starts from the binding of a substrate molecule to the oxidized form of P450, which is then reduced by an electron supplied from NAD(P)H via the P450 reductase system. Molecular oxygen is then bound to the ferrous iron of the heme and is activated depending on the supply of a second electron from NAD(P)H [24]. The spatial orientation of the bound substrate molecule relative to the activated oxygen atom sitting on the heme determines the regio- and stereo-specificity of the oxygenation reactions. P450-catalyzed monooxygenase reactions usually produce hydroxylated products or epoxides. Some P450s show significant ''uncoupling'' of the oxygenase reaction. In the uncoupled reaction, the oxygen molecule bound to the ferrous iron of the heme is released from P450 as superoxide anion, which is harmful to the cells.

Table 1
Heme–thiolate proteins

	Identified as heme–thiolate protein	Spectral peak of reduced form	Function of heme
Cytochrome P450	1974	450 nm with CO	Oxygen activation
Chloroperoxidase (*Caldaryomyces fumago*)	1976	443 nm with CO	Peroxidation
Cystathionine β-synthase	1984	448 nm without CO	Unknown
Nitric oxide synthase	1992	443 nm with CO	Oxygen activation
CooA (*Rhodospirillum rubrum*)	1998	420 nm with CO	CO sensing
Heme-regulated eIF2α kinase	2004	398 nm with NO	NO sensing

Many P450s have been found in animals, plants, and microorganisms. The human genome contains 57 P450 genes. The genomes of some plants contain 200–400 P450 genes. In animals, the major functions of P450s are biosynthesis of cholesterol, catabolism of cholesterol to bile acids, synthesis of various steroid hormones from cholesterol, hydroxylation and epoxidation of arachidonic acid to form HETE and EET, participation in the synthesis of some prostaglandins from arachidonic acid, inactivation of various eicosanoids by hydroxylation, regulation of intracellular concentration of retinoic acid by hydroxylation or epoxidation, etc. Various P450s are expressed tissue-specifically and developmental stage-specifically according to their physiological functions. In addition to these metabolic activities to various endogenous substrates, many P450s catalyze the oxidative metabolism of a wide variety of xenobiotic chemical compounds taken into the animal body from the environment, including food intake. Since lipophilic compounds are not easily excreted out of the animal body via urine or bile, their transformation into more hydrophilic forms by P450-catalyzed reactions is essential for the elimination of those noxious foreign compounds from the animal body. This unique activity of animal P450s seems to have been created during the long history of evolution of animals to metabolize toxic compounds in the plants that they eat, and is now functional in the detoxication of various man-made chemical compounds, including various drugs and environmental pollutants. However, various carcinogenic chemical compounds, including some natural compounds in plants and molds, are activated by P450-catalyzed oxygenase reactions in animal tissue cells to react with nuclear DNA, causing chemical carcinogenesis.

Cytochrome P450 also catalyzes peroxide-dependent oxygenation reactions, which do not require molecular oxygen or the supply of electrons from NAD(P)H [25]. Inorganic peroxides, including hydrogen peroxide and various organic peroxides and hydroperoxides, are utilized to form an activated oxygen atom bound to the heme, which is then incorporated into the substrate molecule.

Whereas most P450s function as monooxygenases, some P450s catalyze the intramolecular transfer of oxygen atoms, such as conversion of prostaglandin H2 to thromboxane A2 by P450 5A1 [26], which does not require the supply of electrons by the P450 reductase system.

Nitric oxide synthase also catalyzes a monooxygenase-type reaction. It is present in eukaryotic organisms and catalyzes the oxidative conversion of L-arginine to L-citrulline and NO depending on the presence of molecular oxygen and supply of electrons from NADPH. Three types of NOS have been purified and characterized from animal tissues: neural NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). They differ in tissue distribution, inducibility, and calmodulin-dependent regulation of the enzymatic activity, but their molecular structures are very similar. The enzyme consists of two domains, a heme-containing catalytic domain and a FAD,FMN-containing reductase domain, linked by a hinge sequence. The latter domain shows a high degree of sequence homology to microsomal NADPH-P450 reductase [19], and supplies electrons from NADPH to the heme in the heme-containing domain for the monooxygenase-type reaction to produce NO from L-arginine. However, no sequence similarity was found between the heme-containing domain and P450s, although the heme has a thiolate anion as the axial ligand, and shows a P450-type optical absorption spectrum upon binding with CO.

Chloroperoxidase of *C. fumago* catalyzes halogenation reactions in the presence of halide anions and hydrogen peroxide. Its physiological function is chlorination of β-ketoadipic acid to δ -chlorolevulinic acid in the biosynthesis of caldariomycin. It has a protoheme with a thiolate anion as the proximal ligand, and combines with carbon monoxide when reduced to show a spectrum almost identical to that of P450 [10,11]. Many haloperoxidases have been purified from various organisms and characterized, but the chloroperoxidase of *C. fumago* is so far the only haloperoxidase that has a heme–thiolate prosthetic group.

Other enzymatic activities of heme–thiolate proteins

Many P450s can catalyze reductive reactions in the absence of molecular oxygen. The oxidation–reduction potential of P450s is about –250 mV, which is low enough to reduce various organic compounds. Reduction of tertiary amine *N*-oxides, arene oxides, azo compounds, aromatic nitro compounds, and quinones by microsomal P450s has been shown by in vitro experiments. Reduction of azo and nitro compounds in the liver, where the oxygen tension is low, has been confirmed. Reductive dehalogenation of some halogenated organic compounds by P450 has also been confirmed.

P450nor of the mold *Fusarium oxysporum* is an interesting soluble P450, whose physiological function is the reduction of nitric oxide to nitrous oxide [27] as a part of the denitrification reaction. NO binds to the ferric heme of P450nor, and then the heme receives two electrons directly from NADH to reduce NO to hydroxylamine radical. Another molecule of NO reacts with the heme-bound hydroxylamine radical to form N_2O, which dissociates from the heme [28]. Direct transfer of electrons from NADH to the heme of P450nor is a remarkable reaction.

Heme of cystathionine β-synthase

An interesting soluble hemoprotein that showed a P450-like 448-nm optical absorption peak upon reduction was purified from the cytosolic fraction of pig liver homogenate and named H-450 in 1976 [13]. The absorption spectrum of reduced H-450 was very similar to that of the carbon monoxide compound of P450, but reduced H-450 showed the 448-nm peak without addition of carbon monoxide [13]. Analysis of the optical absorption spectra and EPR spectra of H-450 in 1984 indicated that the ligand at the 5th coor-

dination position of the protoheme of H-450 is a thiolate anion, and a nitrogenous group, possibly an imidazole group, occupies the 6th coordination position of the heme [14], confirming the heme–thiolate protein nature of H-450. When CO was added to reduced H-450, the Soret peak gradually shifted from 448 to 420 nm [14]. This spectral change indicated that the thiolate anion ligand of the heme is replaced with CO to produce a spectrum that is almost same as the spectrum of the CO compound of a heme–imidazole protein. The amino acid sequence of rat H-450 deduced from the cloned cDNA in 1990 showed that the amino-terminal half of H-450 is similar to bacterial *O*-acetylserine(thiol)-lyase, which catalyzes the synthesis of cysteine from *O*-acetylserine and hydrogen sulfide, whereas the carboxy-terminal half showed no similarity with any known proteins [15]. Two years later, a cDNA of rat cystathionine β-synthase was cloned, and the deduced amino acid sequence was identical with that of H-450 [16].

The role of heme in the enzymatic activity of cystathionine β-synthase is still unknown [29]. Cystathionine β-synthase catalyzes the formation of cystathionine from L-serine and L-homocysteine. It is a PLP-dependent condensation reaction, in which no oxidation–reduction step is involved. The molecular structure of human cystathionine β-synthase obtained by X-ray crystallographic analysis showed that the heme is bound to the amino-terminal region of the protein; His-65 and Cys-52 donate imidazole and thiolate anion ligands, respectively, to the heme [30], which confirmed the conclusion of a previous spectral study [14]. It was found that the cystathionine β-synthase of yeast does not have heme [31], which suggested that the heme is not essential for the activity of the enzyme. It was actually shown that removal of heme from human cystathionine β-synthase did not affect the enzymatic activity [32].

Labile nature of thiolate anion ligand

P450s in eukaryotic cells are all bound to the microsomal membrane or mitochondrial inner membrane, and solubilization of a membrane-bound P450 by detergents results in the conversion of the P450 to a spectrally distinct solubilized form, P420, whose absorption peak in the carbon monoxide difference spectrum is at 420 nm [2,3,33]. This remarkable spectral change is brought about by the displacement of the thiolate anion ligand of the heme with the imidazole group of a nearby histidine residue. Since urea, organic solvents, and some other protein denaturants induce the conversion of P450 to P420, alteration in the conformation of the protein moiety seems to induce the exchange of the heme ligands. In the case of cystathionine β-synthase, the axial ligands of the heme are a thiolate anion and an imidazole group. When CO is added to the reduced enzyme, the thiolate anion ligand of the heme is replaced with CO, whereas the imidazole group remains coordinated to the heme [14]. Coordination of thiolate anion to protoheme is apparently less stable than the imidazole group, in particular when the iron atom of the heme is in the ferrous state.

This labile nature of the thiolate anion ligand in the heme–thiolate proteins explains the functions of some heme–thiolate proteins as the sensors for the detection of some heme-binding gases. In such cases, binding of CO or NO to the heme results in the displacement of the thiolate anion ligand and induces a conformational change of the protein moiety, which results in a change in its enzymatic activity.

Sensing of CO and NO by heme–thiolate proteins

CooA is the product of the *cooA* gene of the purple phototrophic bacterium *R. rubrum*, which can grow on CO as an energy source. Expression of the CO-oxidizing enzyme system of the bacterium is regulated by CooA, which is induced by exogenous CO. It was found in 1996 that CooA contains heme [34]. Mutagenesis study of CooA confirmed that Cys-75 donates the thiolate anion to the ferric heme as an axial ligand, and it is replaced by the imidazole group of His-77 when the heme is reduced and combines with CO [35]. This alteration in the ligand structure of the heme activates CooA to induce the expression of the genes of the CO-oxidizing enzymes.

Heme-regulated eukaryotic initiation factor 2α kinase is a member of the eIF2α kinase family and catalyzes phosphorylation of eIF2α under conditions of heme deficiency to inhibit protein synthesis in reticulocytes. The kinase has two heme-binding sites; one heme is tightly bound and another one is loosely bound. The loosely bound heme is responsible for the regulation of the kinase activity by heme availability [36]. The kinase activity is also activated by NO, and the tightly bound heme participates in the activation. Analysis of the optical absorption, electron spin resonance, and resonance Raman spectra suggested that a thiolate anion is an axial ligand of the tightly bound heme [21]. When the tightly bound heme is reduced and binds with NO, the thiolate anion ligand is replaced with NO accompanied by the activation of the kinase activity [21]. It seems that the displacement of the thiolate ligand of the heme induces some conformational change of the protein concomitant with the activation of the kinase activity.

Although both CooA and heme-regulated eIF2α kinase are heme–thiolate proteins in the oxidized forms, the thiolate ligand is displaced when the heme is reduced and binds with CO or NO. Therefore, they do not show a P450-type optical absorption spectrum in the reduced state, and their heme–thiolate protein nature could be confirmed only by spectral or X-ray crystallographic analyses of the oxidized forms. Detection of heme–thiolate proteins has usually been dependent on the spectroscopic observation of a P450-type 450-nm absorption spectrum of the carbon monoxide compound. It seems likely that more CooA-like or heme-regulated eIF2α kinase-like heme proteins with labile thiolate anion legend exist, and their heme–thiolate protein nature has not been noticed so far.

CooA and heme-regulated eIF2α kinase belong to a class of heme-based sensor proteins that sense particular gases to induce cellular responses to the gases by catalyzing some enzymatic reactions or by regulating transcription of particular genes or translation of mRNAs. Soluble guanylate cyclase (sGC) of animals, which senses NO and produces cyclic GMP, was the first member of this particular hemoprotein group [37]. The heme of sGC has an imidazole group of histidine as the axial ligand [38]. FixL of a nitrogen-fixing bacterium, *Rhizobium meliloti*, is also a heme–imidazole protein, which senses O_2 and regulates the expression of nitrogen fixation enzymes [39]. Both heme–imidazole proteins and heme–thiolate proteins constitute this interesting group of hemoproteins.

Evolutional aspects of heme–thiolate proteins

Comparison of the amino acid sequences including the heme-binding regions of the six members of the heme–thiolate protein family (Table 1) indicates that they have been generated independently during the evolution of living organisms. Cytochrome P450 has evolved from an unknown ancestral form, diversified tremendously to many forms with different substrate specificities, and is now distributed widely among various eukaryotic and prokaryotic organisms. Distribution of NOS is limited to eukaryotic organisms, and it has diversified to a few forms. Cystathionine β-synthase is also a eukaryotic enzyme, although its amino acid sequence seems to suggest its evolutionary relation with a prokaryotic non-heme enzyme, *O*-acetylserine(thiol)-lyase. Heme-regulated eIF2α kinase is an animal enzyme, and its amino acid sequence indicates its relation with some other eIF2α kinases that do not have a heme prosthetic group. *C. fumago* chloroperoxidase and *R. rubrum* CooA are unique proteins and have no similar counterparts in other organisms.

It is conceivable that many new heme–thiolate proteins were generated from various non-heme proteins during the evolution of prokaryotic and eukaryotic organisms owing to fortuitous point mutations in the amino acid sequences that enabled incorporation of heme to the original proteins. Most of the new heme–thiolate proteins thus produced by chance failed to survive and quickly disappeared, whereas a few of them acquired new physiological functions by the addition of heme that were more beneficial to the organisms than the original non-heme proteins, and they survived. We now see the descendants of some such heme–thiolate proteins, fortunate products of molecular evolution of proteins in the incessant progress of biological evolution.

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