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Active-Site Structure Analysis of Recombinant Human Inducible Nitric Oxide Synthase Using Imidazole

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ABSTRACT: Nitric oxide synthase catalyzes the pyridine nucleotide-dependent oxidation of L-arginine to nitric oxide and L-citrulline. It is a specialized cytochrome P_{450} monooxygenase that is sensitive to inhibition by imidazole. Steady-state kinetic studies on recombinant human inducible nitric oxide synthase (rH-iNOS) demonstrate that imidazole and 1-phenylimidazole are competitive and reversible inhibitors versus L-arginine. Structure−activity relationship and pH dependence studies on the inhibition suggest that the neutral form of imidazole may be the preferred species and that the only modifications allowed without the loss of inhibition are at the N-1 position of imidazole. Optical spectrophotometric studies of rH-iNOS with imidazole and 1-phenylimidazole yielded type II difference spectra exhibiting K_d values of 63 ± 2 and 28 ± 3 μM, respectively. These values were in good agreement with the steady-state K_i of 95 ± 10 and 38 ± 4 μM, respectively, and confirms the site of binding is at the sixth axial ligand of the heme. Imidazole (2.2 mM) also perturbed the K_d of L-arginine from 3.03 ± 0.45 to 209 ± 10 μM. The observed increase in the K_d for L-arginine is consistent with imidazole being a competitive inhibitor versus L-arginine. The IC_{50} values of imidazole and 1-phenylimidazole were lower in the absence of exogenous BH_4, and both inhibitors also competitively inhibited the BH_4-dependent activation of the enzyme. These data taken together suggest that the L-arginine, dioxygen, and the BH_4 binding sites are in close proximity in rH-iNOS. Furthermore, these studies demonstrate the usefulness of imidazole compounds as active site probes for recombinant human iNOS.

Nitric oxide (NO•)[1] is a biologically important free radical that mediates a variety of signaling processes in mammals. Its reactivity with iron may well be the molecular basis for its many regulatory functions. The best known example is the maintenance of vascular tone where NO• activates guanylate cyclase via its binding to the heme of the enzyme. The resulting increase of cyclic GMP leads to vasorelaxation. NO• at low to moderate (low micromolar) concentrations is beneficial, but at high concentration (hundreds of micromolar) it becomes destructive, binding to and inhibiting a variety of heme- and iron-containing proteins (Granger & Lehninger, 1982; Nakaki et al., 1990; Lepoivre et al., 1992). The nondiscriminatory inhibition of essential iron containing proteins serves to destroy invading organisms. However, the prolongation of high concentrations of NO• inevitably causes the destruction of the host cells (Mulligan et al., 1991). The constitutive endothelial (NOS 3) and neuronal nitric oxide (NOS 1) synthases are responsible for the generation of NO• at regulatory levels and their activities are controlled by Ca^{2+}/calmodulin. The isoform responsible for the generation of destructive levels of NO• is the inducible nitric oxide synthase (NOS 2) whose activity is independent of Ca^{2+}/calmodulin regulation but contains tightly bound calmodulin (Cho et al., 1992). Generation of excessive levels of NO• by iNOS has been implicated in several inflammatory diseases (Moncada & Higgs, 1993; Cash & Klippel, 1994); thus, a selective inhibitor of iNOS could conceivably modulate such diseases.

It has been demonstrated that NOS is a highly specialized cytochrome P_{450} possessing two hemes per homodimer and carries out two successive hydroxylations of L-arginine (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992). It is unusual that the functional enzyme consists of an intrinsic P_{450} reductase domain along with the P_{450} heme domain in a single polypeptide. Also the enzyme requires BH_4 for maximal activity and stability (Hevel & Marletta, 1992; Baek et al., 1993; Mayer & Werner, 1995). In the isolated enzyme, the iron of the heme is predominantly (∼80%) in the high spin state (McMillan & Masters, 1993), in contrast to the other cytochrome P_{450} enzymes which exist predominantly in the low spin state. Imidazole is a classic inhibitor of

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[1] Abbreviations: NO•, nitric oxide; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; rH-iNOS, recombinant human inducible nitric oxide synthase; rH-ecNOS, recombinant human endothelial nitric oxide synthase; rH-ncNOS, recombinant human neuronal nitric oxide synthase; CaM, calmodulin; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; DEAE, diethylaminoethyl anion exchange chromatography; DMSO, dimethyl sulfoxide; β-NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; BSA, bovine serum albumin; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; 2′, 5′-ADP, 2′, 5′-adenine dinucleotide phosphate; DTT, dithiothreitol; BH_4, 6(*R*)-5,6,7,8-tetrahydrobiopterin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography, V/K_{arginine}, first order rate constant for L-arginine; V_{arginine}, zero order rate constant for L-arginine.

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cytochrome P_{450} and has been demonstrated to be an inhibitor of the constitutive and inducible NOS isoforms from different species (Wolff et al., 1993; Wolff & Gribin, 1994a,b; Mayer et al., 1994). However, studies with the rat and porcine brain NOS yielded conflicting results with respect to the binding of imidazole to the P_{450} heme relative to the binding of L-arginine (Wolff & Gribin, 1994a; Wolff et al., 1994; Mayer et al., 1994). The understanding of how inhibitors bind to the heme active site is paramount in the design of selective inhibitors for the human enzyme. Unfortunately, insufficient amounts of the human iNOS have hampered detailed enzymological analysis. The recent availability of milligram quantities of the recombinant human iNOS (rH-iNOS) has allowed us to initiate biochemical and biophysical studies of this relevant target.[2] Therefore, we have investigated the inhibition of purified rH-iNOS with imidazole to probe the relationship between the dioxygen, L-arginine, and BH_4 binding sites. We report a thorough kinetic and spectrophotometric study of the inhibition of rH-iNOS by imidazole and 1-phenylimidazole. The data suggest that the L-arginine and BH_4 binding sites are in close proximity to the dioxygen binding site of the heme.

MATERIALS AND METHODS

Chemicals. TES, MES, Bis-Tris propane, imidazole, DMSO, L-arginine hydrochloride, L-citrulline, L-ornithine hydrochloride, β-NADPH, BSA, $CaCl_2$, calmodulin, FAD, FMN, and DTT were purchased from Sigma (St. Louis, MO). Citric acid, sodium azide, and potassium phosphate monobasic were purchased from Fisher Scientific (Springfield, NJ). BH_4 was obtained from Dr. B. Schircks (Jona, Switzerland). L-[2,3,4,5-^{3}H]Arginine hydrochloride was purchased from Amersham (Arlington Heights, IL). In-Flow high salt capacity Tru-Count scintillation cocktail was purchased from IN/US Systems Inc. (Fairfield, NJ). All imidazole compounds were purchased from either Sigma or Aldrich or were obtained from the Merck chemical collection.

Human Recombinant NOS Isoforms. rH-iNOS was purified from Baculovirus infected Sf9 cells containing the cDNA for the enzyme. Cell lysate was obtained by homogenation of cells in lysis buffer (20 mM TES, pH 7.4, 1 mM DTT, 10% glycerol, 50 μM BH_4, 50 μM FAD, 50 μM FMN, 25 μg/mL each of antiplatin, aprotinin, leupeptin and chymostatin, 10 μg/ml pepstatin A, 100 μM PMSF, and 50 μM phenanthroline). Preliminary kinetic studies were performed on enzyme purified to greater than 80% purity by a procedure described by Bredt and Synder (1990) employing a 2′,5′-ADP affinity column and a gel filtration column. Most of the studies reported were performed on immunoaffinity purified rH-iNOS. This purification procedure employed an immunoaffinity column constructed by cross-linking an antibody against the carboxy-terminal portion of human iNOS to protein-A agarose followed by a DEAE step. Details of the cloning, expression, and purification of rH-iNOS will be reported elsewhere.[2] The purity of the enzyme was greater than 95% as judged by SDS−PAGE and had a specific activity of 100 nmol/min mg at 37 °C. The constitutive human isoforms were also from Baculovirus infected Sf9 cells containing either the cDNA for the endothelial or the neuronal enzyme. Cell lysate was prepared by the same procedure as that of rH-iNOS except 10 mM CHAPS and 100 mM NaCl were included into the lysis buffer for rH-ecNOS.

Assay for Nitric Oxide Activity. NOS activity was assayed based on a modification of a published procedure (Robertson et al., 1993). Enzyme incubations consisted of 1−5 μg of immunoaffinity purified iNOS in 100 mM TES, pH 7.5, 5 μM FAD, 5 μM FMN, 2 mM $CaCl_2$, 10 μg/mL CaM, 0.5 mM DTT, 0.5 mM NADPH, 0.5 mg/ml BSA, 0.25 μCi of L-[2,3,4,5-^{3}H] arginine, with 10 μM BH_4 (unless otherwise indicated), and with 1 μM Arg (unless otherwise indicated). All reactions were incubated at room temperature (20−25 °C) for 30 min and quenched with 200 mM citric acid, pH 2.2, 0.2 mM L-citrulline, 0.2 mM L-ornithine, 0.2 mM L-arginine, and 0.02% NaN_3. L-Arginine to L-citrulline conversion was quantitated using a radiometric HPLC assay at room temperature on a Zorbax 300-SCX column (25 cm × 4.6 mm i.d.). A linear gradient of 0−60% 0.1 M KH_2PO_4 (pH 4.5) for 2 min, followed by a 6 min wash with 0.1 M KH_2PO_4 (pH 4.5) at a flow rate of 2 mL/min, was used to elute L-[2,3,4,5-^{3}H]Arg and L-[2,3,4,5-^{3}H]Cit. Enzyme activity was linear for 30 min and reported as (% CPM Cit)/(% CPM Cit + % CPM Arg). The HPLC system consisted of two Shimadzu LC-10AD pumps, a Shimadzu SCL-10A gradient controller, a Shimadzu SIL-10A autosampler, and an IN/US β-RAM radioactivity flow monitor. Data were digitized and analyzed on Shimadzu EZCHROM chromatographic software.

Inhibition Studies. The inhibition constants (IC_{50}) of substituted imidazoles were determined by measuring the amount of L-citrulline formed after 30 min in the absence and the presence of increasing concentrations of inhibitor. IC_{50} values were determined graphically by plotting the inhibitor concentration versus the activity using Sigma Plot (Jandel Scientific). The values were calculated by fitting the data to $y = 1/(1 + x/b)$, where x is the inhibitor concentration, y is the relative activity, and b is the IC_{50} value (Segel, 1975). For pH studies, 100 mM TES, pH 7.5, was replaced with 100 mM Bis-tris-propane ranging from pH 6.5 to 9.5. For K_i determinations, time points were taken at 0, 5, 10, 15, and 20 min intervals after initiation of the reactions with enzyme. Initial rates were determined by taking the linear portion of the progress curve at each concentration of L-arginine and a fixed concentration of inhibitor. The data were analyzed and the K_i value obtained from fitting the data to the $v = VA/[K(1 + I/K_i) + A]$ using the Fortran program of Cleland (Segel, 1975; Cleland, 1979).

The cytosolic fraction of murine fibroblast NIH 3T3 cell line expressing rH-iNOS (Tzeng et al., 1995) was used for the examination of the effects of BH_4 on the concentration-dependent inhibition of rH-iNOS by imidazole and 1-phenylimidazole. Assay conditions were the same as that described for IC_{50} determination except that 25 μM L-arginine and 100 nM or 5 μM BH_4 were used.

Diaphorase Activity. The intrinsic diaphorase activity of rH-iNOS was assayed using a modified version of a previous method (Klatt et al., 1992). rH-iNOS was added to a solution that consisted of 100 mM TES (pH 7.5), 500 μM NADPH, 1 μM FAD, 1 μM FMN, 500 μM nitro blue tetrazolium, and imidazole (0−5 mM) in a final volume of 200 μL. Product formation was monitored at 550 nm ($\epsilon = 28.6$ mM^{-1} cm^{-1}) using a microtiter plate reader (Dynatech MR5000) at room temperature. Enough enzyme (0.8 μg) was added

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[2] Calaycay et al., submitted for publication.

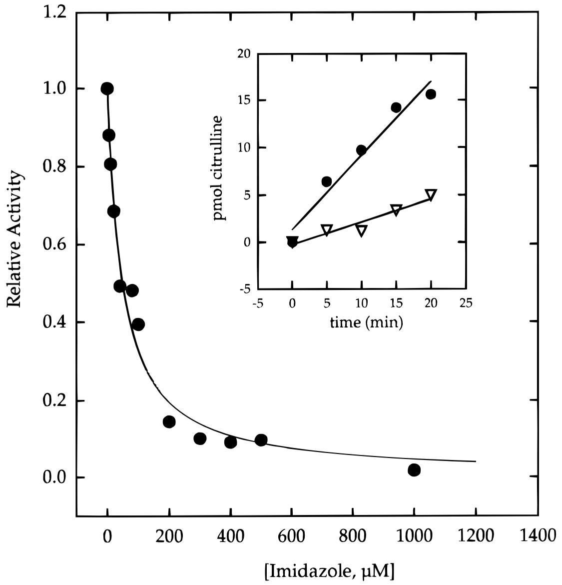


FIGURE 1: Inhibition of rH-iNOS by imidazole. Imidazole inhibited rH-iNOS in a concentration-dependent manner with a calculated IC_{50} of 59 ± 7 μM. Representative progress curves (inset) without added imidazole (●) and with 200 μM imidazole (▽) showed no time dependence on the inhibition; thus imidazole is a reversible inhibitor of rH-iNOS.

to give a linear rate of approximately 0.005−0.010 absorbance units/min in the control sample which did not contain imidazole.

Optical Spectroscopy. All spectra were recorded using a HP8425A diode-array UV/visible spectrophotometer (Hewlett Packard) equipped with a thermostated, multicell-transport cell holder connected to a RM6 Lauda refrigerated water bath. The sample spectrum was blanked against 20 mM TES, pH 7.5, 4 μM BH_4 (unless otherwise indicated), and the absolute absorbance between 190 and 800 nm was recorded at 15 °C. Titration of compounds with the enzyme was accomplished by the step-wise addition of the appropriate stock solution via a syringe at 15 °C and the absolute spectrum recorded at each concentration and normalized at 700 nm. The final added volume was no more than 3% of the initial sample volume. The difference spectrum was generated by the subtraction of the resultant spectrum at each concentration of substrate or inhibitor from the initial spectrum. Binding isotherms were generated graphically by plotting the changes in absorbance at the indicated wavelengths versus the concentration of substrate or inhibitor using Sigma Plot. The dissociation constant (K_d) was calculated by fitting the data to $y = (ax)/(b + x)$, where y is the change in absorbance, x is the concentration of added substrate or inhibitor, a is maximum change in absorbance at infinite concentration of substrate or inhibitor, and b is the dissociation constant, K_d.

RESULTS

Kinetic Studies. Imidazole inhibited rH-iNOS in a concentration-dependent manner and exhibited an IC_{50} value of 59 ± 7 μM (Figure 1). The inhibition was not time-dependent, consistent with it being a reversible inhibitor (Figure 1, inset). The increase in imidazole concentration decreased V/K_{arginine} but not V_{arginine}, indicating that imidazole is a competitive inhibitor of L-arginine (Figure 2). The data gave the best fit to a competitive model with a calculated K_i of 95 ± 10 μM. NOS is a self-sufficient monooxygenase containing a $NADP^+$ reductase domain and a heme hydroxylation domain. The reductase domain has the ability to reduce certain dyes that are independent of the heme domain (Schmidt et al., 1992; Klatt et al., 1992). To test whether imidazole inhibits the reductase domain, the intrinsic diaphorase activity of the enzyme was measured as a function of imidazole concentration. The diaphorase activity was not affected at concentrations up to 1 mM, indicating that imidazole does not affect the reductase domain and suggests the site of imidazole inhibition is the heme domain (data not shown).

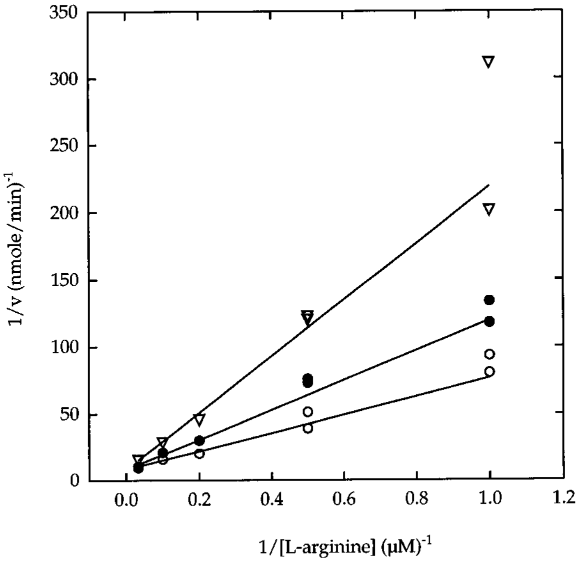
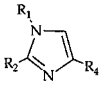


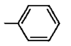
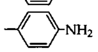
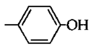
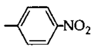
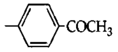
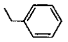
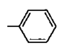
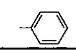
FIGURE 2: Effects of imidazole on the steady-state kinetic parameters of L-arginine. Imidazole at 0 μM (○), 60 μM (●), and 200 μM (▽) inhibited rH-iNOS in a competitive manner with respect to L-arginine. The fit of the data to a competitive model (solid lines) yielded a K_i of 95 ± 10 μM.

Structure−Activity Relationship. A series of imidazole derivatives were tested to identify the structural requirements for inhibition of rH-iNOS. Table 1 showed that the 2- and the 4-positions of imidazole cannot be substituted without a severe loss of inhibition. However, modifications at the 1-position with alkyl and aromatic substituents were well tolerated. One of these compounds, 1-phenylimidazole inhibited rH-iNOS in a competitive manner versus L-arginine and exhibited a K_i of 38 μM (data not shown). Substitutent effects of 1-phenylimidazole were also examined. Hydroxyl and amino groups at the *para* position of the phenyl moiety did not change the inhibition significantly with the *para*-aminophenyl moiety exhibiting slightly better inhibition. However, an electron-withdrawing nitro group had a dramatic effect on the inhibition, increasing the IC_{50} value by 10-fold. Comparison of the IC_{50} of 1-(2-aminoethyl)imidazole (IC_{50} = 22 μM) and 2-(1-imidazol)ethanesulfonic acid (IC_{50} = 4395 μM) indicates that the enzyme disfavors negatively charged substituents at N-1 position.

The Effect of pH on Imidazole Inhibition. The inhibition of rH-iNOS by imidazole and 1-phenylimidazole was examined as a function of pH. Using Bis-Tris propane as a broad pH range buffer, the K_i values for imidazole and 1-phenylimidazole were measured between pH 6.5 and 9.0. Figure 3 shows that, as the pH decreased, the K_i value for imidazole increased. A fit of the data to a titration equation yielded a pK_a value of 7.1 ± 0.2. In the case of 1-phen-

Table 1: Inhibition of rH-iNOS by Substituted Imidazole



Compound	R1	R2	R4	IC50 (μM)
imidazole	H	H	H	59 ± 7 (K_i = 95)
1-phenylimidazole		H	H	33 ± 3 (K_i = 38)
1-(4-aminophenyl) imidazole		H	H	29 ± 4
1-(4-hydroxyphenyl) imidazole		H	H	55 ± 3
1-(4-nitrophenyl) imidazole		H	H	501 ± 67
1-(4-acetophenyl) imidazole		H	H	400 ± 30
2-(1-imidazol)-ethane sulfonic acid	$CH_2CH_2SO_3H$	H	H	4395 ± 520
1-(2-aminoethyl) imidazole	$CH_2CH_2NH_2$	H	H	22 ± 3
1-(2-aminopropyl) imidazole	$CH_2CH_2CH_2NH_2$	H	H	76 ± 3
1-benzylimidazole		H	H	56 ± 6
1-butylimidazole	$CH_2CH_2CH_2CH_3$	H	H	39 ± 1
1-methylimidazole	CH_3	H	H	104 ± 12
2-phenylimidazole	H		H	7900 ± 1100
2-methylimidazole	H	CH_3	H	15,800 ± 1100
2-ethylimidazole	H	CH_2CH_3	H	15,200 ± 600
2-propylimidazole	H	$CH_2CH_2CH_3$	H	1720 ± 100
2-isopropylimidazole	H	$CH(CH_3)_2$	H	1800 ± 100
4-phenylimidazole	H	H		950 ± 70

ylimidazole, however, the K_i was not sensitive to the changes in the pH range examined. Comparison of the pH dependence of the K_i for imidazole to the pH independence of the K_i for 1-phenylimidazole suggests that enzymic groups have minimal contribution on the observed pK_a.

The Effect of BH_4 on Imidazole and 1-Phenylimidazole Inhibition. The effects of exogenous BH_4 concentration on the inhibition of rH-iNOS by imidazole and 1-phenylimidazole were examined. With 1-phenylimidazole, the IC_{50} value at 1 μM L-arginine increased from 12 ± 1 μM with no BH_4 added to 84 ± 8 μM with 100 μM of added BH_4. For imidazole, the IC_{50} value at 1 μM L-arginine increased from 12 ± 1 μM with no BH_4 to 143 ± 9 μM with 100 μM of added BH_4. This suggests that BH_4 can affect the binding of imidazole and 1-phenylimidazole to the enzyme.[3] The effects of BH_4 on the inhibition of imidazole and 1-phenylimidazole were further examined with rH-iNOS expressed in a mouse NIH 3T3 cell line that is deficient in the biosynthesis of BH_4 (Tzeng et al., 1995). Exogenous BH_4 must be added to reconstitute enzymatic activity. This enzyme deficient in BH_4 is useful in that both inhibitor and BH_4 can be added at the same time in the assay and the rate would reflect the ability of the inhibitor to prevent the binding of BH_4. Saturating levels of L-arginine were used in order to minimize the inhibition of L-arginine binding. Using the 3T3 cytosolic fraction expressing rH-iNOS in the absence of added BH_4, the rates of citrulline formation were monitored with varying imidazole or 1-phenylimidazole concentrations. Figure 4 shows that, at 100 nM BH_4 and 25 μM L-arginine in the assay, both imidazole and 1-phenylimidazole inhibited citrulline formation in a concentration-dependent manner (Figure 4A,B). However, at 5 μM BH_4, the inhibition was significantly attenuated for both inhibitors. The inhibition of the enzyme by *N*-nitro-L-arginine was not attenuated as would be expected for an inhibitor of just the L-arginine binding site (Figure 4C). This implies that imidazole and 1-phenylimidazole inhibition of rH-iNOS can be modulated by the presence of exogenous BH_4. The type of inhibition exhibited by imidazole and 1-phenylimidazole was addressed with BH_4 deficient rH-iNOS. The activation of the enzyme by increasing the BH_4 concentration follows a typical titration curve having a K_{act} of 98 ± 32 nM (Figure 5). The presence of 250 μM imidazole or 50 μM 1-phenylimidazole increased the K_{act} by 4- and 8-fold, respectively, but did not significantly affect the V_{max}. This behavior is suggestive of a competitive binding of imidazole and 1-phenylimidazole with respect to BH_4.

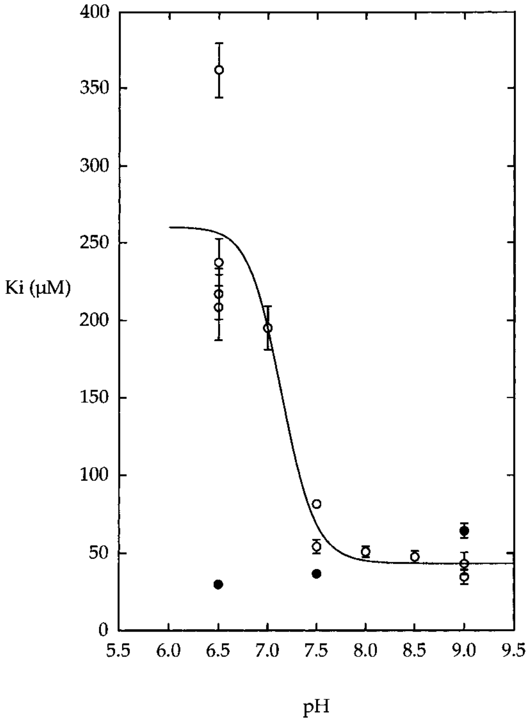


FIGURE 3: pH dependence of the inhibition of rH-iNOS with imidazole and 1-phenylimidazole. The K_i values for imidazole (○) and 1-phenylimidazole (●) were measured as a function of pH. Imidazole showed a marked increase in the K_i value as the pH was decreased. A fit of the data to a titration equation gave a pK_a value of 7.1 ± 0.2. In contrast, the K_i value of 1-phenylimidazole did not change in the same pH range examined (6.5−9.0).

Isoform Selectivity of Imidazole and 1-Phenylimidazole. The inhibition of the constitutive isoforms of NOS were examined for imidazole and 1-phenylimidazole. Table 2 shows imidazole at 10 μM BH_4 and 1 μM L-arginine inhibited the constitutive isoforms with IC_{50} values of 189 ± 22 μM and 175 ± 33 μM for the rH-ecNOS and rH-ncNOS, respectively. For 1-phenylimidazole, the IC_{50} values were 611 ± 63 μM and 429 ± 42 μM for the rH-ecNOS and rH-ncNOS, respectively.

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[3] Using immunopurified rH-iNOS, enzyme inhibition was incomplete in the absence of exogenous BH_4, with 20% of the enzymatic activity not inhibited. Thus a proper fit of the IC_{50} equation was problematic at low BH_4 concentration. Furthermore, the progress curves at low BH_4 concentrations were not linear, making accurate rate determination difficult. The problem can be overcome by using rH-iNOS expressed in a NIH 3T3 cell line that is deficient in BH_4 synthesis (Tzeng et al., 1995).

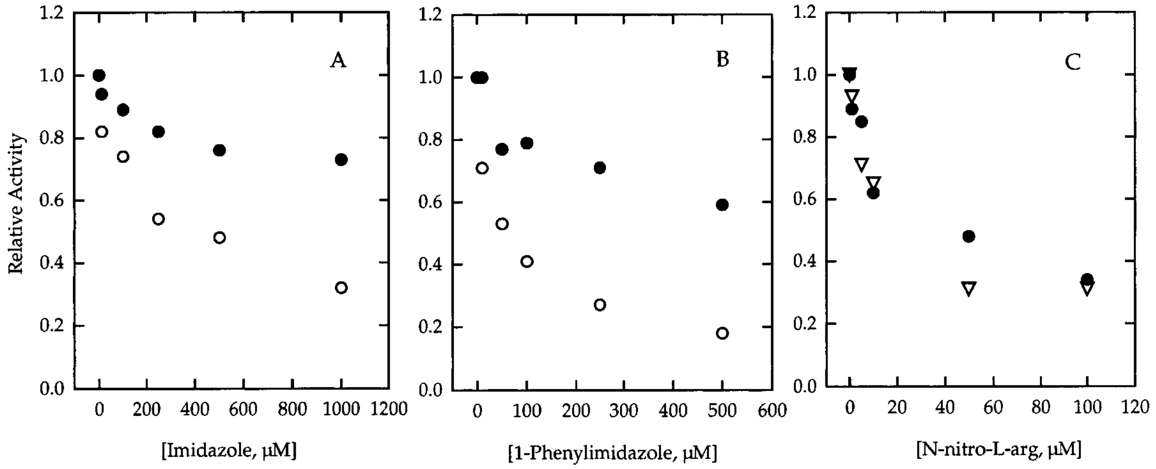

FIGURE 4: Effects of BH_4 on the inhibition of rH-iNOS by imidazole (A), 1-phenylimidazole (B), and *N*-nitro-L-arginine (C). Cell lysate containing rH-iNOS expressed in murine 3T3 cell line deficient in BH_4 biosynthesis was used to examine the effect of BH_4 on the inhibition by these inhibitors. At 25 μM L-arginine concentration and 100 nM BH_4 (○), imidazole, 1-phenylimidazole, and *N*-nitro-L-arginine inhibited the enzyme in a concentration-dependent manner. However, at 5 μM BH_4 (●), the inhibition of the enzyme is significantly attentuated for imidazole and 1-phenylimidazole, but not for *N*-nitro-L-arginine.

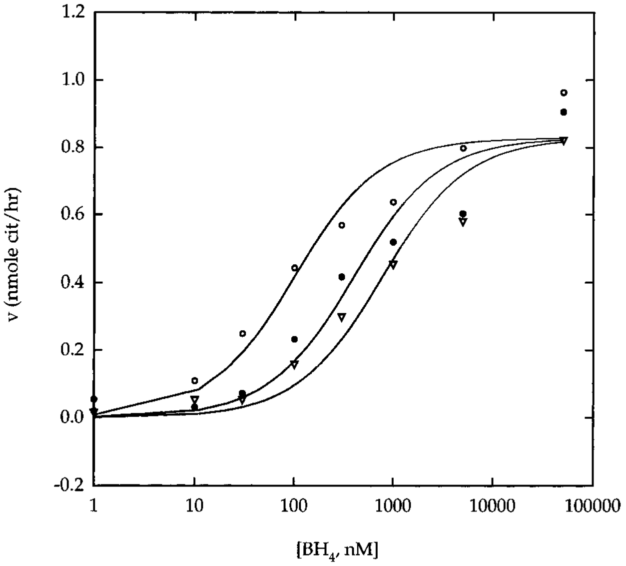

FIGURE 5: Effects of BH_4 activation of rH-iNOS by imidazole and 1-phenylimidazole. The activation of rH-iNOS expressed in 3T3 cells by increasing concentration of BH_4 followed saturation kinetics exhibiting a K_{act} for BH_4 of 98 ± 32 nM (○). Imidazole (●) and 1-phenylimidazole (▽) increased the K_{act} for BH_4 but do not significantly affect the V_{max}. The data gave the best fit for a competitive model (solid lines).

Table 2: Inhibition of Human NOS Isoforms by 1-Phenylimidazole and Imidazole[a]

human isoform	IC_{50} (μM) 1-phenylimidazole	IC_{50} (μM) imidazole
rH-iNOS	33 ± 3	59 ± 7
rH-ecNOS	611 ± 63	189 ± 22
rH-ncNOS	429 ± 42	175 ± 33

[a] rH-iNOS is immunoaffinity/DEAE purified; the cNOSs were from spin-filtered lysates. BH_4 concentration was 10 μM in all cases.

Spectroscopic Studies. rH-iNOS exhibited a typical Soret absorbance having a maximum at 400 nm with other absorbance features at 450 nm due to the flavins.[2] The sequential addition of increasing concentrations of imidazole to rH-iNOS caused an incremental red shift of the Soret maximum from 400 to 430 nm. This concentration-dependent absorbance change is typical of type II difference spectra and is characterized by a peak at 430 nm and a trough at 390 nm (Figure 6). This indicates that imidazole binding causes the population of high spin heme iron to convert to a low spin state upon binding of imidazole (McMillan & Masters, 1993). Measurements of the magnitude of the type II spectral change as a function of imidazole concentration allowed for the determination of the K_d for imidazole to be 63 ± 2 μM (Figure 6, inset). This is in good agreement with the K_i for imidazole of 95 μM determined from steady-state kinetics. Spectral titration of the free enzyme with L-arginine yielded a type I difference spectrum with a peak at 390 nm and a trough at 422 nm, similar to the rat ncNOS (McMillan & Masters, 1993). The calculated K_d value for L-arginine is 3.0 ± 0.4 μM, similar to the steady-state K_m of 2.3 μM (Figure 7A). The addition of increasing concen-

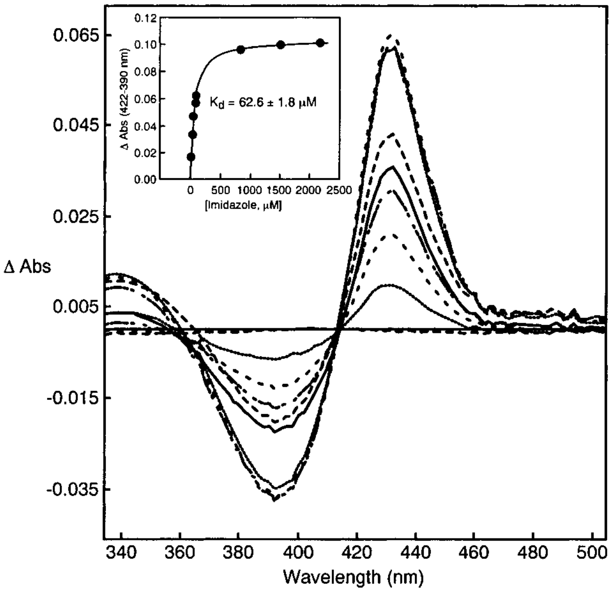

FIGURE 6: Spectral perturbation of the Soret upon binding of imidazole to rH-iNOS. Imidazole elicited a concentration dependent optical absorbance change of the enzyme bound heme. The difference spectra were characteristic of a type II spectral change that had a peak at 422 nm and a trough at 390 nm. A plot of the magnitude of the absorbance change at 422 nm and 390 nm as a function of imidazole concentration yielded a saturation curve with a calculated K_d for imidazole of 63 ± 2 μM (inset).

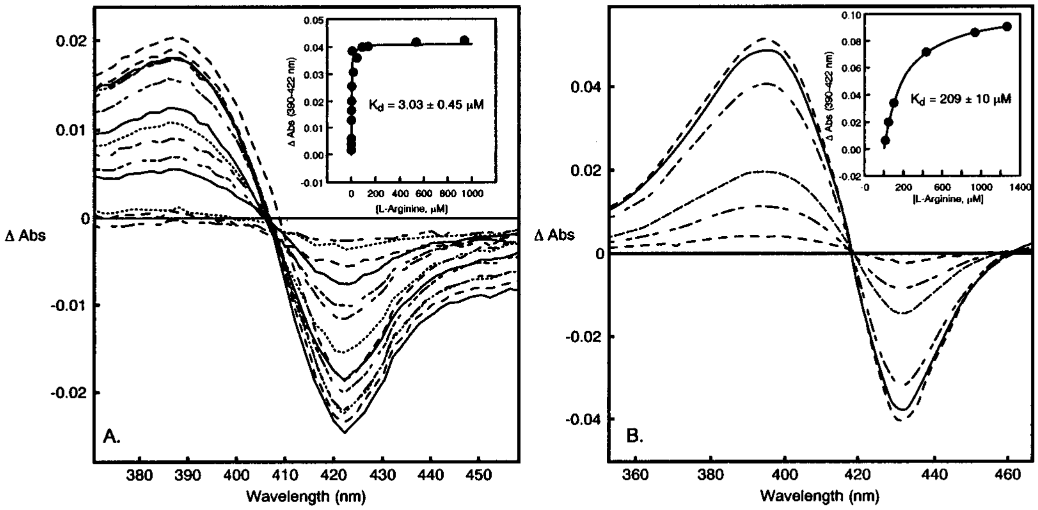

FIGURE 7: Spectral perturbation of the Soret upon binding of L-arginine to rH-iNOS. (A) L-Arginine caused a concentration-dependent optical absorbance change of the enzyme-bound heme. The difference spectra had characteristics of a type I spectral change with a peak at 390 nm and a trough at 422 nm. A plot of the magnitude of the absorbance change at 390 and 422 nm as a function of L-arginine concentration yielded a titration curve with a calculated K_d of 3.03 ± 0.45 μM (inset). (B) L-arginine also caused a type I spectral change of the enzyme-bound heme in the presence of 2.2 mM imidazole, but the apparent K_d for L-arginine under these conditions was 209 ± 10 μM (inset).

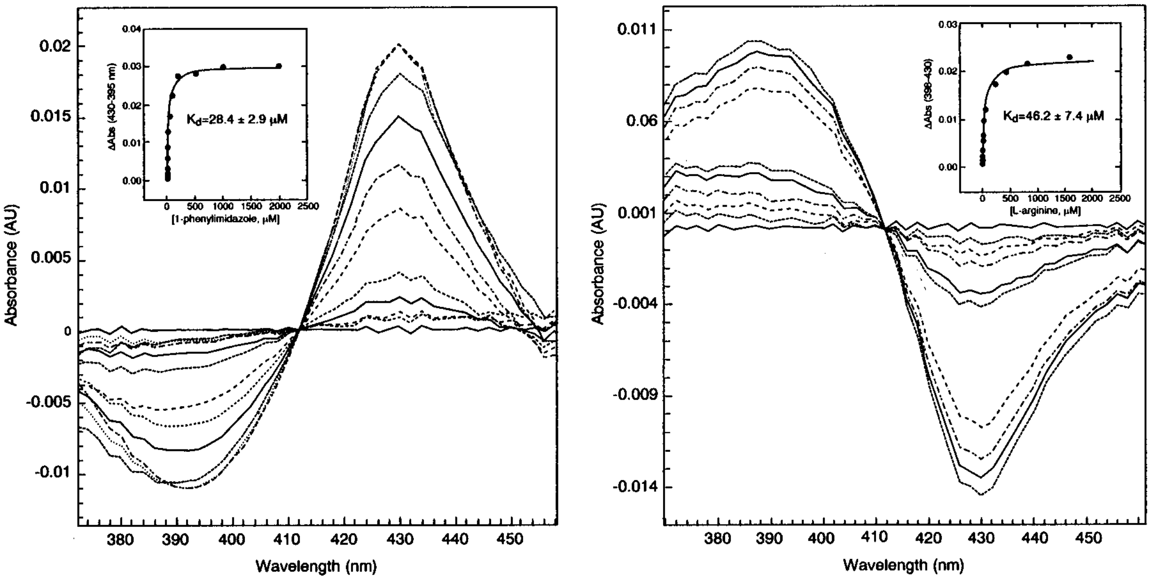

FIGURE 8: Spectral perturbation of the Soret upon binding of 1-phenylimidazole to rH-iNOS. (A) 1-phenylimidazole caused a type II spectral change with a calculated K_d of 28 ± 3 μM (inset). (B) In the presence of 1.6 mM 1-phenylimidazole, L-arginine caused a type I difference spectra with an apparent K_d of 46 ± 7 μM.

tration of L-arginine to the imidazole–enzyme complex also resulted in a type I spectral change (Figure 7B). However, the measurement of the magnitude of the type I spectral change as a function of L-arginine concentration gave a K_d value for L-arginine of 209 ± 10 μM (Figure 7B, inset). Thus the K_d for L-arginine was 70-fold higher in the presence of 2.2 mM imidazole compared to the value in the absence of imidazole.

The addition of 1-phenylimidazole to rH-iNOS also caused a type II difference spectrum (Figure 8A). The K_d for 1-phenylimidazole was 28 ± 3 μM at 4 μM BH_4 (Figure 8A, inset). This compares well with the steady-state K_i of 38 ± 4 μM. The titration of the 1-phenylimidazole-enzyme complex with L-arginine gave a type I difference spectrum. The apparent K_d for L-arginine is 46 ± 7 μM in the presence of 1.6 mM 1-phenylimidazole (Figure 8B). This value was ~15-fold higher than the value of 3 μM measured in the absence of inhibitor (Figure 7A). The perturbation of the K_d value of L-arginine by imidazole and 1-phenylimidazole is in accord with the competitive nature of these inhibitors observed from steady-state kinetics.

DISCUSSION

Imidazole is a classical inhibitor of cytochrome P_{450} enzymes. It exerts its inhibitory effect via the direct ligation to the heme iron at the dioxygen binding site (Lipscomb, 1980; Shiro et al., 1989; Banci et al., 1994). The rH-iNOS has been shown to possess a cytochrome P450-like heme in the active site as does the murine iNOS counterpart (White & Marletta, 1992). Studies reported here demonstrate imidazole is a competitive inhibitor of L-arginine having a K_i of 95 μM. Spectrophotometric binding studies demonstrate that imidazole binds to the enzyme causing a type II optical difference spectrum with a K_d of 63 μM, indicative of its interaction with the heme iron at the sixth axial ligand site (McMillan & Masters, 1993). Saturating concentration of imidazole (2.2 mM) also perturbs the binding affinity of L-arginine, increasing the K_d by about 70-fold, from 3 to 209 μM. These data are in accord with the steady-state kinetic results and indicate that both imidazole and L-arginine bind to a mutually exclusive site. The 2-fold more potent inhibitor, 1-phenylimidazole, is also a competitive inhibitor versus L-arginine based on the same type of analysis.

The competitive behavior of imidazole with respect to L-arginine suggests a link between the binding site of imidazole (dioxygen site) and L-arginine, but the two compounds perturb the active site heme differently. The binding of L-arginine to the enzyme induces a blue-shift in the Soret of the imidazole–enzyme complex from 422 to 396 nm, indicating a change in the coordination state of the heme ferric iron upon binding. The 430 nm species is a six-coordinate low spin heme with imidazole as the sixth axial ligand, and the 396 nm species is the five-coordinated high spin heme (McMillan & Masters, 1993). Upon binding of L-arginine to the imidazole–rH-iNOS binary complex, the ligation of imidazole to the ferric iron is disrupted as evident from the spectral shift. The observed competitive nature of imidazole with respect to L-arginine suggests that imidazole is subsequently displaced from the active site upon L-arginine binding. This change in coordination state of the heme as a result of substrate binding is similar to the binding of the substrate, camphor, to the low spin six-coordinate heme of P_{450}cam (Poulos et al., 1985, 1986). In this case a water molecule is the sixth axial ligand and is displaced upon substrate binding.

Imidazole has been reported to inhibit NOS from different species (Wolff et al., 1993; Wolff & Gribin, 1994a,b; Mayer et al., 1994). The rat neuronal cNOS has been extensively characterized by kinetic and spectrophotometric methods (Wolff et al., 1993; McMillan & Masters, 1993). For this enzyme, imidazole is a noncompetitive inhibitor versus L-arginine exhibiting a K_i of 200 μM. A noncompetitive pattern with a K_i of 48 μM has also been observed for the murine macrophage iNOS based on steady-state kinetic studies (Wolff & Gribin, 1994a). The noncompetitive patterns for these two enzymes suggest that imidazole is not displaced from the active site by the binding of L-arginine, although the optical spectroscopic data suggest a disruption of the sixth axial ligand (imidazole) leading to a five-coordinate high spin heme (McMillan & Masters, 1993). Thus a ternary complex between the enzyme, imidazole, and L-arginine exists. On the other hand, competitive patterns versus L-arginine have been observed for the bovine endothelial and porcine neuronal cNOSs with K_i values of 50 and 198 μM, respectively (Wolff & Gribin, 1994a; Wolff et al., 1994; Mayer et al., 1994). These observed variations in the kinetic parameters as well as in the type of inhibition exerted by imidazole strongly suggest that there are microheterogeneities at the heme hydroxylation site among these enzymes from different species. Furthermore, in the case of the rat brain cNOS and murine iNOS, there may exist interactions that prevent the displacement of imidazole from the active site, even though the ligation to the heme is disrupted upon binding of L-arginine. These interactions are absent in rH-iNOS as well as in the porcine neuronal and bovine endothelial cNOSs. These observed differences in the inhibition profile of imidazole derivatives between rH-iNOS and murine macrophage iNOS are noteworthy and have been observed for another inhibitor, aminoguanidine (Wolff & Lubbeskie, 1995; Garvey et al., 1994). Thus extrapolations of inhibition data derived from the murine macrophage iNOS to the human iNOS should be made with caution.

Imidazole is the only inhibitor of NOS in which the mode of binding has been firmly established, based on the wealth of kinetic and spectroscopic data. Thus, it is an attractive candidate for probing the active site heme domain of rH-iNOS. A combination of structure–activity relationship (SAR) studies and pH profiles of inhibition with imidazole and its derivatives suggest the structural and electronic requirements for optimal inhibition. The pH dependence of the inhibition of the enzyme indicates an ionizable group is deprotonated for maximal inhibition. However, in the case of 1-phenylimidazole, the K_i value was 2-fold lower than imidazole and exhibited no pH dependence. Differences in the mode of binding cannot account for this observation, since spectroscopic studies indicate the binding of 1-phenylimidazole to the enzyme is qualitatively indistinguishable from that of imidazole. Thus, one can rule out significant involvement of enzymic groups to the observed pK_a. The identity of this ionizable group is unknown at this point; however, an assignment of the observed pK_a to that of imidazole seems reasonable. This would also explain why enzyme inhibition with 1-phenylimidazole is pH independent since its pK_a is 5.8 (Elguero et al., 1968), 1.1 pH unit lower than imidazole (pK_a = 6.9). Thus at pH 6.5, the neutral form of 1-phenylimidazole predominates over the protonated form by 5 to 1; in contrast to imidazole where, at the same pH, the protonated form predominates over the unprotonated form by 2.5 to 1. If this is correct, then this pK_a effect emphasizes the importance of the protonation state of the N-3 nitrogen of imidazole in the inhibition of rH-iNOS. Consistent with this hypothesis is the observation that 1-(*p*-nitro)phenylimidazole is a weak inhibitor. This may be probably due to the electron withdrawing ability of the nitro group that decreases the electron density at N-3 resulting in the reduced ability to bind to the heme. Finally, alkyl substituents at the 2- or the 4-position probably interfere with iron binding due to steric reasons. Similar SAR results have been observed with imidazole and its derivatives in the inhibition of microsomal cytochrome P_{450} enzymes and provide additional evidence that the heme of rH-iNOS is cytochrome P_{450}-like (Rogerson et al., 1977).

The requirement for BH_4 for stability and maximal activity is a unique property of NOS and sets it apart from all P450 enzymes. Although the relationship between BH_4 and protein dimer integrity has been established for murine and human iNOS (Baek et al., 1993; Tzeng et al., 1995), the mechanistic link between this cofactor and NO formation relative to the P_{450} heme is unclear. For rH-iNOS, imidazole and 1-phenylimidazole inhibition can be affected by exogenous BH_4. Furthermore, both imidazole and 1-phenylimidazole behave as competitive inhibitors with respect to enzyme activation by exogenous BH_4. This competitive behavior of imidazole with both L-arginine and BH_4 has not been observed previously with any inducible NOS isoforms and suggests the BH_4 binding site is very close to the dioxygen site (Table 3). Exogenous BH_4 had no effect on the inhibition of the enzyme by *N*-nitro-L-arginine, indicating that the guanidino moiety does not overlap with the BH_4 site. A speculative model of the heme active site of rH-iNOS is shown in Figure 9. The close convergence of both

Table 3: Inhibition Parameters of Imidazoles for NOS Isoforms from Different Species

inhibitor	NOS isoform	inhibition pattern L-Arg	 BH_4	K_i (μM)
imidazole	bovine ncNOS	NC^{a}	NC	200^{c}
	murine iNOS	NC	NC	48^{d}
	bovine ecNOS	C^{b}	NC	50^{e}
	human iNOS	C	C	95^{f}
1-phenylimidazole	bovine ncNOS	NC	NC	38^{c}
	murine iNOS	NC	C	0.7^{d}
	bovine ecNOS	C	C	50^{e}
	human iNOS	C	C	38^{f}

[a] NC, noncompetitive. [b] C, competitive. [c] From Wolff et al. (1993). [d] From Wolff et al. (1994a). [e] From Wolff et al. (1994b). [f] This report.

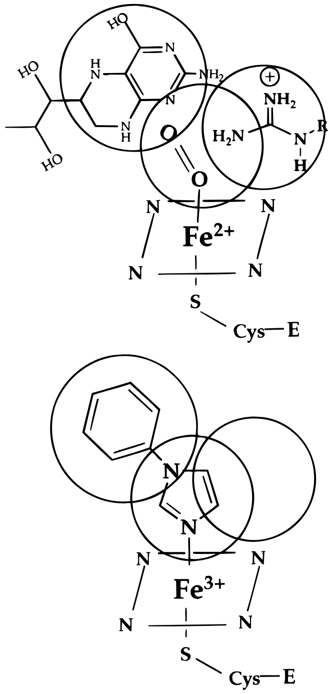

FIGURE 9: Proposed arrangement of the three binding sites in rH-iNOS.

the BH_4 and L-arginine sites into the dioxygen site of the heme is the notable feature and is consistent with the data presented here. We suggest that the variations in the inhibition parameters of imidazole observed with different NOS's are due to the difference in the spatial arrangement of the BH_4 and L-arginine sites relative to the P450 heme. The close proximity of the L-arginine binding site to the heme site makes mechanistic sense since hydroxylation would require the heme. However, the close proximity of the BH_4 site to the heme brings into question the catalytic role of BH_4 in the hydroxylation of L-arginine. Additional studies are needed to answer this question. The intimate arrangement of the three substrate sites in rH-iNOS may have implications in the design of multisubstrate site inhibitors.

An important issue in the development of an inhibitor for iNOS is isoform selectivity. 1-Phenylimidazole is 20-fold selective for rH-iNOS over rH-ecNOS. This selectivity, although modest, suggests isoform variations between the active sites of the human iNOS and ecNOS. It is tempting to speculate the existence of a specific interaction(s) between rH-iNOS and the phenyl ring that gives rise to the observed selectivity. The use of a photoaffinity probe such as 1-(4-azidophenyl)-imidazole may be useful in the identification of amino acid residues that interact with the phenyl ring. These experiments are in progress.

In summary, we have described the detailed kinetic and spectroscopic studies of human iNOS with respect to imidazole inhibition. These studies have provided initial insights into the relative spatial arrangements of the substrate binding sites relative to the P450-like heme of this enzyme. The wide spectrum of inhibition patterns observed for the imidazole class of inhibitors reflects the differences in the relative spatial arrangements of the L-arginine, dioxygen, and BH_4 binding sites among NOS isoforms from different species.

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