Histidine-132 Does Not Stabilize a Distal Water Ligand and Is Not an Important Residue for the Enzyme Activity in Heme Oxygenase-1†

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ABSTRACT: Heme oxygenase is a key enzyme in the oxygen-dependent heme catabolism pathway. In order to clarify the role of highly conserved His132 in heme oxygenase isoform-1, we have prepared 30 kDa truncated rat heme oxygenase mutants in which His132 has been replaced by Ala, Gly, and Ser. The expressed recombinant mutant proteins were isolated in inclusion bodies and were recovered from the lysis pellet by dissolution in urea followed by dialysis. The solubilized fraction obtained, however, was composed of a mixture of a functional enzyme and an inactive fraction. The inactive fraction was removed by Sephadex G-75 gel filtration column chromatography, as it eluted out of the column at the void volume. The gel filtration-purified heme oxygenase mutants have spectroscopic and enzymatic properties identical to those of wild type. The hemin complex of the H132A mutant exhibits a transition between a high-spin acid form and a low-spin alkaline form with a pK_a value of 7.6 identical to that in the wild-type complex. These results demonstrate that His132 in heme oxygenase does not link to the coordinated water molecule and is not an essential residue for the enzyme activity. These results are in accordance with our previous preliminary results [Ito-Maki, M., Ishikawa, K., Mansfield Matera, K., Sato, M., Ikeda-Saito, M., & Yoshida, T. (1995) *Arch. Biochem. Biophys. 317*, 253−258] but contradict a recent report that His132 is the distal base linked to the coordinated water molecule and an important residue for enzyme catalysis [Wilks, A., Ortiz de Montellano, P. R., Sun, J., & Loehr, T. M. (1996) *Biochemistry 35*, 930−936]. Prolonged storage of the solubilized fraction from the inclusion bodies of the mutants, H132S in particular, results in an increase in the void volume fraction with a concomitant decrease of the 30 kDa fraction. We infer that His132 plays a structural role in stabilization of the heme oxygenase protein.

Heme oxygenase (HO),[1] an amphipathic microsomal protein, catalyzes the regiospecific oxidative degradation of iron protoporphyrin IX (heme hereafter) to biliverdin, CO, and Fe in the presence of NADPH−cytochrome P-450 reductase, which functions as an electron donor (Tenhunen et al., 1969; Kikuchi & Yoshida, 1980; Maines, 1988). In the catalytic cycle of HO, the enzyme first binds 1 equiv of heme, resulting in the formation of the heme−enzyme complex, which exhibits optical absorption spectral properties similar to those of myoglobins and hemoglobins (Yoshida & Kikuchi, 1978a, 1979). The first electron donated from the reductase reduces the ferric heme iron to the ferrous state, and a molecule of oxygen binds to form a metastable oxy form (Yoshida et al., 1980). Electron donation to the oxy form initiates the three stepwise oxygenase reactions where heme is converted to the ferric iron−biliverdin complex through α-hydroxyheme and verdoheme (Scheme 1). Electron donation from the reductase converts the iron−biliverdin complex to ferrous iron and biliverdin (Yoshida & Kikuchi, 1978a). Heme, therefore, participates both as a prosthetic group and as a substrate, a property unique to heme oxygenase (Yoshida & Kikuchi, 1978b).

Recent studies by us and others using truncated water soluble forms of recombinant HO preparations have demonstrated that, in the heme−enzyme complex, a neutral form of the imidazole group of histidine (His25 in the HO isoform-1 sequence) is the axial heme ligand (Takahashi et al., 1994a,b, 1995; Ito-Maki et al., 1995; Sun et al., 1994). However, the nature of the distal residue is yet to be identified. Our previous studies have shown the presence of an ionizable group with a pK_a value of 7.6, which links with the coordinated water molecule, in the distal pocket of the ferric heme−enzyme complex, clearly demonstrating the presence of a distal base residue in HO (Takahashi et al., 1994a). Spectroscopic properties of the heme−HO complex are similar to those of myoglobins and hemoglobins in which a histidine residue is the distal residue. Thus, a histidine could be a reasonable candidate for the distal base in the HO complex. His84, -119, and -132 are the three conserved His residues in the HO isoform-1 sequence in addition to

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[1] Abbreviations: HO, heme oxygenase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

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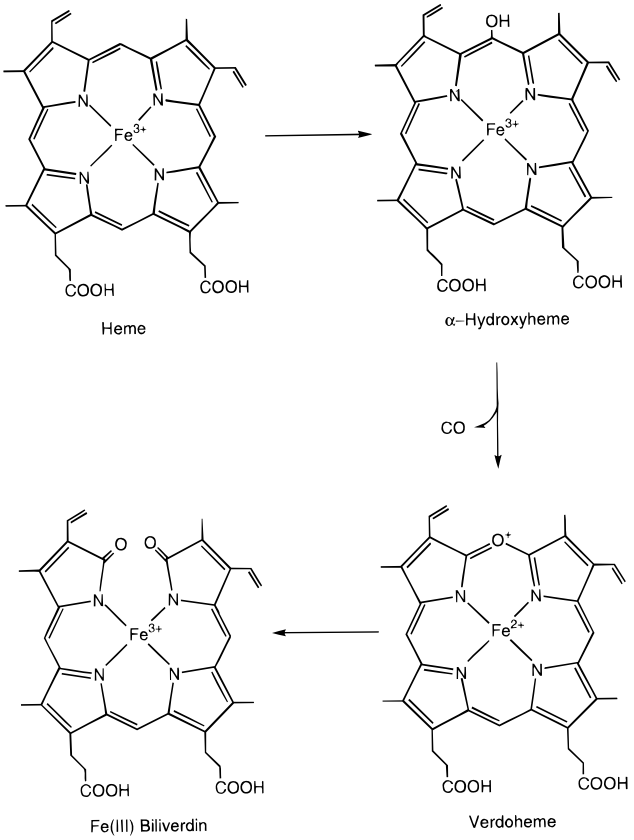
Scheme 1: Intermediates in the Reaction of the Heme Oxygenase-Catalyzed Conversion of Iron Protoporphyrin IX to the Biliverdin−Iron Complex



the proximal heme ligand, His25. Initial studies have shown that His84 and His119 are not essential for the enzyme activity; therefore, they are unlikely to be functionally important residues (Ishikawa et al., 1992). Recent mutagenesis studies on His132 have resulted in conflicting conclusions. Our mutagenesis study (Ito-Maki et al., 1995) has revealed that the mutation of the conserved His132 to Ala does not alter the specific enzyme activity when ascorbic acid was used as an electron donor and has led us to the conclusion that His132 is not essential for the enzyme catalysis despite the fact that His132 is located in the middle of a highly conserved amino acid stretch encompassing positions 126−154 in the rat, human, mouse, chicken, and pig HO-1 proteins (Shibahara et al., 1985; Yoshida et al., 1988; Kageyama et al., 1988; Evans et al., 1991; Suzuki et al., 1992). However, this conclusion was challenged by Wilks et al. (1996), who claimed that the enzyme activity was lowered by 50−60% in the H132G and H132A mutants and by 80% upon the His132 to Ser replacement. They also reported that His132 was required for catalytic turnover of the enzyme with hydrogen peroxide.

The nature of the distal residue is significant in controlling the reactivities of hemoproteins. Distal amino acid residues, in concert with the proximal iron ligand, play key roles in oxygen activation in peroxidase and cytochrome P-450 enzymes (Dawson, 1988). Oxygen affinities of ferrous hemoproteins are governed by the distal pocket amino acids' polarity, steric hindrance, and hydrogen bonding effects (Springer et al., 1994). These properties are relevant in terms of HO enzyme activity, because molecular oxygen first binds to the HO ferrous heme iron and then the bound oxygen is activated to initiate the heme catabolism reactions. Amino acid residues in the distal heme pocket are significant in the molecular oxygen binding to the heme−HO complex (Takahashi et al., 1995). Therefore, resolution of the contradictory results on His132 is of vital importance for understanding the active site structure of HO. By use of a T7 promoter-based expression system with BL21 as a host cell, we are now able to prepare the 30 kDa HO His132 mutant proteins, H132A, H132G, and H132S, in sufficient amount to carry out both enzymatic and spectroscopic studies. We report here that replacement of His132 by Ala, Ser, and Gly does not alter the enzyme catalytic turnover with either NADPH−cytochrome P-450 reductase or hydrogen peroxide. We also show that optical absorption and EPR spectroscopic properties of the H132A−heme complex are essentially the same as those of the wild-type complex and therefore conclude that His132 is not a significant residue for the enzyme function; however, it might be an important residue for stability of the HO protein.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. The sources of reagents are as follows: restriction endonucleases, T4 polynucleotide kinase, Taq DNA polymerase, and a pTV118N sequencing primer, P7 (3′-CAGCACTGACCCTTTTGGGACCGC-5′), from Toyobo; restriction enzymes from New England Biolabs; T4 DNA ligase from Boehringer; DyeDeoxy Terminator Cycle Sequencing kit from Perkin-Elmer; ampicillin, hemin, Triton X-100, sodium deoxycholate, and bovine serum albumin from Sigma; desferrioxamine from Ciba-Geigy; nitrocellulose membranes from msi; Sephadex G-75 from Pharmacia; DE-32 from Whatman; peroxidase-conjugated sheep IgG against rabbit IgG from Cappel; synthetic oligonucleotides from Sawady Technology; and BL21 and pT7BlueT vector from Novagen. A prokaryotic expression vector, pMW172, was a generous gift from K. Nagai (MRC Laboratory of Molecular Biology, Cambridge, U.K.). Rabbit antibody was raised against HO-1 purified from rat liver treated with $CdCl_2$ as described previously (Ishizawa et al., 1983).

Construction of Expression Plasmids of the Soluble Rat HO-1. (a) pMWΔRHO1 and pMWΔH132A. An expression vector, which contains a HO-1 cDNA coding amino acid residues from 1 to 263 with Ser262 → Arg and Ser263 → Leu mutations, was constructed in pMW172. A PCR was carried out with a rat HO-1 expression vector, pTVΔRHO1 (Ishikawa et al., 1992), as a template and a synthetic nucleotide X (5′-AACAGCATATGGAGCGCCCACAGCTCGA-3′) and P7 as primers. The nucleotide X corresponds to the nucleotide sequence positions −8 to +20 of pTVΔRHO1, except that ACC at positions −3 to −1 has been changed to CAT to create a new *Nde*I restriction site. The target fragment was digested with *Nde*I and *Hin*dIII and cloned into pMW172 to construct pMWΔRHO1. For construction of pMWΔH132A, a similar procedure was employed, except that pTVΔH132A (Ishikawa et al., 1992) was used as a template for the PCR.

(b) pMWΔH132S and pMWΔH132G. PCR for construction of pMWΔH132S in which His132 was replaced by Ser was performed using pTVΔRHO1 as a template DNA with oligonucleotide X and an oligonucleotide (3′-CACCGGAGGCGTATATGGGCGATGGACCCACTGGAG-5′) as a mutagenic primer. The underlined bases are the complementary

codon for Ser132. The target fragment was subcloned into pT7BlueT-Vector which was digested with *Eco*RV followed by the addition of a single 3′-T residue at each end. The *Nde*I/*Bst*EII fragment cutout from this subclone was ligated into pMWΔRHO1 predigested with *Nde*I and *Bst*EII. For construction of pMWΔH132G, the same procedure was employed except that the oligonucleotide of 3′-CACC GGCCGCGTATATGGGCGATGGACCCACTGGAG-5′ (the underlined bases are the complementary codon for Gly132) was used as a mutagenic primer. The entire coding sequences of these plasmids were determined by the cycle-sequencing method with an Applied Biosystems 373A DNA sequencer.

Expression and Purification of 30 kDa Soluble HO-1. A fresh single colony of *Escherichia coli* strain BL21 transformed with pMWΔRHO1 was precultured overnight at 37 °C in 10 mL of Luria-Bertani medium. Then, 100 μL of the preculture was used to inoculate 500 mL cultures of the same medium. The cells were grown at 37 °C for 16 h. Cells were harvested, washed, and lysed as described previously (Ito-Maki et al., 1995). The lysed cells were briefly sonicated until they lost their viscosity and were then centrifuged at 10500*g* for 1 h. The resulting supernatant was used to prepare the soluble 30 kDa HO-1 protein following the purification method described previously (Ito-Maki et al., 1995) except that the DEAE-cellulose column chromatography step of the original procedure has been omitted in the present method. The 30 kDa fragment has been shown to be catalytically active (Wilks & Ortiz de Montellano, 1993; Takahashi et al., 1994; Ito-Maki et al., 1995), and we therefore designate the 30 kDa water soluble form as wild type for the purpose of discussion. The wild-type enzyme prepared by this new method was homogeneous as judged by the SDS−PAGE which showed only one band at 30 kDa, and its enzymatic and spectroscopic properties were the same as those of our previous wild-type preparation (Takahashi et al., 1994a; Ito-Maki et al., 1995).

Extraction of ΔH132A Mutants of the Soluble HO from Inclusion Bodies. Culture conditions for bacteria transformed with the expression plasmids for the mutants were the same as those for the wild-type enzyme. To 10 g of harvested cells in 100 mL of 50 mM Tris-HCl (pH 7.2) containing 2 mM EDTA was added 10 mg of lysozyme, and the mixture was stirred for 1 h at 4 °C. The mixture was then sonicated 10 times at maximum power with a Branson SONIFIER 250 for 30 s with 4 min intervals and centrifuged at 8000*g* for 20 min. The precipitates were suspended in 100 mL of 0.5 M sucrose with a Hitachi HG 30 homogenizer, followed by centrifugation at 8000*g* for 20 min. Precipitates were treated with 100 mL of 20 mM sodium phosphate buffer (pH 7.4) containing 1% Triton X-100 and 1% sodium deoxycholate. The mixture was left for 1 h and centrifuged at 8000*g* for 20 min. The resulting precipitates were solubilized by homogenization in 10 mL of 30 mM Tris-HCl buffer (pH 7.2) containing 30 mM NaCl, 1 mM DTT, 5 mM 2-mercaptoethanol, and 8 M urea, followed by dialysis for 24 h against 2 L of 20 mM sodium phosphate buffer (pH 7.4) containing 1 mM DTT and 5 mM 2-mercaptoethanol; the buffer was renewed three times. No SH-protecting reagents were added to the final buffer. After dialysis, the sample was centrifuged at 100500*g* for 30 min and the supernatant was used as the soluble protein.

Steady-State Activity Assay. We used the following two assay systems.

(a) NADPH−Cytochrome P-450 System. This assay method is similar to that described previously (Yoshida & Kikuchi, 1978a) with minor modifications. The standard reaction mixture contained in a final volume of 1.5 mL of 0.1 M potassium phosphate buffer (pH 7.4) an appropriate amount of HO, 30 nmol of hemin, 0.15 mg of bovine serum albumin, partially purified biliverdin reductase (5 nmol/min), 1 μmol of NADPH, and 1 unit of NADPH−cytochrome P-450 reductase. The reaction was initiated by the addition of NADPH, and the reaction was carried out for 10 min at 37 °C. The absorbance at 468 nm was recorded in the difference between the standard and control systems; the latter system contained all components except NADPH. The value of 43.5 mM^{-1} cm^{-1} was used for the extinction coefficient of bilirubin at 468 nm. One unit of enzyme was defined as the amount of enzyme catalyzing the formation of 1 nmol of bilirubin per hour under these conditions. NADPH−cytochrome P-450 reductase was solubilized from rat liver microsomes with Triton X-100 and purified by the method of Yasukochi and Masters (1976). Partially purified biliverdin reductase was prepared from the cytosolic fraction of porcine spleen by the method of Noguchi et al. (1979).

(b) Ascorbic Acid System. Reaction conditions are similar to those of the NADPH−cytochrome P-450 system, except that the assay mixture includes 150 μmol of sodium ascorbate and 15 μmol of desferrioxamine in place of NADPH and NADPH cytochrome P-450 reductase.

Spectroscopy. Light absorption spectra were recorded by Hewlett-Packard 8453 and Hitachi U-3210 spectrophotometers at 20 °C. EPR spectra were recorded by a Bruker ESP-300 spectrometer operating at 9.45 GHz equipped with an Oxford liquid helium flow cryostat as described previously (Takahashi et al., 1994a). The microwave frequency was monitored by a frequency counter (HP-5350), and an NMR gauss meter (Bruker ER-035M) was used to determine the magnetic flux density. Far-UV CD spectra (260−160 nm) were measured as described previously (Ito-Maki et al., 1995).

RESULTS AND DISCUSSION

Expression and Purification of the His132 Mutants. In an earlier work (Ishikawa et al., 1992) where HO was expressed in JM109 cells using a pTV118N-based expression vector, the roles of His132 could not be assessed due to the extremely low expression of H132A. By increasing the bacterial culture in the subsequent study (Ito-Maki et al., 1995), we were able to purify H132A to examine its enzyme activity, although the amount of the purified enzyme we obtained was still small. When ascorbic acid was used as an electron donor, the H132A mutant retained enzyme activity, but under the NADPH−cytochrome P-450 reductase system, a lowered enzyme activity was observed. To obtain a large amount of H132A mutant, we employed here another expression vector having T7 promotor, pMW172, and *E. coli* strain BL21 which lacks ompT and lon proteases.

The harvested cells transformed with pMWΔH132A were brown, while the cells expressing the wild-type enzyme were dark green. Figure 1 shows the SDS−PAGE of the sonicated BL21 cells transformed with pMWΔH132A (lane 3) and its

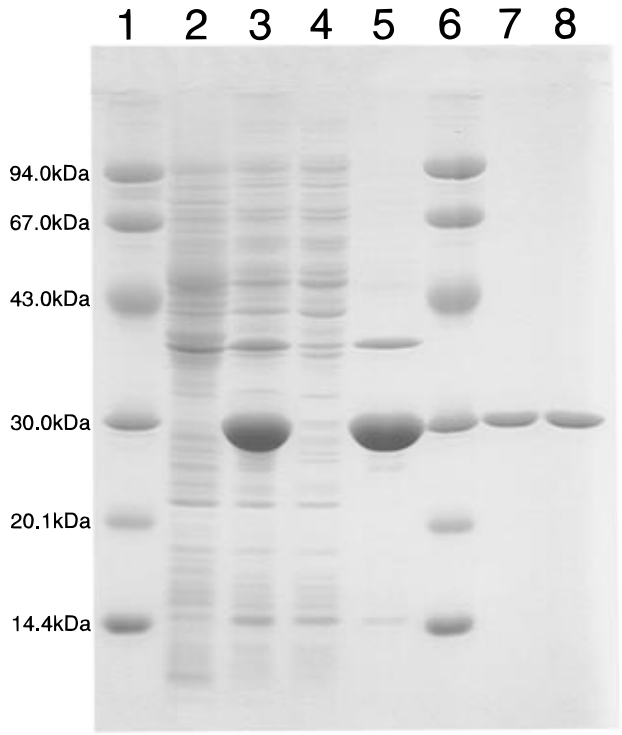


FIGURE 1: SDS−PAGE analysis of expression of H132A in *E. coli* and its intracellular localization, and purified H132A: lane 1, molecular mass markers; lane 2, sonicate of BL21 cells without pMWΔH132A (80 μg of total protein); lane 3, sonicate of BL21 harboring pMWΔH132A expression vector (80 μg of total protein); lane 4, supernatant fraction of lane 3 (36 μg of total protein); lane 5, precipitate fraction of lane 3 (44 μg of total protein); lane 6, molecular mass markers; lane 7, renatured inclusion body (6 μg of protein); and lane 8, 6 μg of the final preparation of the H132A mutant after Sephadex G-75 column chromatography. For electrophoresis, a 10% polyacrylamide gel was used, and the gel was stained with Coomassie brilliant blue.

soluble (lane 4) and precipitate (lane 5) fractions. The presence of large bands at 30 kDa in lanes 3 and 5 and its complete absence in lane 4 imply that the expressed H132A mutant protein was recovered exclusively in the precipitate fraction. The expressed H132A seemed to accumulate in the host cells as an inclusion body. This is different from the expression of the wild-type fragment which was fully recovered in the soluble fraction. Soluble H132A obtained after extraction from inclusion bodies and renaturation was apparently pure as judged from the SDS−PAGE (lane 7). We found that the specific activity of the soluble H132A for heme degradation was about $^1/_5$ of that of the wild-type enzyme, suggesting that the H132A mutant has lowered activity or the soluble fraction contains a considerable amount of nonfunctional H132A protein. In order to assess the possibility of the presence of nonfunctional H132A protein, we subjected the soluble fraction to gel filtration column chromatography with Sephadex G-75. The elution profile of the G-75 column chromatography showed the presence of two protein peaks (data not shown); one fraction eluted at the void volume and the other one at the same elution volume as the wild-type protein. The latter fraction has a molecular mass of 30 kDa (lane 8 of Figure 1). Western blotting analysis showed both fractions cross-reacted with the native HO antibody (data not shown). The far-UV CD spectrum of the 30 kDa fraction (data not shown) was indistinguishable from that of the wild-type enzyme (Ito-Maki et al., 1995), suggesting that H132A is properly folded.

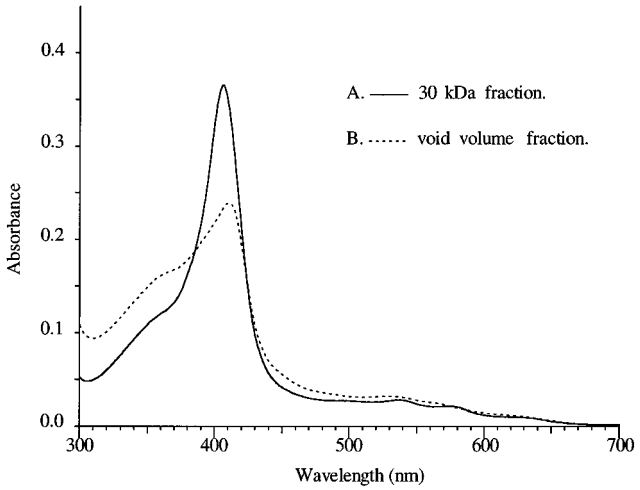


FIGURE 2: Light absorption spectra of the heme complex of the 30 kDa fraction (solid line) and that of the void volume fraction (broken line). Measurements were carried out in 0.1 M phosphate buffer at pH 7.4 and 20 °C. The heme concentration was 2.3 μM.

The void volume fraction became turbid, and its CD spectrum could not be obtained.

The void volume and 30 kDa fractions were examined by their reactions with hemin. The 30 kDa fraction forms a 1:1 complex with hemin as observed for the wild-type enzyme (Yoshida & Kikuchi, 1978; Ito-Maki et al., 1995), and the complex exhibits an optical absorption spectrum identical to that of the wild-type complex as shown in Figure 2. When hemin was added to the void volume fraction keeping the same hemin:protein ratio (w/w) as that of the 30 kDa fraction, a new optical absorption spectrum was observed (Figure 2). The spectrum, which has two Soret bands at 408 and 350 nm, is quite different from that of the heme complex of the 30 kDa fraction but is similar to that of hemin nonspecifically bound to proteins, such as albumin. On the basis of these observations, we concluded that the 30 kDa fraction represents the H132A mutant of heme oxygenase. The specific heme degradation activity of the 30 kDa fraction was comparable to that of the wild-type enzyme. Similar results have been obtained for two other His132 mutants, H132S and H132G. Both H132S and H132G were expressed as inclusion bodies, and their 30 kDa fractions from the G-75 gel filtration chromatography had the same heme degradation activities as that of the wild-type enzyme. These support our recent conclusion that His132 is not important for the heme degradation activity of the enzyme (Ito-Maki, 1995) but are in contrast to the results of Wilks et al. (1996), who claimed that the His132 mutants have specific activity of less than 50% of that of the wild-type enzyme.

In our new expression system, H132A, H132S, and H132G were obtained as an inclusion body, and renaturation and purification procedures described in this report have yielded enzymatically active preparations of the HO His132 mutants. Since inclusion bodies are known to be resistant against bacterial proteolysis, and since there was a reasonable amount of mRNA for H132A present in our previous expression system with a plasmid of pTV118N and JM109 cells (K. Ishikawa and T. Yoshida, unpublished observations), we think that the low level of expression of the H132A protein in our previous studies (Ishikawa et al., 1992; Ito-Maki et al., 1994) was due to bacterial proteolysis. H132A appears

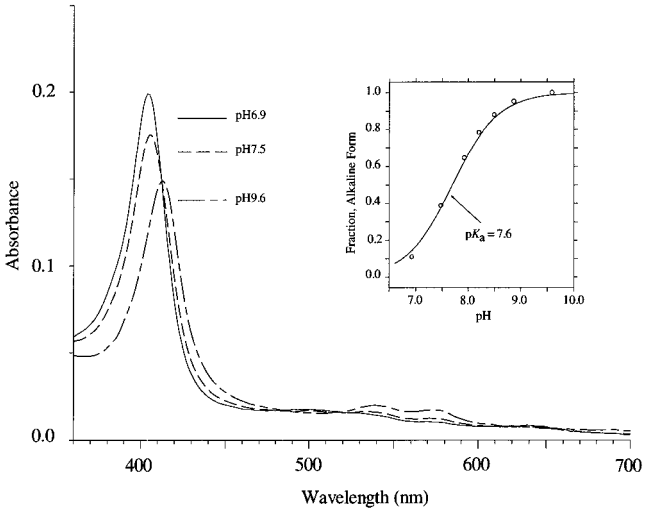


FIGURE 3: Light absorption spectra of the ferric heme−heme oxygenase complex (1.3 μM) at pH 6.9, 7.5, and 9.6 and 20 °C. (inset) The fraction of the alkaline form calculated from the pH-dependent changes in the absorbance at 404 nm. The symbols are experimental values, and the curve is drawn by a least-squares fitting to the $n = 1$ Hendersen−Hasselbach equation.

to be more susceptible to bacterial proteolysis than the wild-type enzyme. Prolonged storage of the solubilized fraction from the inclusion bodies of these H132 mutants at 5 °C, H132S in particular, results in an increase in the void volume fraction with a concomitant decrease of the 30 kDa fraction. These observations have led us to propose that His132 in HO-1 is significant for stability of the HO protein.

Spectroscopic Properties of the Heme Complex. Figure 3 illustrates the pH-dependent changes in the light absorption spectrum of the ferric heme−H132A complex between pH 6 and 10. At pH 6, the heme−H132A complex exhibits a typical hexacoordinate high-spin spectrum with a Soret peak at 404 nm and bands at 500 and 631 nm in the visible region; the spectrum is indistinguishable from that of the wild-type heme complex. At alkaline pH, the high-spin spectrum is replaced by a spectrum with bands at 413, 540, and 575 nm which is identical to the wild-type spectrum in alkaline pH. This pH-dependent spectral change is reversible between pH 6 and 10, and the pK_a value of the change is estimated to be 7.6 as shown in the inset of Figure 3. These features, including spectral shape, peak positions, and the pK_a value of the spectral transitions, are identical to those of the wild-type heme complex (Takahashi et al., 1994a). The light absorption spectra of the heme complexes of H132S and H132G are the same as those of the wild-type complex (data not shown). These results do not agree with those of Wilks et al. (1996), who claimed that the optical absorption spectra of the same set of the mutant complexes were different from that of the wild-type complex.

The active site structure of the H132A−heme complex has been further examined by EPR spectroscopy. Figure 4A compares the EPR spectrum of the ferric heme−H132A complex with that of the wild-type complex at pH 6. Both show typical high-spin EPR spectra, indicating that the His132 → Ala replacement does not alter the ferric heme iron electronic structure of the enzyme complex at pH 6.

In contrast to the results of Wilks et al. (1996), our optical absorption and EPR results clearly demonstrate that the electronic structure of the ferric heme iron in the mutant complexes is the same as that in the wild-type complex. The optical absorption spectra of the His132 mutant complexes reported by Wilks et al. (1996) had broad bands around 400 and 350 nm in the Soret region. Wilks et al. claimed that the His132 → Ala, → Gly, and → Ser mutations removed the coordinated water, resulting in a pentacoordinate high-spin species. However, the optical absorption spectra of the His132 mutants reported by Wilks et al. (1996) are quite different from those of the well-established five-coordinate ferric hemoproteins, such as distal His mutant myoglobins, *Aplysia* myoglobin, horseradish peroxidase, and cyanogen bromide-modified myoglobin (Bracete et al., 1992; Giacometti et al., 1981; Ikeda-Saito et al., 1992). Optical absorption

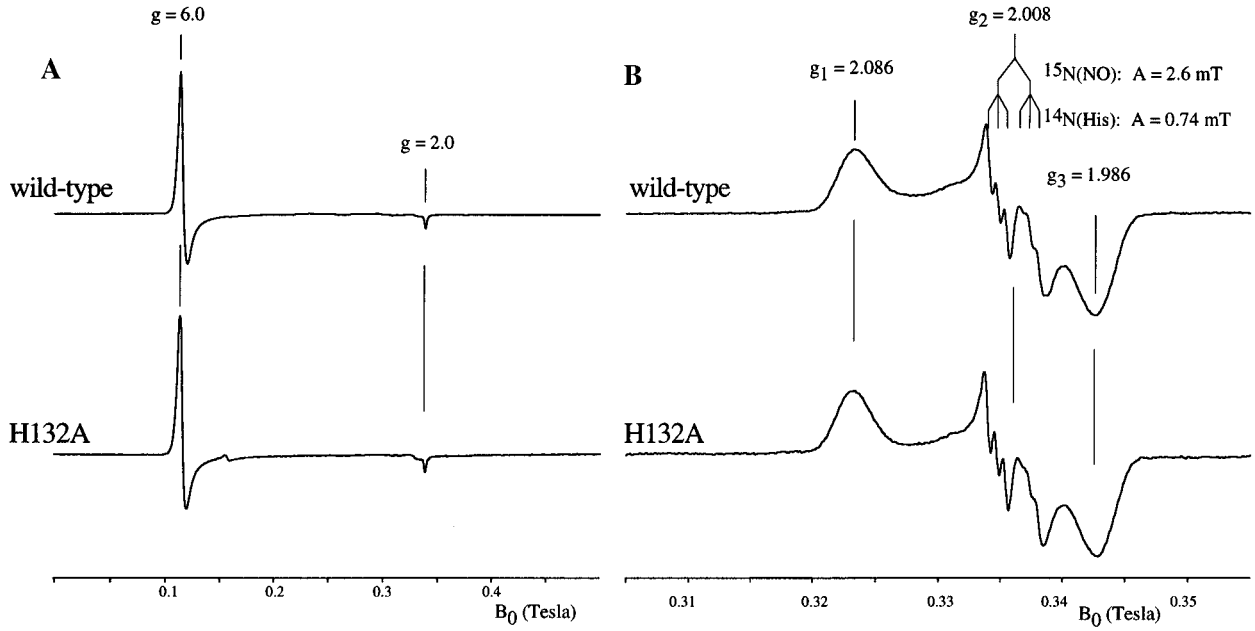


FIGURE 4: EPR spectra of the ferric form (A) and ferrous ^{15}NO complexes (B) of the heme complexes of wild type (top) and H132A (bottom). For the ferric form, measurements were carried out at 6 K with an incident microwave power of 1 mW with a field modulation of 1 mT at 100 kHz. For the ^{15}NO complexes, measurements were carried out at 30 K with an incident microwave power of 0.2 mW with a field modulation of 0.2 mT at 100 kHz.

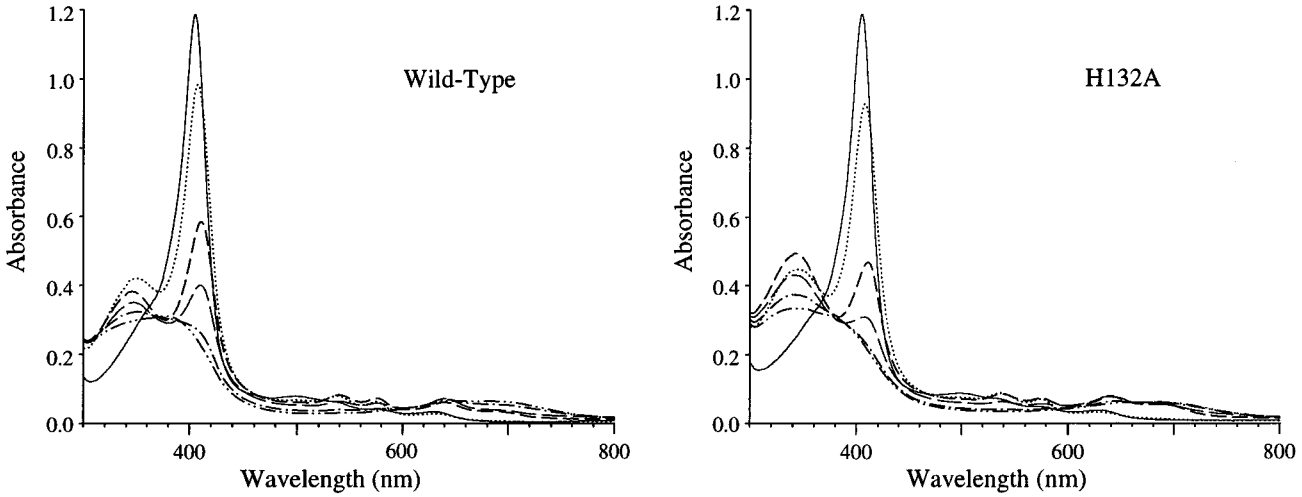


FIGURE 5: Enzymatic degradation of the heme group in heme oxygenase for wild type (left panel) and H132A (right panel). Experimental conditions were as follows: [HO−heme] = 7.6 μM, [NADPH−cytochrome P-450 reductase] = 50 nM, and [NADPH] = 60 μM. Reactions were initiated by addition of NADPH to the 1 mL optical cuvette containing the heme−HO complex and NADPH−cytochrome P-450 reductase in 0.1 M phosphate buffer at pH 7.0. The spectra were recorded before the addition of NADPH (—) and at the following times after the addition of NADPH: immediately (•••), 5 min (- - -), 10 min (— —), 20 min (—•—), and 30 min (—••—).

spectra of these five-coordinate hemoproteins have a broad but well-defined Soret band around 400 nm but do *not* exhibit a large absorption band near 350 nm as seen in the His132 mutants of Wilks et al. (1996). The optical absorption spectra reported by Wilks et al. do not appear to arise from a single pentacoordinate ferric high-spin species but rather look like a mixture of several species, including free hemin and/or hemin nonspecifically bound to proteins.

Our previous studies on the hemin−HO complex have established that the ferric heme iron electronic and coordination structures reversibly change from a water-bound hexacoordinate high-spin state at neutral pH to a hydroxide-bound hexacoordinate low-spin state iron at alkaline pH with a pK_a value of 7.6 (Takahashi et al., 1994a,b). The spectra of the acid and alkaline forms of the ferric heme−H132A complex are found to be identical to those of the wild-type complex between pH 6 and 11. This infers that water and hydroxide are the sixth ligand for the acid and alkaline forms of the mutant complex, as is the case for the wild-type complex. The same pK_a value observed for the mutant complex demonstrates that His132 is not linked to the coordinated water, since acid−base transitions observed in ferric hemoproteins with a water ligand are considered to be linked to the ionization of a distal amino acid residue which forms a hydrogen bond with the bound water ligand. If His132 were linked to the coordinated water as proposed by Wilks et al. (1996), then the optical spectrum of the hemin−H132A complex would not show the pH-dependent change shown in Figure 3. In addition, the EPR results further support the fact that the His132 → Ala replacement has not altered the electronic and coordination structures of the heme iron since pentacoordinate high-spin ferric hemoproteins usually exhibit a rhombic high-spin EPR signal (Ikeda-Saito et al., 1992).

In order to further evaluate the possible effect of the His132 mutation, ferrous forms of the heme−H132A complex were also examined by optical absorption for the ligand-free and CO forms and by EPR for the NO complex. The optical absorption spectra of the ligand-free ferrous form and the ferrous CO complex were found to be the same as those of the wild-type complexes (data not shown). Figure 4B compares the EPR spectrum of the ferrous ^{15}NO complex of H132A with that of the wild-type complex that was reported previously (Takahashi et al., 1994a). These two spectra were identical. EPR spectroscopy of ferrous NO complexes of hemes and hemoproteins directly detects an unpaired electron spin residing on the heme−NO system and thus serves as a sensitive probe for the nature of the heme iron ligand trans to the bound NO and the ligand coordination geometry (Kon, 1967; Yoshimura, 1996). Therefore, the EPR results further support our conclusion that the His132 is not located very close to the bound ligand in the distal heme pocket of the heme−HO complex.

Catalytic Turnover of the H132A Mutant. Addition of NADPH and NADPH−cytochrome P-450 reductase converts the hemin bound to the H132A mutant into biliverdin. Figure 5 shows the spectral changes during catalytic turnover of the heme complexes of wild type and H132A. It is apparent that the rate and the mode of the conversion by H132A are essentially the same as those of wild type, supporting the observations that our His132 mutant preparations have enzyme activity very similar to that of the wild-type enzyme. Finally, reaction of hydrogen peroxide with the heme−H132A complex was examined. Wilks and Ortiz de Montellano (1993) showed that the reaction of the heme−HO complex with hydrogen peroxide converted the bound hemin into a verdoheme species. According to Wilks et al. (1996), H132A, H132G, and H132S did not react with hydrogen peroxide to yield verdoheme species. However, as shown in Figure 6 for H132A, addition of 3 equiv of hydrogen peroxide to the enzyme complex converts the heme group in H132A to verdoheme as reported for the wild-type complex (Wilks & Ortiz de Montellano, 1993). On the basis of these observations, we conclude that the enzyme activity is independent of the His132 → Ala, → Ser, and → Gly mutations.

Comparison with the Results of Wilks et al. (1996). It is intriguing to consider why the same set of the His132 HO mutants prepared by Wilks et al. (1996) had enzymatic and spectroscopic properties distinctly different from those of our mutant preparations. Molecular biology procedures including PCR mutagenesis were carried out by the standard methods, and the mutations were confirmed by DNA sequencing.

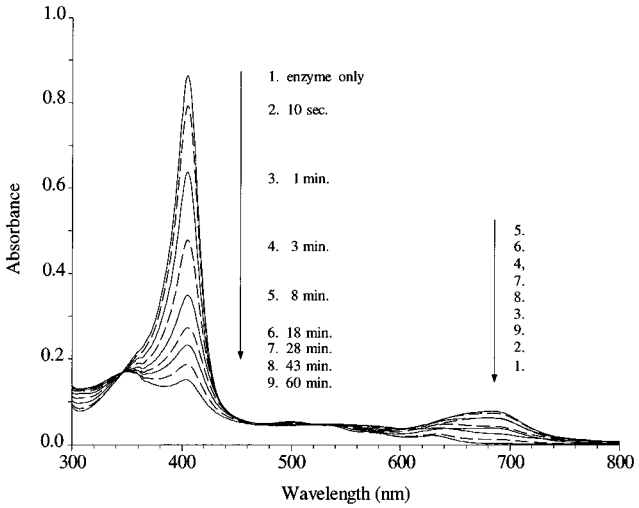


FIGURE 6: Reaction of the heme−H132A complex with hydrogen peroxide in 0.1 M phosphate buffer at pH 7.0 and 20 °C. Reaction was initiated by addition of hydrogen peroxide (18 μM final concentration) to the 2 mL optical cuvette containing the heme−H132A complex (6 μM). Spectra were recorded by the Hewlett-Packard spectrophotometer with a 1 s scan at the time indicated after the addition of hydrogen peroxide.

Possibilities of contamination or coexpression of the wild-type protein in the mutant enzyme preparations are highly remote. We have been using rat isoform-1, while human isoform-1 was used by Wilks et al. (1996). The amino acid sequence around His132 is quite similar between these two proteins (Shibahara et al., 1985; Yoshida et al., 1988); hence, it is highly unlikely that the difference of species, rat *versus* human, is the cause of the discrepancies in the spectroscopic and enzymatic properties. It is possible that the preparations used by Wilks et al. (1996) contained a significant amount of the nonfunctional fraction of the mutant proteins, since gel filtration chromatography, a required step for removal of the nonfunctional fraction in our experimental procedure, was not employed in their preparation method. Although the data are not shown, we have found that the recombinant His132 → Ala, → Ser, and → Gly mutant proteins purified by ion exchange (DEAE-cellulose) chromatography contain some of the nonfunctional form of the enzyme. As described above, the nonfunctional void volume fraction of the H132A protein does not stoichiometrically bind hemin, exhibits a light absorption spectrum different from that of the wild-type hemin complex, and lacks the HO enzyme activity. The altered optical absorption spectra, weak hemin binding, and reduced enzyme activity reported by Wilks et al. (1996) could be accounted for by the presence of nonfunctional fractions in their preparations. Diminished enzyme activity of H132S observed by Wilks et al. (1996) could also be explained by its spontaneous conversion to the nonfunctional void volume fraction. Whatever the reason for the apparent discrepancies, the H132A, H132G, and H132S mutant enzymes prepared by our method exhibit spectroscopic and enzymatic properties essentially identical to those of the wild-type enzyme. Hence, we conclude that His132 is not the distal base linked to the coordinated water molecule in the hemin complex of HO, and it is not an important residue for the enzyme catalysis.

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