Mutation of the Five Conserved Histidines in the Endothelial Nitric-oxide Synthase Hemoprotein Domain

NO EVIDENCE FOR A NON-HEME METAL REQUIREMENT FOR CATALYSIS*

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Five conserved histidine residues are found in the human endothelial nitric-oxide synthase (NOS) heme domain: His-420, His-421, and His-461 are close to the heme, whereas His-146 and His-214 are some distance away. To investigate whether the histidines form a non-heme iron-binding site, we have expressed the H146A, H214A, H420A, H421A, and H461A mutants. The H420A mutant could not be isolated, and the H146A and H421A mutants were inactive. The H214A mutant resembled the wild-type enzyme in all respects. The H461A mutant had a low-spin heme, but high concentrations of L-Arg and tetrahydrobiopterin led to partial recovery of activity. Laser atomic emission showed that the only significant metal in NOS other than calcium and iron is zinc. The activities of the NOS isoforms were not increased by incubation with Fe^{2+}, but were inhibited by high Fe^{2+} or Zn^{2+} concentrations. The histidine mutations altered the ability of the protein to dimerize and to bind heme. However, the protein metal content, the inability of exogenous Fe^{2+} to increase catalytic activity, and the absence of evidence that the conserved histidines form a metal site provide no support for a catalytic role for a non-heme reductase-active metal.

The nitric-oxide synthase (NOS) isoforms are self-sufficient monoxygenases that utilize O_2, NADPH, and L-Arg as substrates to synthesize NO, NADP^+, and citrulline (1–6). The bi-domain structure of NOS contains a C-terminal reductase domain that binds NADPH, FAD, and FMN and an N-terminal domain that binds heme, H_2, and L-Arg. The two domains are connected by a CaM consensus binding sequence. Binding of CaM to this interdomain hinge triggers the electron transfer from the FMN to the heme required to oxidize L-Arg to NO and citrulline. Three distinct mammalian NOS isoforms have been cloned and characterized: nNOS (NOS-I), iNOS (NOS-II), and eNOS (NOS-III). The activities of nNOS and eNOS are regulated by the cellular Ca^{2+} levels. However, CaM binds essentially co-translationally to iNOS, and its activity is primarily regulated at the transcriptional level rather than by the Ca^{2+} concentration (1–6).

A high degree of sequence similarity exists among the NOS isoforms, in accord with their similar cofactor requirements and enzymatic properties (1–6). In this context, one of the unresolved mechanistic questions concerns the role of H_2 in the NOS catalytic cycle. Two lines of evidence argue for a redox role for the pterin in NOS catalysis: (a) redox-inactive H_2B analogs emulate the structural effects of H_2 (dimer stabilization, increased substrate affinity, heme low- to high-spin shift), but do not sustain catalysis (7, 8), and (b) low-temperature experiments suggest that H_2B provides the electron necessary to reduce the oxoferrheme intermediate to the species that oxidizes arginine to N-hydroxylarginine (9).

A precedent for a redox role of H_2B is provided by the aromatic amino acid hydroxylases, in which H_2B reacts with O_2 to form a 4α-hydroperoxy complex. Reaction of this peroxo intermediate with a non-heme iron atom generates the Fe^{4+} = O species actually involved in amino acid hydroxylation (10). The crystal structure of tyrosine hydroxylase shows that the iron is coordinated by a “2-His-1-carboxylate facial triad” (11), a motif also present in other non-heme iron proteins (12). In tyrosine hydroxylase, these metal-coordinating residues are His-331, His-336, and Glu-376, with His-331 as the axial ligand and two water molecules at equatorial positions completing a square pyramidal geometry.

Perry and Marletta (13) have reported that iNOS and nNOS are purified with roughly equivalent amounts of bound zinc and copper, respectively, but after desalting can be reconstituted with ferrous chloride to give proteins with one non-heme iron atom per monomer. This iron reconstitution reportedly increases the NO-synthesizing activity of both nNOS and iNOS (13). These authors argued that the DHH motif found in all the NOS enzymes (Asp-419, His-420, and His-421 in human eNOS) resembles the IHDAHT motif in enzymes such as lysyl hydroxylase that catalyze iron-dependent hydroxylations. In lysyl hydroxylase, the aspartate and the second histidine coordinate to the non-heme iron atom (14). Perry and Marletta proposed that the aspartate and the second histidine in NOS similarly coordinate Fe^{2+}. The increased catalytic activity upon incubation with Fe^{2+} was ascribed to binding of the metal close to H_2B. They furthermore found the nNOS H652A mutant (equivalent to the human eNOS H421A mutant) to be inactive and argued that the loss of activity was due to the role of this histidine in coordinating to the divalent metal. By analogy with lysyl hydroxylase, another of the proposed metal ligands in NOS would be His-692, which corresponds to His-461 in human eNOS.

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domain indicates that the NOS enzymes have a metal-binding site at the monomer-monomer interface consisting of a Zn$^{2+}$ coordinated to four cysteine residues, two from each monomer (15, 16). Crystallization of the iNOS heme domain can result in loss of the zinc and the formation of a disulfide bridge involving Cys-109 of the two monomers (16, 17). No evidence was found in the crystal structures for the additional histidine-dependent metal-binding site invoked by Perry and Marletta (13). Moreover, incubation of eNOS with 10 mM FeSO$_4$ failed to identify new metal-binding sites (15).

We report here the site-directed mutagenesis of the five conserved histidines in the human eNOS heme domain, determination of the metal content of NOS expressed in Escherichia coli and purified by affinity chromatography, and analysis of the effect of exogenous Fe$^{2+}$ and Zn$^{2+}$ on the activity of human eNOS and nNOS.

EXPERIMENTAL PROCEDURES

Materials and General Methods—nNOS was coexpressed with human CaM and purified as reported (18, 19). Expression of the eNOS heme domain, consisting of residues 1–521 plus a six-His tag, in pCwori will be reported separately (2). ρ-Arg was obtained from Aldrich, 2',5'-ADP-Sepharose and 2'-AMP were from Sigma, and H$_B$ was from Alexis Biochemicals (San Diego, CA). The Superdex HR200 gel filtration column was from Amersham Pharmacia Biotech; Vent polymerase and restriction enzymes were from New England Biolabs Inc. (Beverly, MA); and the pGEM-T vector and isopropyl-$β$-thiogalactopyranoside were from Promega (Madison, WI). DNA purification kits and Nif$^{-}$NTA-agarose were purchased from QIAGEN Inc. (Chatsworth, CA). Bacto-yeast extract, Bacto-Tryptone, and Bacto-yeast extract, Bacto-Tryptone, and NTA-agarose were purchased from QIAGEN Inc. (Chatsworth, CA). Alexis Biochemicals (San Diego, CA). The Superdex HR200 gel filtration column was from Amersham Pharmacia Biotech; Vent polymerase and restriction enzymes were from New England Biolabs Inc. (Beverly, MA); and the pGEM-T vector and isopropyl-$β$-thiogalactopyranoside were from Promega (Madison, WI). DNA purification kits and Nif$^{-}$NTA-agarose were purchased from QIAGEN Inc. (Chatsworth, CA). Bacto-yeast extract, Bacto-Tryptone, and E. coli DH5a cells were from Life Technologies, Inc., and BL21 DE3-competent cells were from Novagen (Madison, WI).

Construction of the His-to-Ala Mutants—The five His-to-Ala mutants were constructed by overlap polymerase chain reaction, with two complementary mutagenic primers that introduced a new restriction site (silent mutations) to facilitate screening of the clones. The reverse mutagenic primer was used together with the wild-type primer to construct the mutant with the mutation.

Expression and Purification of the Mutant Proteins—The five His-to-Ala mutants were coexpressed with human CaM and purified as reported (18, 19). Expression of the eNOS heme domain, consisting of residues 1–521 plus a six-His tag, in pCwori will be reported separately (2). ρ-Arg was obtained from Aldrich, 2',5'-ADP-Sepharose and 2'-AMP were from Sigma, and H$_B$ was from Alexis Biochemicals (San Diego, CA). The Superdex HR200 gel filtration column was from Amersham Pharmacia Biotech; Vent polymerase and restriction enzymes were from New England Biolabs Inc. (Beverly, MA); and the pGEM-T vector and isopropyl-$β$-thiogalactopyranoside were from Promega (Madison, WI). DNA purification kits and Nif$^{-}$NTA-agarose were purchased from QIAGEN Inc. (Chatsworth, CA). Bacto-yeast extract, Bacto-Tryptone, and E. coli DH5a cells were from Life Technologies, Inc., and BL21 DE3-competent cells were from Novagen (Madison, WI).

Addition of 100 μM H$_B$ induced a progressive increase in the high-spin component and a progressive decrease in the low-spin component of the spectrum. The first difference spectrum was recorded 1 min after H$_B$ addition, and successive spectra were recorded every 5 min at 25°C. The spectra were plotted, and the $A_{390 \text{-nm}} - A_{410 \text{-nm}}$ values were calculated (20). The spin change ($A_{390 \text{-nm}} - A_{410 \text{-nm}}$) was then plotted against the log of the molecular mass, where $A$ is the maximum absorbance value and $k$ is a kinetic refolding constant.

FPLC Assays—Aliquots of the mutant proteins (200 μl, ~0.5 mg/ml) were analyzed at 25°C on an LCC 500-Plus FPLC system equipped with two P-500 pumps, a Superdex HR200 column, and a monitor set at 280 nm. The flow rate was 0.5 ml/min, and the buffer consisted of 50 mM Hepes, pH 7.0, and 100 mM NaCl. When desired to promote dimer formation, the buffer included 1 mM ρ-Arg and 0.5 μM H$_B$. The column void volume was determined with dextran blue, and the total volume with potassium ferricyanide. The column was calibrated with the following proteins (Sigma): thyroglobulin (689 kDa), apoferritin (443 kDa), β-galactosidase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and horse myoglobin (17 kDa).

The calibration curve was obtained by plotting the $(v_e - v_o)/(v_e - v_i)$ ratio against the log of the molecular mass, where $v_e$ is the elution volume of the protein, $v_i$ is the void volume of the column, and $v_o$ is the total bed volume of the column. The elution positions of the dimer ($10.5$ ml) and monomer ($11.5$ ml) were used to confirm (19).
The absence of a Soret band shift in the presence of high pH is due to another ligand or the proximal thiolate ligand has been observed by Ernest Appelhans of Garratt-Callahan Co. (Millbrae, California). The metal content in NOS samples was determined by inductively coupled plasma atomic emission spectroscopy (15). The metal content in NOS samples was determined by inductively coupled plasma atomic emission spectroscopy (15). Metal Determination—The metal content in NOS samples was determined by inductively coupled plasma atomic emission spectroscopy (15).

RESULTS

Characterization of the H146A Mutant—His-146 is located far from the active site in the α2 helix of eNOS (Fig. 1) (15). Expression of the H146A mutant and elution from Ni²⁺-NTA-agarose yielded a protein with a Soret absorption maximum at 408 nm despite the presence of the 150 mM imidazole used to elute the protein from the column (Fig. 2, trace 1). Addition of up to 30 mM L-Arg plus 30 μM H₄B did not induce a low-to-high-spin shift in the Soret maximum. The absence of a distinct 427 nm absorption peak in the H146A mutant in the presence of imidazole indicates that either the heme distal site is occupied by another ligand or the proximal thiolate ligand has been lost. The absence of a Soret band shift in the presence of high concentrations of L-Arg and H₄B confirms that major alterations have occurred in the heme environment. When the H146A mutant was reduced with NADPH under a CO atmosphere, the reduced CO difference spectrum exhibited only a cytochrome P420 peak (Fig. 2, inset).

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The H214A mutant is indistinguishable from wild-type eNOS (Fig. 3). The Soret maximum at 395 nm (Fig. 3, trace 1) obtained in the presence of l-Arg and H4B indicates that the heme is in the high-spin state in the presence of these factors. Addition of 30 mM imidazole to this protein induced a high- to low-spin shift, and, consequently, the likelihood that the nNOS H652A mutant, equivalent to the H421A mutant studied here (13), for which the 450 nm Fe2⁺-CO complex could be obtained only when the iron was reduced with dithionite. This difference is not surprising given the higher stability of the eNOS than nNOS dimers and, consequently, the likelihood that the nNOS H652A mutant is primarily in the monomeric state.

The NO-synthesizing activity of the H214A mutant (230 nmol min⁻¹ mg⁻¹) is slightly higher than that of wild-type human eNOS (200 nmol min⁻¹ mg⁻¹). The cytochrome c-reducing activity of the H214A mutant in the presence of CaM (2500 nmol min⁻¹ mg⁻¹) is similar to that of the wild-type enzyme (3000 nmol min⁻¹ mg⁻¹).

Characterization of the H420A and H421A Mutants—His-420 and His-421 are located at the beginning of the a7β helix (Fig. 4 (left panel)) (15) in a region close to the heme site proposed to undergo a large rearrangement upon dimerization (17). The imidazole side chains of His-420 and His-421 are hydrogen-bonded to Asp-397 in the neighboring subunit and thus participate in interactions that stabilize the dimer structure.

We were unable to obtain the full-length H420A mutant, apparently because it is proteolysed in the E. coli expression system. Heme insertion in the isolated protein was imperfect, as the protein eluted from the Ni2⁺-NTA-agarose column did not exhibit the usual low-spin spectrum due to the imidazole complex. Its absorption maximum was at 406 nm and did not shift to a high-spin spectrum in the presence of large concentrations of l-Arg and H4B (data not shown).

Characterization of the H421A Mutant—His-461 in human eNOS (His-463 in bovine eNOS) is located at the end of the a11b helix, although it does not interact directly with H4B, it contributes to the electrostatic interactions that stabilize the pterin moiety (Fig. 1) (15). The band at 415 nm, and even large concentrations of l-Arg and H4B did not induce a low- to high-spin shift (data not shown). The purified H421A mutant had NO-synthesizing activity, but had wild-type cytochrome c-reducing activity (2700 nmol min⁻¹ mg⁻¹).

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The H461A mutant purified in the absence of H$_4$B and l-Arg was almost completely in the low-spin state (Fig. 5, trace 1). This contrasts with the wild-type protein, which, even in the absence of the cofactor and substrate, is predominantly in the high-spin state (18, 19, 22). Incubation of the H461A mutant with 1 mM l-Arg in the absence of H$_4$B engendered a moderate low- to high-spin shift (Fig. 5, trace 2), but even prolonged incubation with up to 10 mM l-Arg did not produce a complete shift to the high-spin state. Binding of H$_4$B thus appears to be required for a complete shift in the spin state. The spin state shift in the mutant appears to involve a slow refolding process keyed to the residues that bind H$_4$B and l-Arg because only after incubation of the mutant with 10 mM l-Arg plus 100 μM H$_4$B for 30 min at 30 °C did we obtain a high-spin absorption spectrum identical to that of the wild-type protein (Fig. 5, trace 3). However, even under these optimal conditions, the H461A mutant was less active than the wild-type enzyme (see below).

FIG. 5. Characterization of the H461A mutant. The left panel shows the electronic absorption spectrum of H461A eNOS after elution from the 2',5'-ADP-agarose column in the complete absence of l-Arg and H$_4$B (trace 1). Trace 2 was obtained after incubation of the same sample with 1 mM l-Arg in the absence of H$_4$B for 15 min. Subsequently, this mutant was incubated with 10 mM l-Arg plus 300 μM H$_4$B for 30 min at 25 °C (trace 3). The right panel shows the FPLC profile of the H461A mutant on a Superdex HR200 gel filtration column monitored at 280 nm. The two traces show the maximum (70:30) and minimum (40:60) dimer/monomer (M) ratios observed for the H461A mutant in a total of six different preparations. In both cases, 10 mM l-Arg and 100 μM H$_4$B were present.

FIG. 6. Refolding of the heme environment of the eNOS H461A mutant upon incubation with H$_4$B. The purified H461A mutant was incubated with 10 mM l-Arg before addition of H$_4$B (final concentration) was added, and the spectroscopic changes were monitored as described under “Experimental Procedures.” The first difference spectrum was recorded 1 min after addition of H$_4$B, and spectra were subsequently recorded every 5 min at 25 °C (A). A plot of $A_{390\text{nm}}$ versus incubation time is given in B. The curve was fitted to a single exponential equation (solid line) as described under “Experimental Procedures.” The curve shown is representative of six different experiments.
calcium and iron, significant amounts of only zinc. Full-length nNOS contained, in addition to calcium and iron, a substoichiometric amount of zinc (relative to iron) and traces of copper and nickel. iNOS purified by the normal procedure contained a variety of metals, but when purified with EDTA-treated buffer washes, contained only calcium, iron, and small amounts of zinc and magnesium. No other metal apart from iron and calcium appeared to be present in significant amounts. In particular, copper was not found as a significant constituent, in contrast to an earlier report that copper is a major constituent of nNOS when it is isolated (13).

Incubation of eNOS and nNOS with Divalent Cations—Fe$^{2+}$ reportedly increases NOS activity (13). Zn$^{2+}$, which is present in the eNOS structure (15, 16), has been shown at high concentrations to inhibit the enzyme (23, 24). We compared the effect of these two divalent cations on NOS activity (Fig. 8). In our hands, 4 mM Fe$^{2+}$ had no effect on eNOS activity and only slightly enhanced the nNOS and inhibited the iNOS activities. Addition of 40 mM Fe$^{2+}$ decreased the activities of eNOS and iNOS and caused little change in that of nNOS. Larger concentrations of Fe$^{2+}$ (>80 mM) decreased all three NOS activities. Zn$^{2+}$ is a potent NOS inhibitor, and 40 mM Zn$^{2+}$ almost suppressed the eNOS, nNOS, and iNOS activities. This inhibition of NOS by Zn$^{2+}$ was observed previously by other investigators (23, 24) and was tentatively attributed to defective reduction of the heme iron in the presence of the divalent cation (23).

**DISCUSSION**

The roles of the five histidines conserved among all the known mammalian NOS sequences have been examined by site-directed mutagenesis. These studies have a direct bearing on the report that the activity of NOS is increased by divalent cations that coordinate to one or more of these histidine residues (13). This proposed histidine-dependent metal-binding site is distinct from the zinc site at the dimer interface (15, 16), in which the metal is coordinated to four cysteine residues.

Three of the five conserved histidines (His-146, His-420, and His-421) are part of secondary structural elements that are required for the enzyme either to fold properly or to assemble into the catalytically active eNOS dimer. His-146 is distant from the active site (Fig. 1), but its replacement with an alanine produces an inactive protein with an improperly incorporated heme group (Fig. 2). These long-distance effects presumably reflect a critical role of His-146 in the folding of the α2 and α1 helices.

The DHF motif containing His-420 and His-421 lies at the dimer interface and is involved in multiple hydrogen bonds that implicate it as a critical region in the monomer–monomer interface (Fig. 1) (13). Mutation of either of the two histidine residues in the DHF motif destabilizes the protein. The H420A mutant is apparently misfolded and is sufficiently sensitive to proteolytic digestion that the intact protein could not be isolated. The purified H421A mutant has a low-spin heme Soret maximum and is both unstable and inactive. However, the dimeric form of the protein retains the heme prosthetic group and forms an Fe$^{2+}$-CO complex when reduced with NADPH (Fig. 4). The CO complex is formed only by the lower (versus wild-type) fraction of the protein in the dimeric state, as electron transfer to the iron reportedly occurs from the reductase domain of one monomer to the heme domain of the other (21). This behavior contrasts with that observed when His-652, the equivalent residue in nNOS, was mutated (13). The inability of NADPH to reduce the His-652 mutant of nNOS led to the proposal that loss of a divalent metal coordinated to the histidine residue interrupted the electron transfer pathway. The present results with the eNOS H421A mutant make this explanation untenable, at least as a general one. Our data indicate that the inactivity of the eNOS H421A mutant, and presumably its nNOS H652A counterpart, is due to the formation of a defective heme environment and dimer interface, as evidenced by an increase in the proportion of the protein in the monomeric state. Although the His-420/His-421 pair from each of the eNOS monomers faces the corresponding His-420/His-421 pair of the other monomer, the arrangement of these four imidazole side chains does not seem to be appropriate for metal coordination since no metal was bound in this region when the eNOS heme domain was incubated with 10 mM FeSO$_4$ (15).3 His-214 does not fulfill an apparent major catalytic or structural role because the H214A mutant displays a wild-type phenotype (Fig. 3).

Mutation of His-461, which binds to H$_4$B, alters the mono-

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3 T. Poulos, personal communication.
mer-monomer interaction, the heme chromophore (Fig. 5), and the NO-releasing activity of eNOS. Indeed, one of the electrostatic interactions necessary for proper binding of H$_4$B involves a hydrogen bond through a water molecule to one of the imidazole nitrogenos of His-461. This stabilizing interaction implicates the heme residue of one subunit in binding the pterin molecule of the other subunit (15). In contrast to wild-type eNOS, which binds H$_4$B almost instantaneously (18), the H461A mutant binds H$_4$B slowly even in the presence of 10 mM l-Arg. This suggests that H$_4$B binding requires gradual refolding of the biotpterin site and dimer interface (Fig. 5). This reorganization of the biotpterin-binding site, which is accompanied by a low- to high-spin shift of the iron, occurs over a period of ~30 min at 25 °C (Fig. 6). When reconstituted with H$_4$B, the H461A mutant has ~25% of the catalytic activity of the wild-type enzyme. The lower activity of fully reconstituted H461A eNOS, despite its full conversion to the high-spin state, stems, at least in part, from a subtle disruption of the monomer-monomer interface. Direct evidence for this is provided by FPLC analysis, which shows that the dimer/monomer ratio for the H461A mutant is lower than that of the wild-type protein (Fig. 5, right panel). A comparable alteration in the binding of H$_4$B has been reported for the nNOS C331A mutant, which requires prolonged incubation with l-Arg and H$_4$B to bind the biotpterin and to reconstitute the catalytic activity (25). Sequence comparisons suggest that Cys-331 is one of the cysteines in nNOS that coordinate to the Zn$^{2+}$ cation. The structural alteration due to defective binding of Zn$^{2+}$ is presumably overcome by the slow binding of l-Arg and H$_4$B (25).

Metal analysis of the eNOS, nNOS, and iNOS samples indicates that the only significant metal associated with the proteins, apart from calcium and iron, is zinc. Zinc is present in the eNOS heme domain at a ratio of ~0.6 eq relative to the total iron; in nNOS at a ratio of ~0.3 eq; and in iNOS at ratios of ~0.7 and 0.2 eq in the enzyme isolated with normal and EDTA-treated buffers, respectively (Fig. 7). The zinc content roughly correlates with the stability of the homodimer to dissociation because the eNOS homodimer is the most stable and the iNOS homodimer the least stable among the isoforms. It is likely that the monomeric enzyme does not retain the zinc because the zinc-binding site, as shown by crystal structures of eNOS and iNOS (15, 16), is at the dimer interface and is composed of two thiolate groups from each of the monomers in the dimer. Thus, a completely dimeric enzyme would be expected to have 0.5 eq of zinc relative to heme iron, close to the value found for the eNOS heme domain and iNOS purified without exposure to EDTA. Monomerization of the protein should decrease the proportion of bound Zn$^{2+}$, as found for nNOS and EDTA-treated iNOS. The metal content studies provide no support for the presence of a catalytic metal other than the heme iron, although it has not been possible to quantitate the iron content sufficiently well to determine whether any non-heme iron is present.

The metal independence of the activities of eNOS and nNOS found here differs from the report that the activities of nNOS and iNOS increase upon incubation with Fe$^{2+}$ (13). In our hands, the maximum increase in NO production by nNOS upon incubation with iron, as judged by the hemoglobin assay, is only 18% (Fig. 7). No increase in the eNOS and iNOS activities was observed upon incubation with 4 μM iron, and a reproducible decrease in these activities was observed with 40 μM Fe$^{2+}$. The reason for the discrepancy in our findings and those of Perry and Marletta (13) is unclear. One difference in the two experiments is that Perry and Marletta measured activity by measuring the conversion of [14C]arginine to [14C]citrulline, whereas we determined the production of NO by the hemoglo-

**REFERENCES**

1. Griffith, O. W., and Stuehr, D. J. (1995) *Annu. Rev. Physiol.* 57, 707–736
2. Knoves, R. G., and Moncada, S. (1994) *Biochem. J.* 298, 249–258
3. Masters, B. S., McMillan, K., Sheta, E. A., Nishimura, J. S., Roman, L. J., and Martasek, P. O. (1986) *FASEB J.* 10, 552–558
4. Marletta, M. A. (1994) *Cell* 78, 927–930
5. Breed, D. S., and Snyder, S. H. (1994) *Annu. Rev. Biochem.* 63, 175–195
6. MacMicking, J., Xie, Q. W., and Nathan, C. (1997) *Annu. Rev. Immunol.* 15, 323–350
7. Pfeiffer, S., Gorren, A. C., Pitters, E., Schmidt, K., Werner, E. R., and Mayer, B. (1997) *Biochem. J.* 328, 349–352
8. Mayer, B., Wu, C., Gorren, A. C., Pfeiffer, S., Schmidt, K., Clark, P., Stuehr, D. J., and Werner, E. R. (1997) *Biochemistry* 36, 8422–8427
9. Bec, N., Gorren, A. C., Voelker, C., Mayer, B., and Lange, R. (1998) *J. Biol. Chem.* 273, 13502–13508
10. Chen, D., and Frey, P. A. (1998) *J. Biol. Chem.* 273, 25594–25601
11. Goodwill, K. E., Sabatier, C., and Stevens, R. C. (1998) *Biochemistry* 37, 13437–13445
12. Hegg, E. L., and Que, L., Jr. (1997) *Eur. J. Biochem.* 250, 625–629
13. Perry, J. M., and Marletta, M. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 11101–11106
14. Pirskanen, A., Kaimio, A.-M., Myllyla, R., and Kivirikko, K. I. (1996) *J. Biol. Chem.* 271, 939–9402
15. Raman, C. S., Li, H., Martasek, P., Kral, V., Masters, B. S. S., and Pouls, T. (1998) *Cell* 95, 939–950
16. Fischmann, T. O., Hruza, A., Niu, X. D., Faseettia, J. D., Lunn, C. A., Delphin, E., Prongay, A. J., Reichert, P., Landell, D. J., Narula, S. K., and Weber, P. C. (1999) *Nat. Struct. Biol.* 6, 233–242
17. Crane, B. R., Arvai, A. S., Ghosh, D. K., Wu, C., Getzoff, E. D., Stuehr, D. J., and Tainer, J. A. (1998) *Science* 279, 2121–2126
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18. Rodríguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R. (1996) J. Biol. Chem. 271, 11462–11467
19. Rodríguez-Crespo, I., and Ortiz de Montellano, P. R. (1996) Arch. Biochem. Biophys. 336, 151–156
20. Huhmer, A. F. R., Nishida, C., Ortiz de Montellano, P. R., and Schöneich, C. (1997) Chem. Res. Toxicol. 10, 618–626
21. Siddhanta, U., Presta, A., Fan, R., Wolan, D., Rousseau, D. L., and Stuehr, D. J. (1998) J. Biol. Chem. 273, 18950–18958
22. Martásek, P., Liu, Q., Liu, J., Roman, L. J., Gross, S. S., Sessa, W. C., and Masters, B. S. S. (1996) Biochem. Biophys. Res. Commun. 219, 359–365
23. Persechini, A., McMillan, K., and Masters, B. S. S. (1995) Biochemistry 34, 15091–15095
24. Demura, Y., Aminishima, S., Ishizaki, T., Okamura, S., Miyamori, I., and Matsukawa, S. (1998) Free Radical Biol. Med. 25, 314–320
25. Martásek, P., Miller, R. T., Liu, Q., Roman, L. J., Salerno, J. C., Migita, C. T., Raman, C. S., Gross, S. S., Ikeda-Saito, M., and Masters, B. S. S. (1998) J. Biol. Chem. 273, 34799–34805
26. Boucher, J.-L., Genet, A., Vadon, S., Delaforge, M., Henry, Y., and Mansuy, D. (1992) Biochem. Biophys. Res. Commun. 187, 880–886
27. Boucher, J.-L., Genet, A., Vadon, S., Delaforge, M., and Mansuy, M. (1992) Biochem. Biophys. Res. Commun. 184, 1158–1164
28. Nagase, S., Takemura, K., Ueda, A., Hirayama, A., Aoyagi, K., Kondoh, M., and Royama, A. (1987) Biochem. Biophys. Res. Commun. 133, 156–153
29. Sennequier, N., Boucher, J.-L., Battioni, P., and Mansuy, D. (1995) Tetrahe- dron Lett. 34, 6059–6062
30. Everett, S. A., Dennis, M. F., Patel, K. B., Stratford, M. R. L., and Wardman, P. (1996) Biochem. J. 317, 17–21
31. Venema, V. J., Ju, H., Zou, R., and Venema, R. C. (1997) J. Biol. Chem. 272, 28187–28190