Neuronal PAS domain protein 2, which was recently established to be a heme protein, acts as a CO-dependent transcription factor. The protein consists of the basic helix-loop-helix domain and two heme-containing PAS domains (PAS-A and PAS-B). In this study, we prepared wild type and mutants of the isolated PAS-A domain and measured resonance Raman spectra of these proteins. Upon excitation of the Raman spectrum at 363.8 nm, a band assignable to Fe=O-S stretching was observed at 334 cm⁻¹ for the ferric wild type protein; in contrast, this band was drastically weaker in the spectrum of C170A, suggesting that Cys¹⁷⁰ is an axial ligand of the ferric heme. The Raman spectrum of the reduced form of wild type was mainly of six-coordinate low spin, and the ν₁₁ band, which is sensitive to the donor strength of the axial ligand, was lower than that of reduced cytochrome c₉, suggesting coordination of a strong ligand and thus a deprotonated His. In the reduced forms of H119A and H171A, the five-coordinate species became more prevalent, whereas no such changes were observed for C170A, indicating that His¹¹⁹ and His¹⁷¹, but not Cys¹⁷⁰, are axial ligands in the ferrous heme. This means that ligand replacement from Cys to His occurs upon heme reduction. The ν_{Fe=CO} versus ν_{CO} correlation indicates that a neutral His is a trans ligand of CO. Our results support a mechanism in which CO binding disrupts the hydrogen bonding of His¹⁷¹ with surrounding amino acids, which induces conformational changes in the His¹⁷¹-Cys¹⁷⁰ moiety, leading to physiological signaling.

In recent years, a variety of heme-containing gas sensor proteins have been discovered in different species, from bacteria to mammals (1–3). In these proteins, a change in the concentration of gas molecules such as NO, O₂, or CO is detected by a heme group and transduced to the functional domain as a signal, leading to modulation of protein activity. The heme-based gas sensor proteins discovered so far are listed in Table 1. NO is a signaling molecule involved in vasodilation and neuronal transmission (4, 5). Soluble guanylate cyclase (sGC)¹ is a well known heme-based NO sensor protein. Upon binding of NO to sGC, the iron-histidine bond in the N-terminal region, the sole covalent linkage between the heme and protein, is cleaved. The bond cleavage induces conformational changes, resulting in a 400-fold increase in GC activity in the C-terminal region (6). Heme-regulated eukaryotic initiation factor 2α kinase (eIF2α) also forms a five-coordinated NO-heme complex via Fe-His bond cleavage, resulting in activation by NO (7). The O₂-sensing proteins identified so far include FixL (8), DOS (9), PDEA1 (2), and HemAT (10, 11). The sensory domains of FixL and Ec DOS belong to the PAS² superfamily. FixL is a heme-based oxygen sensor involved in the regulation of expression of nitrogen fixation genes in response to O₂ concentration (8, 12, 13). Under low O₂ concentrations, FixL is autophosphorylated at a histidine residue and transfers it to FixJ, whereas a high concentration of O₂ suppresses kinase activity (8). Ec DOS is also an O₂ (and/or redox) sensor protein identified in Escherichia coli that exhibits phosphodiesterase activity in an O₂-dependent (and/or redox-dependent) manner (9, 14). CooA was the first CO sensor protein identified from a purple nonsulfur photosynthetic bacterium, Rhodospirillum rubrum (15, 16). When CO binds to heme, the accompanying conformational changes induce binding of CooA to its target DNA (17). Neuronal PAS domain protein 2 (NPAS2) was the second heme-based CO sensor protein discovered but the first one identified in mammals (18). It acts as a transcription factor for clock genes in a CO-dependent manner.

NPAS2 is a member of the basic helix-loop-helix (bHLH)-PAS family, including BMAL1 and Clock (19). As illustrated schematically in Fig. 1, the protein consists of the N-terminal bHLH domain and two PAS domains (PAS-A and PAS-B), structural modules that are present in widespread components of signal transduction proteins from organisms in all kingdoms of life (20). Both PAS-A and PAS-B bind one heme, although the protein can work as a transcriptional activator without heme (18). The PAS domains of NPAS2 display significant

¹ The abbreviations used are: sGC, soluble guanylate cyclase; NPAS2, neuronal PAS domain protein 2; bHLH, basic helix-loop-helix; RR, resonance Ramam; HRI, heme-regulated eukaryotic initiation factor 2α kinase; Mb, myoglobin; 6cLS, six-coordinate low spin; 6cHS, six-coordinate high spin; 5cHS, five-coordinate high spin; WT, wild type.

² PAS is an acronym formed from the names of proteins in which imperfect repeat sequences were initially recognized: PER (the Drosophila period clock protein), ARNT (vertebrate aryl hydrocarbon receptor nuclear translocator), and SIM (Drosophila single-minded protein).
sequence similarity to Clock. The bHLH, PAS-A, and PAS-B domains are 84, 69, and 90% identical, respectively, to the corresponding domains of Clock sequence (21). Both NPAS2 and Clock form heterodimers with BMAL1 and activate the expression of per and cry genes, which are negative regulatory components of the circadian clock. NPAS2 performs the same function as Clock but is expressed in a different region of the body. Specifically, NPAS2 is expressed primarily in the forebrain (22, 23), whereas Clock is present in the suprachiasmatic nucleus (21, 24).

Both proteins bind to the same DNA sequence as heterodimers with BMAL1 (25, 26). DNA binding of NPAS2-BMAL1 is modulated by the concentration ratio of oxidized and reduced NAD. NAD(P)H enhances the DNA binding ability of NPAS2-BMAL1 by interacting with the bHLH domain, whereas NAD(P)⁺ inhibits DNA binding (26). Furthermore, CO molecules produced by heme oxygenase-2 also modulate DNA binding of NPAS2-BMAL1 in the presence of a heme bound to the PAS domains, as reported by Gilles-Gonzalez and McKnight (18). At low micromolar levels of CO, heme forms a complex with CO, resulting in inhibition of DNA binding (26). Furthermore, CO-bound NPAS2 was expressed in E. coli (Sendai, Japan). The cloning vector, pBluescript SK II(+) was acquired from Takara Bio Inc., Toyobo, New England BioLabs (Beverly, MA), and Nippon Roche (Tokyo, Japan). The pBluescript-PASA plasmid was digested with NdeI restriction sites, and those for 3′ and 5′-ends were generated by reverse transcription-PCR using RNA isolated from mouse livers. The primers employed for reverse transcription-PCR were 5′-CGGGATCCCATATGTCATTCCTCAGTAACG-3′ and 5′-GCAAGCTTGGTTGACCTTATTTCTAAGAATCGC-3′. Primers for the 5′-ends contained BamHI and Sacl restriction sites, and those for 3′-ends contained Sall restriction sites for subcloning. PCR products were digested with BamHI and Sacl and inserted in the corresponding sites of the cloning vector, pBluescript SK II(+). The plasmids obtained were confirmed by sequencing of the nucleotide sequence by Sanger’s method using an automatic sequencer, DSQ-2000L (Shimadzu Co., Kyoto, Japan). The pBluescript-PASA plasmid was digested with NdeI and Sacl and subcloned into the E. coli expression vector, pET28a(+), which introduces a His₈ tag at the N terminus of expressed proteins.

To create mutants of the PAS-A domain, PCR-based mutagenesis was performed using the QuickChange mutagenesis kit from Stratagene with pET28 containing wild type PAS-A as a template. The desired mutation was confirmed by sequencing.

**EXPERIMENTAL PROCEDURES**

**Materials**—Mouse livers were obtained from C57BL/6 mice. The mRNA purification and reverse transcription-PCR kits were purchased from Amersham Biosciences and Roche Applied Science, respectively. Oligonucleotides were synthesized at Nihon Gene Research Laboratory (Sendai, Japan). The cloning vector, pBluescript SK II(+)+, and an expression vector, pET28a(+), were purchased from Toyobo (Osaka, Japan) and Novagen (Darmstadt, Germany), respectively. E. coli competent cells, XL1-blue (for cloning) and BL21 (for protein expression), were purchased from Novagen and Stratagene (La Jolla, CA), respectively. Restriction and modifying enzymes for DNA recombination were obtained from Takara Bio Inc., Toyobo, New England BioLabs (Beverly, MA), and Nippon Roche (Tokyo, Japan).13C18O and13CO were acquired from Cambridge Isotope Laboratories, Inc. (Andover, MA).54Fe-labeled heme was purchased from Frontier Scientific (Logan, UT). Other chemicals were from Wako Pure Chemicals (Osaka, Japan).

**Construction of the Isolated PAS-A Domain of NPAS2—**His-tagged expression plasmids of the isolated PAS-A domain of NPAS2 containing amino acid residues 78–240 were generated by subcloning into the pET28a(+) expression vector. cDNA encoding the PAS-A domain was generated by reverse transcription-PCR using RNA isolated from mouse livers. The primers employed for reverse transcription-PCR are 5′-CGGGATCCCATATGTCATTCCTCAGTAACG-3′ and 5′-GCAAGCTTGGTTGACCTTATTTCTAAGAATCGC-3′. Primers for the 5′-ends contained BamHI and Sacl restriction sites, and those for 3′-ends contained Sall restriction sites for subcloning. PCR products were digested with BamHI and Sacl and inserted in the corresponding sites of the cloning vector, pBluescript SK II(+). The plasmids obtained were confirmed by sequencing of the nucleotide sequence by Sanger’s method using an automatic sequencer, DSQ-2000L (Shimadzu Co., Kyoto, Japan). The pBluescript-PASA plasmid was digested with NdeI and Sacl and subcloned into the E. coli expression vector, pET28a(+), which introduces a His₈ tag at the N terminus of expressed proteins.

**Protein Expression and Purification—**The His-tagged PAS-A domain was expressed in E. coli BL21(DE3)-CodonPlus harboring each expression vector. Protein expression was induced at A₆₀₀ = 0.6 by the addition of isopropyl β-D-thiogalactopyranoside (final concentration of 50 μM). Cells were further incubated for 20–24 h after the addition of...
isopropyl β-D-thiogalactopyranoside. E. coli cells expressing the PAS-A domain were suspended in buffer A (50 mM sodium phosphate buffer, pH 7.8, 50 mM NaCl, 2 mM mercuric captoethanol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 2 mM 2-mercaptoethanol). Cells were crushed by pulsed sonication for 2 min (3 times with 2-min intervals) on ice using the ULTRASONIC DISRUPTOR UD-201 (Tomy Seiko, Tokyo, Japan) and centrifuged at 35,000 rpm for 35 min at 4 °C. Purification of the PAS-A domain using a probe pulse. Raman-scattered light was dispersed with a 0.5-m Spex polychromator (SPEX750M; Jobin Yvon) equipped with a liquid nitrogen-cooled CCD detector (Spec10:400BLN; Roper Scientific). The excitation wavelengths employed were 413.1 and 568.2 nm from a single polychromator (SPEX750M; Jobin Yvon) and the nature of its interactions in the heme pocket. Fe(II)-OH stretching (ν3,OH) bands have been observed for alkaline ferric myoglobin (Mb), hemoglobin, and horseradish peroxidase in the 450–555 cm⁻¹ region, which shift upon isotope substitution of solvent (D₂O and H₂O-D) (30–32). However, for the ferric PAS-A domain, no isotope-sensitive bands were observed in the 450–555 cm⁻¹ region (data not shown). This finding suggests that the sixth ligand of the heme iron in the ferric state is not a hydroxide at pH 8.0, although the results do not necessarily exclude the possibility of water coordination in the 6cHS species.

RESULTS

Sort-exciton Resonance Raman Spectra of the PAS-A Domain—Fig. 2 depicts the RR spectra of ferric (A and D), ferrous (B and E), and CO-bound (C and F) PAS-A domains of NPAS2 at pH 8.0 in the low (left) and high (right) frequency regions. It is well established that the Raman spectra of heme proteins in the high frequency region comprise porphyrin in-plane modes, which are sensitive to oxidation, spin, and coordination states of the heme iron (27–29). The oxidation state marker band, ν3, of the oxidized protein appeared at 1373 cm⁻¹, typical of the ferric state of heme proteins (Fig. 2, spectrum D). The spin and coordination marker band, ν5, is composed of an intense 1504 cm⁻¹ peak with a small shoulder at 1490 cm⁻¹ and a weak 1471 cm⁻¹ peak, suggesting the co-existence of six-coordinate low spin (6cLS), five-coordinate high spin (5cHS), and six-coordinate high spin (6cHS) hemes in the ferric form. The 6cLS species (1504 cm⁻¹) is dominant at neutral pH. A mixture of five- and six-coordinate species is also observed in the ferrous form (Fig. 2, spectrum E), for which ν3 bands observed at 1471 and 1493 cm⁻¹ correspond to the 5cHS and 6cLS species, respectively. The addition of CO to the ferrous protein shifts the ν3 band from 1360 to 1372 cm⁻¹, indicating the formation of CO-bound heme. The frequencies of the marker bands of NPAS2 are compared with those of other heme proteins in Table II.

Low frequency RR spectra of the PAS-A domain are additionally illustrated in Fig. 2 (left panel). Spectra in this region are very useful for identifying a ligand, since metal-ligand vibrations directly demonstrate the presence of a particular ligand and the nature of its interactions in the heme pocket. Fe(II)-OH stretching (ν3,OH) bands have been observed for alkaline ferric myoglobin (Mb), hemoglobin, and horseradish peroxidase in the 450–555 cm⁻¹ region, which shift upon isotope substitution of solvent (D₂O and H₂O-D) (30–32). However, for the ferric PAS-A domain, no isotope-sensitive bands were observed in the 450–555 cm⁻¹ region (data not shown). This finding suggests that the sixth ligand of the heme iron in the ferric state is not a hydroxide at pH 8.0, although the results do not necessarily exclude the possibility of water coordination in the 6cHS species.
Generally, the Fe-His stretching mode ($v_{\text{FeHis}}$) is evident in the 200–250 cm$^{-1}$ region for the ferrous five-coordinate species (33). However, RR spectra of the ferrous PAS-A domain excited at 413.1 and 441.6 nm exhibited a weak feature at around 220 cm$^{-1}$, probably due to the predominance of 6cLS species. The CO-bound form (Fig. 2, inset) shows a prominent band at 334 cm$^{-1}$ for the wild type (WT) protein ($v_{\text{Fe-S}}$), which is assigned to the Fe-CO stretching mode ($v_{\text{Fe-CO}}$), as examined later.

Propionate bending modes, $\delta(C_3C_6C_1)$, for the PAS-A domain in the ferrous and CO-bound forms were observed at 382 and 379 cm$^{-1}$, respectively (Fig. 2, spectra B and C). The frequency of the propionate bending mode is correlated with the strength of the hydrogen bond between heme propionate and surrounding amino acid residues (34–36). For instance, the heme-propionate group of Mb is hydrogen-bonded to His97 and Ser92, and its $\delta(C_3C_6C_1)$ mode appears at 376 cm$^{-1}$. Disruption of this hydrogen bond in H97F and H97A/S92A mutants results in a downshift to 366 and 365 cm$^{-1}$, respectively (36). Therefore, the higher frequencies of $\delta(C_3C_6C_1)$ for the PAS-A domains in both ferrous and CO-bound forms suggest strong hydrogen bonding between the heme propionate group and nearby amino acid residues.

Near UV-excited Resonance Raman Spectra of the PAS-A Domain—With near UV excitation for the 5cHS heme possessing Cys as a proximal ligand, the Fe-S stretching mode is intensity-enhanced upon excitation within the Fe-S charge transfer band and in fact is observed around 350 cm$^{-1}$ for P450 and nitric-oxide synthase (37, 38). A low frequency RR spectrum of the ferric PAS-A domain at pH 8.0 excited at 363.8 nm is shown in Fig. 3. A broad band was observed around 340 cm$^{-1}$ for the wild type (WT) protein (spectrum A). When the band is fit with two Gaussian bands, the calculated Raman shifts were located at 334 and 347 cm$^{-1}$. With decreasing pH from 8.0 to 7.0, the intensity of the 334 cm$^{-1}$ band decreased drastically, whereas the 347-cm$^{-1}$ band was little affected (spectrum B). At pH 7.0, Soret-excited RR spectrum showed the disappearance of the 5cHS species as shown by the loss of the $v_{\text{Fe-S}}$ band at 1491 cm$^{-1}$ (spectra E and F, left inset). Since the Fe-S stretching mode is observable for the 5cHS species, the intensity decrease in the 334-cm$^{-1}$ band at pH 7.0 suggests that the band is derived from the 5cHS species.

Among four cysteine residues contained in the PAS-A domain, only Cys170 is inferred to be close to the heme-binding region, and accordingly, we replaced Cys170 with Ala to confirm Cys coordination. The corresponding Raman spectrum of the C170A mutant is depicted by spectrum C. The Raman spectrum of the C170A mutant is similar to that of WT protein at pH 7.0 (spectrum B). Furthermore, the band at 334 cm$^{-1}$ was upshifted by −2.0 cm$^{-1}$ in the $^{54}$Fe-labeled probe (Fig. 3, right inset, spectrum H). Although the 334-cm$^{-1}$ band did not disappear completely in the C170A mutant and WT at pH 7.0, it is presumably due to a porphyrin mode of 6cLS heme present around 330 cm$^{-1}$ and accidentally overlapped with the Fe-S stretching mode. These results indicate that a major part of the Raman band at 334 cm$^{-1}$ is derived from the Fe-S stretching mode, and Cys170 is an axial ligand of the ferric heme in the PAS-A domain. On the other hand, the 347-cm$^{-1}$ band was assigned to the porphyrin $v_{\text{Fe}}$ mode (35), since the 347-cm$^{-1}$

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**TABLE II**

| Coordination | Reference |
|-------------|-----------|
| Cys| Ref. 61 |
| His| Ref. 62 |
| Met| Ref. 65 |
| Cys| Ref. 66 |
| His| Ref. 67 |
| His| Ref. 68 |
| His| Ref. 69 |
| His| Ref. 70 |
| His| Ref. 71 |
| His| Ref. 72 |
| His| Ref. 73 |
| His| Ref. 74 |
| His| Ref. 75 |
| His| Ref. 76 |
| His| Ref. 77 |

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His is a trans ligand of CO in the CO-bound PAS-A domain.

Resonance Raman Spectra of the Photodissociated CO-bound PAS-A Domain—Due to the predominance of the 6cLS species, the Fe-His stretching mode was weak in RR spectra of the ferrous form (Fig. 2, spectrum B). Although the hemoproteins with six-coordinate structure in the ferrous form do not exhibit the Fe-His mode, the photoproduct formed transiently by CO dissociation from the CO-heme complex under high laser power conditions, which adopts the 5cHS state, displays the Fe-His mode in some cases. To analyze the Fe-His stretching mode, the RR spectrum of the photoproduct of the CO-heme complex was recorded. Unfortunately, the Fe-His stretching mode of the PAS-A domain was not identified with continuous wave excitation at 413.1, 422.6, and 441.6 nm, even with high laser power (>20 milliwatts), probably due to rapid rebinding of photodissociated CO. An example of excitation at 441.6 nm is depicted in spectrum A in Fig. 4. On the other hand, a single color pulse excitation with 10-ns width was applied to get the spectra shown in Fig. 5. The left inset shows high frequency RR spectra of WT at pH 8.0 (E) and pH 7.0 (F) excited at 413.1 nm. The right inset shows low frequency RR spectra of WT with 56Fe-labeled heme (G) and 54Fe-labeled heme (H) excited at 363.8 nm.

There is an inverse linear correlation between the frequencies of the \( v_{\text{Fe-CO}} \) and \( v_{\text{C-O}} \) stretching modes of heme proteins and model compounds (39, 40). The corresponding frequencies of the PAS-A domain fall on the same line as that for proteins with proximal histidine, as illustrated in Fig. 6. This finding suggests that the trans ligand of iron-bound CO is a neutral histidine, in accordance with the frequencies of \( v_{\text{Fe-His}} \) detected for transient CO photoproducts (Fig. 4, spectrum B).

Resonance Raman Spectra of the Mutant PAS-A Domain—To determine the coordination structure of the heme, histidine and cysteine residues were replaced with Ala. In total, six mutants (H119A, H138A, H148A, H171A, H217A, and C170A) were generated. Although absorption spectra of the mutant PAS-A domain changed slightly, CD spectra showed that the protein folds remain unchanged after the mutation. Since \( v_{\text{Fe-His}} \) is sensitive to the spin and coordination states of the heme iron (29), the \( v_{\text{Fe-His}} \) regions of Raman spectra of the mutant PAS-A domains in the ferric (left panel) and ferrous (right panel) states are depicted in Fig. 7. As described earlier, the \( v_{\text{Fe-His}} \) band of the ferric WT PAS-A domain consists of three peaks at 1471, 1490, and 1504 cm\(^{-1}\), and their relative intensities are altered by mutation without changes in frequency (Supplemental Fig. 1S and Table IS). Mutation of His\(^{119}\) and Cys\(^{170}\) to Ala significantly increased the intensity of the 1490 cm\(^{-1}\) band (5cHS), compared with that of the 1504 cm\(^{-1}\) band (6cLS), whereas...
H148A and H171A induced a moderate increase. This finding indicates an increase in the 5cHS species at the expense of the 6cLS species for these mutants. This spectral change is understandable if His119 and Cys170 are axial ligands of heme in the 6cLS species of the ferric PAS-A domain. Cys170 ligation is consistent with the observed Fe-Cys stretching mode by 363.8-nm excitation (Fig. 3). On the other hand, the intensity of the 1471-cm \(^{-1}\) band increased significantly for ferric H138A and moderately for H119A and H148A mutants. The data signify that mutation of three His residues results in an increase in the 6cHS species, thus allowing coordination of a weak ligand to the heme iron. These observations are compatible with coordination of His119 or His138 and Cys170 to the ferric iron in the PAS-A domain.

In contrast to the ferric form, ferrous C170A and H119A mutants displayed no significant differences from WT regarding the Fe-CO mode. In all of the spectra, the 5cHS species at 1471 cm \(^{-1}\) is the main component. A new band appeared at 1501 cm \(^{-1}\) for the ferrous H119A and H171A mutants, for which the 1493-cm \(^{-1}\) band appeared weaker than that of WT (Fig. 7A). This frequency is similar to that observed previously for four-coordinate ferrous hemes (41, 42), suggesting that His119 and His171 are axial ligands in 6cLS species of the ferrous form.

Fig. 8 shows the Fe-CO stretching mode of WT and the above mutants in the CO-bound forms. Except for the H217A mutant whose \(v_{\text{Fe-Co}}\) mode increased by 2 cm \(^{-1}\), none of the His and Cys mutants exhibited a change in \(v_{\text{Fe-Co}}\) frequency. Thus, the polarity around iron-bound CO as well as the trans ligand of CO is not affected by the mutation. The specific axial histidine that is replaced by CO in the 6cLS species and the His serving as the trans ligand of CO remain to be identified.
The frequency of $\nu_{11}$ was not affected by mutation at His$^{148}$ and His$^{217}$ but upshifted by 2–3 cm$^{-1}$ for H119A, H138A, and H171A mutants. Unexpectedly, the C170A mutant displayed the largest high frequency shift of $\nu_{11}$. Presumably, Cys$^{170}$ is not an axial ligand of the ferrous heme (discussed below) but interacts directly or indirectly with the axial histidine so strongly as to deprotonate it. Therefore, its removal affects the donation of the His residue.

Time-resolved Resonance Raman Spectra after CO Photodissociation—To investigate the conformational dynamics induced by CO binding to the PAS-A domain, we measured nanosecond time-resolved RR spectra. Fig. 10 (left) illustrates the raw time-resolved RR spectra in the low frequency region of the CO-bound PAS-A domain after photolysis. The spectrum at the bottom was observed in the absence of the pump beam (hereafter referred to as “probe-only” spectrum). The probe-only spectrum was almost identical to the continuous wave-excited RR spectrum for the CO-bound PAS-A domain as shown in Fig. 2 (spectrum C). This implies that the probe pulse is weak enough to protect the sample from photolysis. In the spectrum for $\Delta t = 0$, a new band appeared, which was identical to that observed in Fig. 4 (spectrum B). This band is located at 220 cm$^{-1}$ and is more apparent in the difference spectra obtained by subtracting the probe-only spectrum from the pump-probe spectra depicted on the right.
The band gradually decayed and completely disappeared at \( \sim 500 \mu s \). Since the intensity decrease of this band is in parallel with the increase of the 496-cm\(^{-1}\) negative band, which is the Fe-CO stretching mode, the 220-cm\(^{-1}\) band is assigned to the Fe-His stretching mode of the transiently formed five-coordinate species. This means that the photodissociated CO rebinds to the heme in 500 \( \mu s \). Vinyl and propionate bending modes were also observed at 413 and 379 cm\(^{-1}\), respectively, in the raw time-resolved RR spectra (left panel), but these bands exhibited no shifts during CO rebinding (right panel). This implies that the structural changes of heme propionate and vinyl side chains are not involved in CO binding, in contrast to that observed for sGC (47).

**DISCUSSION**

**Coordination Structure of the PAS-A Domain—**RR spectra in the high frequency region provide information on the coordination and spin state of heme (29). The present Raman spectra demonstrate that both the ferric and ferrous PAS-A domains consist of a mixture of five- and six-coordinate heme (Fig. 2). Since a potent Fe-S vibration appeared at 334 cm\(^{-1}\) upon excitation at 363.8 nm, which was dramatically weakened by replacement of Cys170 with alanine (Fig. 3), Cys170 is considered to be one of the axial ligands of ferric heme. The observed increase in the five-coordinate species for the C170A mutant (Fig. 7A) is consistent with this conclusion.

On the other hand, Cys170 is not one of the axial ligands in the ferrous form, indicating replacement of the axial ligand. In high frequency RR spectra of the ferrous H119A and H171A mutants, a new \( v_3 \) band appeared at 1501 cm\(^{-1}\), whereas the C170A mutant displayed an identical spectrum to that of WT (Fig. 7B). The frequency of the new \( v_3 \) band, which is typical of the ferrous five-coordinate species, increased in both mutants. Therefore, replacement of His119 or His171 with Ala converts the five- or six-coordinate heme to four- or five-coordinate heme. These results suggest that His119 and His171, not Cys170, are axial ligands of heme in the WT ferrous form.

**Fig. 11.** Sequence alignment of the PAS-A domain with other heme PAS proteins (FixL and Ec DOS). Cys170 and His171 of the PAS-A domain are located in the early G strand following the FG loop. In Ec DOS, Met95, an axial ligand of the ferrous heme, exists in the FG loop, and is replaced by O\(_2\) (50, 51). Arg220 in Bradyrhizobium japonicum FixL, which corresponds to Met95 in Ec DOS, is located at the same position as the distal histidine of Mb and forms a hydrogen bond with iron-bound O\(_2\) (52, 53). Accordingly, Cys170 and His171 of the PAS-A domain are present in the so-called distal heme pocket, and either of them may be the sixth ligand of heme.

The H138A mutant exhibited a slightly different Raman spectrum in the ferric state, compared with that of WT (Fig. 7A). The intensity of the \( v_3 \) band at 1470 cm\(^{-1}\), which is derived from the 6cHS species, increased at the expense of the 1503-cm\(^{-1}\) band of the 6cLS species in this mutant. This increase in the 6cHS species for the H138A mutant may be explained by
the coordination of a water molecule to heme instead of His\textsuperscript{138}. Furthermore, a sequence alignment of the PAS-A domain with FixL and EcDOS shows that His\textsuperscript{138} is in the F\textsubscript{β} helix in which proximal histidine (His\textsuperscript{77} for EcDOS and His\textsuperscript{194} for FixL) is located (Fig. 11). Therefore, His\textsuperscript{138} may be possibly an axial ligand of heme in the ferric state, similar to His\textsuperscript{119}. However, it is unlikely that both axial ligands are replaced upon heme reduction as discussed below. Therefore, it seems more plausible at this point that His\textsuperscript{119} is an axial ligand of the ferric heme, which is retained upon heme reduction (Fig. 12).

Conformational Change of NPAS2 upon CO Binding and Signal Transduction Mechanism—

It is well established that correlation between the Fe-C and C-O stretching frequencies provides information on the proximal amino acid in the CO-bound form (40). The frequencies of $v_{\text{Fe-CO}}$ at 497 cm\textsuperscript{-1} and $v_{\text{C-O}}$ at 1962 cm\textsuperscript{-1} for the PAS-A domain fall in the region observed for heme proteins containing a neutral imidazole ligand (Fig. 6). Therefore, a CO adduct of the PAS-A domain has a neutral histidine as an axial ligand in the CO-bound form. The frequencies of the Fe-His stretching modes reflect the electrostatic properties of the axial histidine governed by the hydrogen-bonding network (33). For example, cytochrome c peroxidase, in which proximal histidine forms a strong hydrogen bond with the adjacent Asp\textsubscript{235}, has a frequency of $v_{\text{Fe-His}}$ at 246 cm\textsuperscript{-1} (57). Elimination of this hydrogen bond by the Asp\textsubscript{235} mutation significantly decreases the value to 205 cm\textsuperscript{-1} (58, 59). The $v_{\text{Fe-His}}$ value at 220 cm\textsuperscript{-1} for the PAS-A photoproduct of the CO-complex is similar to those of deoxy-Mb and R-type deoxy-hemoglobin, which possess relatively weak hydrogen bonding coordinates heme even after the intrinsic axial ligand is removed.

Conformational Change of NPAS2 upon CO Binding and Signal Transduction Mechanism—
between axial histidine and surrounding amino acid residues. Accordingly, the $v_{\text{Fe-His}}$ frequency of the photodissociated PAS-A domain at 220 cm$^{-1}$ signifies that the axial histidine of the CO-bound PAS-A domain is in the neutral form and has no or weak hydrogen bonding. This finding is consistent with results derived from the correlation plot.

In contrast to the observed neutral histidine coordination in the CO-bound form, the $v_{C=O}$ frequency of the native ferrous form suggests a different heme environment. The $v_{C=O}$ band is a good $\pi$-electron density marker and is sensitive to the donor strength of the axial ligand, especially in the ferrous state (43). The $v_{C=O}$ mode of the bis(imidazole) complex of iron(II)-protoporphyrin (Fe(II)PP(ImH)$_2$) is observed at 1533 cm$^{-1}$, which is significantly downshifted upon ionization of one or both coordinated imidazoles ($v_{C=O}$ = 1526 cm$^{-1}$ for Fe(II)PP(ImH)(Im$^-$) and $v_{C=O}$ = 1517 cm$^{-1}$ for Fe(II)PP(ImH)$_2$), since the deprotonated imidazole increases the back-donation from the $d_{z^2}$ orbital of the heme iron to the $e_g^*$ porphyrin orbital. The increase in electron density of the $e_g^*$ orbital with anti-bonding character about the $C_p-C_p$ bond results in reduction of the $v_{C=O}$ frequency. The $v_{C=O}$ value of the PAS-A domain (1533 cm$^{-1}$) is lower than that of reduced cytochrome $c$ (1540 cm$^{-1}$) (46), which implies that one of the coordinated histidines is strongly hydrogen-bonded in the ferrous form. Therefore, the electrostatic character of the iron-coordinated His is different between the ferrous and CO-bound forms.

Although mutational analysis revealed that His$^{119}$ and His$^{171}$ are probable axial ligands in the ferrous form, it is not clear which His is replaced by CO, since the Fe-C stretching modes remain unaffected by replacement of His$^{119}$ and His$^{171}$ with Ala (Fig. 8). In view of the finding that the $v_{C=O}$ band shifts from 1533 to $\sim$1539 cm$^{-1}$ when Cys$^{170}$ is replaced with Ala, we propose that Cys$^{170}$ acts as a hydrogen bond acceptor of His$^{171}$ (Figs. 9 and 12). If His$^{171}$ is replaced by CO and His$^{119}$ is retained, release of His$^{171}$ from the heme iron would trigger conformational changes relevant to signaling (Fig. 12A). On the other hand, if His$^{119}$ is replaced by CO and His$^{171}$ is retained, the Fig. 6 data indicate that the hydrogen binding between His$^{171}$ and Cys$^{170}$ would be disrupted upon CO binding (Fig. 12B). Sequential alignment with Ec DOS and FixL suggests that the former case is more plausible as shown in Fig. 11. However, in any case, His$^{171}$ appears to play a crucial role in signal transduction for dissociation of NPAS2 from BMAL1 accompanied by CO binding.

Comparison of the Signal Transduction Mechanism with Those of Other Sensory PAS Proteins—In FixL, conformational changes at the distal site are important for signal transduction to the kinase domain, whereas the proximal site is not included in the signaling pathway (1, 60). The x-ray crystal structure of the B. japonicum FixL heme domain shows that hydrogen bonding between heme 7-propionate and Arg$^{220}$ is disrupted upon O$_2$ binding. Instead, Arg$^{220}$ forms a hydrogen bond with heme-bound O$_2$ (52, 53). This type of hydrogen bond rearrangement in the distal site induces conformational changes to deactivate the kinase domain. The frequency of $\delta(C_p)_{(p)}C_{(p)}$ of the ferrous PAS-A domain of NPAS2 is slightly downshifted from 382 to 379 cm$^{-1}$ upon CO binding (Fig. 2), suggesting that the strength of hydrogen bonding of the heme propionate is weakened by CO binding. However, the frequency of $\delta(C_p)_{(p)}C_{(p)}$ at 379 cm$^{-1}$ still indicates strong hydrogen bonding. Moreover, the frequency does not change during the CO rebinding process in time-resolved RR spectra (Fig. 10). Although we cannot rule out the possibility of rearrangement of hydrogen bonding upon CO binding, it seems that hydrogen bonding of the propionate group is not essential for the signal transduction mechanism of the PAS-A domain.

In Ec DOS, the sixth ligand of the reduced heme is Met$^{55}$ (50, 51). In the presence of O$_2$, Met$^{55}$ is replaced by O$_2$ and Arg$^{7}$, which corresponds to Arg$^{220}$ in B. japonicum FixL, orients to the heme distal pocket from the protein surface to form a hydrogen bond with iron-bound O$_2$ (50). Thus, the conformational changes accompanied by O$_2$ ligation are localized to the FG loop, which serves as a trigger for initial signal transduction in Ec DOS. This mechanism indicates that His$^{171}$, located close to the FG loop of the PAS-A domain, may play the same role as Met$^{55}$ in Ec DOS.

In both FixL and Ec DOS, changes in the heme distal pocket initiate signal transduction, as discussed above. His$^{171}$ in the PAS-A domain, which is located in the early G strand, may be involved in the signal transduction pathway. Our results thus collectively implicate a conserved mechanism of signal transduction in the heme PAS superfamily.

In summary, RR and mutational studies demonstrate that Cys$^{170}$ and His$^{119}$ are axial ligands of the ferric heme in the PAS-A domain. Cys$^{170}$ may be replaced by His$^{171}$ upon reduction of heme. In the ferrous heme, one of the axial histidines (possibly His$^{171}$) is deprotonated, whereas a neutral histidine coordinates to heme in the CO-bound form. These results suggest that CO binding alters the structure around the protoporphyrin, which triggers a signal to the dimer interface with BMAL1.
