Expression and Immunoaffinity Purification of Human Inducible Nitric-oxide Synthase

INHIBITION STUDIES WITH 2-AMINO-5,6-DIHYDRO-4H-1,3-THIAZINE*

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Recombinant human inducible nitric-oxide synthase (rH-iNOS) was expressed in the baculovirus system and purified by a novel immunoaffinity column. rH-iNOS and its native counterpart from cytokine-stimulated primary hepatocytes exhibited similar molecular mass of 130 kDa on SDS-polyacrylamide gel electrophoresis, recognition by antipeptide antibodies, specific activities, and IC50 values for inhibitors. The active dimeric form exhibited a specific activity range of 114–260 nmol/min/mg at 37 °C and contained 1.15 μmol of calmodulin/monomer. The enzyme exhibited a Soret absorbance maximum at 446 nm, indicating a P450-type heme. Imidazole induced a type II difference spectrum, reversible by l-Arg. 2-Amino-5,6-dihydro-4H-1,3-thiazine (ADT) was competitive versus l-Arg (Ki = 22.6 ± 1.9 μM), reversed the type II difference spectrum induced by imidazole (Kd = 17.7 μM), altered the CO-ferrous absorbance of rH-iNOS. l-Arg did not perturb the CO-ferrous adduct directly, but it partially reversed the ADT-induced absorbance shift, indicating that both bind similarly to the protein but interact differently with the heme.

Nitric oxide (NO) is involved in the regulation of diverse biological functions (for reviews see Refs. 1–3). Three nitric-oxide synthases (NOS,1 EC 1.14.13.39), termed neuronal NOS (n-cNOS or NOS1), endothelial cell NOS (ec-cNOS or NOS3), and inducible NOS (iNOS or NOS2), are capable of catalyzing the production of NO, citrulline, and NADP+ from Arg, molecular oxygen, and NADPH. Each isoform consists of an amino-terminal heme domain that binds the cofactor tetrahydrobiopterin (BH4) and the substrate l-Arg, a consensus calmodulin (CaM)-binding domain, and a carboxyl-terminal reductase domain that binds NADPH as well as the flavins FAD and FMN (4–8). Spectral studies have demonstrated that NOS inhibitors can interact with the heme domain (7–9). For n-cNOS, the binding of CaM has been demonstrated to facilitate the transfer of electrons from the reductase to the heme domain (9, 10). Physical evidence suggests that the active form of the enzymes is a dimer (11, 12).

n-cNOS and ec-cNOS are constitutively expressed under normal conditions, and their activities are regulated by the binding of CaM at elevated intracellular Ca2+ concentrations (1–3). The expression of iNOS can be induced by inflammatory stimuli in a wide variety of cell types, including macrophages, hepatocytes, chondrocytes, and smooth muscle cells (13–16). Unlike the cNOS isoforms, the activity of iNOS is reported to be independent of elevations in intracellular Ca2+ concentrations (17). For murine iNOS, it has been demonstrated that CaM tightly binds the enzyme even at very low Ca2+ concentrations (18). Therefore, once expressed, iNOS is believed to generate sustained levels of NO in vivo. The sustained generation of NO by iNOS may contribute to the pathology of a number of inflammatory diseases, e.g. septic shock, inflammatory arthritis, type 1 diabetes, and inflammatory bowel disease (1, 3). Potent, isozyme-specific NOS inhibitors will aid in defining the role of iNOS in vivo and perhaps provide effective anti-inflammatory agents for use in human disease.

A number of NOS inhibitors have been characterized kinetically with respect to rodent iNOS (19–23). However, rodent and human iNOS differ in several respects. For example, unlike cNOS enzymes, which are 90–95% identical among mammalian species, the amino acid identity of murine and human iNOS is approximately 80% (14); human iNOS is significantly more sensitive to EGTA inhibition than murine iNOS (14, 24); and the inhibitor, aminoguanidine, has been reported to be

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1 The abbreviations used are: NOS, nitric-oxide synthase; iNOS, inducible NOS; rH-iNOS, recombinant human iNOS; n-cNOS, neuronal constitutive NOS; ec-cNOS, endothelial cell constitutive NOS; CaM, calmodulin; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BH4, tetrahydrobiopterin; PAG, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; RP-HPLC, reverse phase HPLC; PBS, phosphate-buffered saline; ADT, 2-amino-5,6-dihydro-4H-1,3-thiazine; MS, mass spectrometry; LC-ESI MS, liquid chromatography electrospray ionization; MALDI-TOF MS, matrix-assisted laser desorption ionization time of flight; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; RIA, radioimmuno assay.
20-fold more active against murine iNOS as compared with human iNOS (25). Since sources of native human iNOS are limited, the expression of a recombinant human iNOS that faithfully represents its native counterpart is essential for the characterization of inhibitors intended for clinical use.

To provide an abundant source of human iNOS, conditions were determined in the baculovirus system for expressing rH-iNOS with properties similar to its native counterpart. This system differs from other reports of rH-iNOS expression (26, 27) in that the enzyme is provided an endogenous source of the key cofactor BH4, and does not require exposure of the enzyme to exogenous inhibitors. Milligram quantities of highly stable rH-iNOS were purified by a novel immunoaffinity purification protocol that avoided the use of NADPH. The immunoaffinity-purified rH-iNOS was characterized and used to probe the mechanism of inhibition of a potent cyclic isothiourea.

EXPERIMENTAL PROCEDURES

Materials—The following reagents were obtained from Sigma: hemin chloride, dimethyl sulfoxide, [6-aminolevulinic acid-1, l-Arg, l-Val, NADPH, FAD, FMN, TES, Tris- HCl, diithiothreitol, glycerol, antipain, aprotinin, leupeptin, chymotrypsin, pepstatin A, phenylmethylsulfonyl fluoride, nitro blue tetrazolium, NAD, methionine, NADP, NADPH, l-arginine, selenomethionine, nitroblue tetrazolium tablets, and EGTA.

For rH-iNOS, an expression kit (Pharmingen, San Diego, CA) was used for the separation of the analytes using Buffer A (0.02 M sodium citrate, pH 2.2) and analyzed by HPLC. The procedure for the expression of recombinant human c-nNOS, the full-length cDNA was cloned into the EcoRI site of baculovirus and recombinant human n-cNOS protein was performed similarly to rH-iNOS.

HPLC-based Enzyme Assay—The generation of l-[3H]citrulline from l-[3H]Arg was assayed under the following conditions: 50 μM containing 20 μM TES buffer, pH 7.4, 10% glycerol, 100 μM bovine serum albumin, 2 μM diithiothreitol, 5 μM FAD, 5 μM FMN, 250 μM NADPH, 5 μM BH4, 1–25 μM cold l-Arg, 0.0003 μCi (1.0 μCi/2.8 × 10−3 mmol) [3H]Arg and enzyme (2–5). Reactions were incubated at either room temperature or 37°C; aliquots were transferred into 100 μl of stop solution (0.02 M sodium citrate, pH 2.2) and analyzed by HPLC. The HPLC separation has been described previously (33, 34). The procedure was performed with a PolyPrep RP-18 preparative exchange column (2.1 × 30 mm) from Applied Biosystems (Foster City, CA) was used for the separation of the analytes using Buffer A (0.02 M sodium citrate, pH 2.2, with 5% acetonitrile) and Buffer B (0.2 M sodium citrate, pH 3.0, with 5% acetonitrile) with a flow rate of 0.75 ml/min and under the following elution conditions: 0–1 min, 15% B; 1.1–2.5 min, 90% B; 2.6–4.5 min, 15% B. Injections were made every 4.5 min. The radioactivity detector was programmed to deliver a 1:3 (v/v) ratio of effluent to scintillation liquid.

Enzyme activity in crude recombinant enzyme preparations was determined after the removal of endogenous l-Arg by spin-filtration through Bio-Rad P-30 resin, preequilibrated in lysis buffer. In crude native human hepatocyte preparations, activity was determined in the presence of 60 μM Val to prevent interference due to endogenous arginase.

For Km determination, the l-Arg concentration was varied from 0.5 to 40 μM. The data were fit to a hyperbolic function using Sigma Plot (version 4.17, Jandel Scientific, Cotra Madera, CA), and values were reported with standard errors. For IC50 measurements, enzyme activity was monitored under the standard assay protocol at 1 or 5 μM l-Arg in the presence of varying concentrations of inhibitor.

Generation of Antipeptide Antibodies—Three peptides were synthesized with the following sequences: immungen peptide A (Cys-Arg-Val-Osn-Ser-Leu-Glu-Met-Ser-Ala-Leu), probe peptide B (Tyr-Arg-Ala-Ser-Leu-Glu-Met-Ser-Ala-Leu), and consensus peptide C (Arg-Cys-Asp-Arg-Val-Phe-His-Glu-Ala-Leu). Peptides contained the 7-residue carboxyl terminus of human iNOS plus a multipurpose tetrapeptide extension (Cys-Arg-Nle-Orn). The Cys was used to couple the peptide to thyroglobulin using the sulfo-N-hydroxysulfosuccinimide ester technique (35). The incorporation of Arg and Orn was intended to improve solubility, while norleucine was used to determine coupling efficiency by amino acid analysis.
antibody production using this peptide conjugate was undertaken in rabbit. Recombinant Diagnostic Activities (Berkertix, Schwalbach/Ts., Germany) described previously (36). Peptide B, which was used for the specific elution of the enzyme from the immunoaffinity column, also contained the 7-residue carboxyl terminus of human iNOS plus an amino-termini tripeptide, Tyr-Arg-Ala. Peptide C was selected from a region of high amino acid sequence identity (residues 695–705 in rat n-cNOS) among all NOS isoforms and was used as an immunogen to generate a common antibody. The acetylated peptide B was prepared by dissolving the peptide in 1.0 ml of a 70:30 mixture of MeOH/acetic anhydride (v/v), incubated at 25°C for 10 min, diluted with an equal volume of H2O and evaporated to dryness. Reconstitution in H2O and evaporation was repeated twice to remove excess acetic acid. The resulting powder was dissolved in 0.075 M phosphate-buffered saline (PBS)-azide, pH 7.0.

**Characterization of Recombinant Human iNOS**

The wash steps consisted sequentially of 8 column volumes of affinity column using a Hamilton syringe pump at a flow rate of 3 ml/min. The diluted sample was then pumped (1 ml/min) onto 0.5 × 10-cm glass columns, each packed with 1 ml of Tosohaas TSK-GE XLH-Sepharose 5FF ion exchange resin (Analytical Sales and Service, Macherey-Nagel & Co, Düren, Germany). Gradient elution was performed according to a previously described method (11) on a Tosohaas TSK-GEL GMPW column (60 cm × 7.5 mm inner diameter) in running buffer containing 0.2 mM NaCl at a flow rate of 0.4 ml/min. Column volumes were calculated according to Laemmli (37). Samples were visualized by electrophoretic fractionation by ADP-Sepharose affinity chromatography of rH-iNOS was also purified using conventional 2',5'-ADP-Sepharose affinity purification (39), using a 0.5 mM NaCl wash, and eluting with 5 mM NADPH in 20 mM TES, pH 7.4, 1 mM CHAPS, 10% glycerol, and 10 μM BH4.

**RP-HPLC Analysis—Samples for mass spectrometry, microsequencing, amino acid analyses, and stoichiometry determinations were chromatographed using an ABI 130A HPLC system with a 2.1 × 10-cm C18 reverse phase column of a 9:1 (v/v) mixture of 2.5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, each prepared at 10 mg/ml in deionized H2O, on the sample target surface prior to the addition of a 0.5-μl sample.**

**SDS-PAGE Analysis and Protein Concentration—** SDS-PAGE was performed according to Laemmli (37). Samples were visualized by either Coomassie Blue or silver staining or transferred to 0.45 μm nitrocellulose for immunoblot analysis. The nitrocellulose membranes were blocked with 3% casein and incubated with primary anti-peptide A antibody at a 1:20,000 dilution, followed by an alkaline phosphatase-conjugated anti-rabbit IgG-Fc, and visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Crude protein concentrations (1:400,000) were diluted with an equal volume of 0.075 M PBS with 0.02% NaN3, 0.1 mM BH4, 0.01M NaCl. The antipeptide A antiserum is specific for iNOS and does not detect recombinant human ec-cNOS or recombinant human n-cNOS on immunoblots (data not shown). 100 μl of the antiserum at a dilution of 1:10,000 was mixed with 100 μl of buffer, standard, or an unknown sample and incubated overnight at 4°C. A typical purification involved infusing 700–900 ml of the antiserum at a flow rate of 3 ml/min. The washed samples were then centrifuged at 18,000 rpm for 10 min. The supernatant was added to a 100-ml bed of ImmunoPure IgG Orientation Kit, Pierce). The peptide bound to the antiserum was then precipitated with 100 μl of matrix solution of a 9:1 (v/v) mixture of 2.5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid, each prepared at 10 mg/ml. All mass spectra were recorded using a Finnigan Mat Vision 2000 MALDI-TOF mass spectrometer (Finnigan Corp., San Jose, CA).

**Cofactor Analysis—** The amount of insect CaM was determined by first separating CaM from rH-iNOS by RP-HPLC. The isolated insect CaM peak was hydrolyzed for amino acid analysis. Values for the amino acids Asp, Asn, Glu, Ala, Val, Ile, and Leu were determined and corrected for the expected (i) bovine CaM, purified from bovine brain, 95.5% pure, and (ii) spiked norleucine from the amino acid hydrolysate (0.92). These amino acids were chosen after it was determined experimentally that their levels remained stable between 24 and 72 h of hydrolysis. Based on the known amino acid composition of Drosophila melanogaster CaM, the values for each of these amino acids were used to separate calculate the amount of CaM. The seven calculated values for CaM were then averaged, and the mean value was used to determine the stoichiometric ratio with rH-iNOS. The values for these amino acids were also subtracted from the corresponding amino acid values obtained from direct hydrolysis of the starting material. Using the known amino acid composition of rH-iNOS, each of these subtracted values was then used to separately calculate the amount of rH-iNOS monomer in the complex. The mean value was determined and used to calculate the stoichiometric ratio of rH-iNOS to CaM.

**Pterin Analysis—** An aliquot of the immunoaffinity-purified enzyme was prepared using a DEA ESI-MS analysis was performed using a Finnigan TSQ-700 LC-ESI mass spectrometer (San Jose, CA), as described previously (40). A titreric digest of rH-iNOS was separated on a C8 reverse phase column (2.1 × 100 mm) at a flow rate of 50 μl/min with 0.075% aqueous trifluoroacetic acid and a gradient of 2–80% acetonitrile over 40 min. The effluent was fed directly to the electrospray interface of a Finnigan MAT TSQ-700 quadrupole mass spectrometer (41). The effluent was fed directly to the electrospray interface of a Finnigan MAT TSQ-700 quadrupole mass spectrometer (41).

**SDS-PAGE Analysis and Protein Concentration—** SDS-PAGE was performed according to Laemmli (37). Samples were visualized by either Coomassie Blue or silver staining or transferred to 0.45 μm nitrocellulose membranes, which were blocked with 3% casein and incubated with primary anti-peptide A antibody at a 1:20,000 dilution, followed by an alkaline phosphatase-conjugated anti-rabbit IgG-Fc, and visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. "Characterization of Recombinant Human iNOS"
Optical Spectroscopy—All spectra were recorded using a HP8452A diode-array UV/visible spectrometer (Hewlett Packard, Palo Alto, CA) with a thermostatted, multiecell-transport cell holder attached to a circulating water bath. Sample spectra were blanked against 20 mM TES, pH 7.5, 4 µM BH4, and the absolute absorbance between 190 and 800 nm was recorded at 15 °C. The imidazole-ferric low spin spectrum of rH-iNOS was generated by the addition of 2 mM imidazole unless otherwise specified. Subsequently, 2 mM L-Arg was added to generate the high spin heme spectrum.

Titration of ADT (for structure, see Fig. 4) against the ferric form of rH-iNOS was accomplished by the stepwise addition of the appropriate stock solution via a syringe at 15°C. The absolute spectrum was 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. By plotting the changes in absorbance at the indicated wavelengths versus the concentration of substrate or inhibitor and fitting the data to Equation 1, the dissociation constant (Kd) can be calculated.

\[ y = (a \cdot x)(b + x) \quad \text{(Eq. 1)} \]

In Equation 1, \( y \) is the change in absorbance, \( x \) is the concentration of added inhibitor, \( a \) is the maximum change in absorbance at an infinite concentration of inhibitor, and \( b \) is the dissociation constant, \( K_d \).

The addition of ADT to the CO-ferrous adduct of rH-iNOS was the same as described above for the ferrocene form of the enzyme but in a septum-sealed cuvette under argon. All transfers were done via a gas-tight syringe. The ADT solution (1 mM) was rendered anaerobic by septum-sealed cuvette under argon. All transfers were done via a gas-

RESULTS

Expression of Active rH-iNOS—rH-iNOS was expressed in SF9 cells using a recombinant baculovirus system. Time course studies indicated that total iNOS activity in S-100 lysates, as measured by conversion of L-[^3H]Arg to L-[^3H]citrulline; rH-iNOS protein levels were measured in a competitive RIA. B and C, comparison of the relative specific activities of rH-iNOS during the time course of infection. In B, the ratio of enzyme activity to RIA units was used as a measure of specific activity in the S-100 lysates. No ratio was calculated for the earliest time point, since the RIA value was below the limit of detection. In C, the relative specific activity of rH-iNOS was estimated by immunoblot analysis using the peptide C antipeptide antibody. Samples of native murine iNOS, native human iNOS, or rH-iNOS, harvested at the times indicated and each containing equal amounts (1 pmol/min) of iNOS activity, were loaded.

FIG. 1. Expression and purification of rH-iNOS. A, time course of iNOS activity and protein expression. Enzyme activity was determined by the conversion of L-[^3H]Arg to L-[^3H]citrulline; rH-iNOS protein levels were measured in a competitive RIA. B and C, comparison of the relative specific activities of rH-iNOS during the time course of infection. In B, the ratio of enzyme activity to RIA units was used as a measure of specific activity in the S-100 lysates. No ratio was calculated for the earliest time point, since the RIA value was below the limit of detection. In C, the relative specific activity of rH-iNOS was estimated by immunoblot analysis using the peptide C antipeptide antibody. Samples of native murine iNOS, native human iNOS, or rH-iNOS, harvested at the times indicated and each containing equal amounts (1 pmol/min) of iNOS activity, were loaded.

Expression of Active rH-iNOS—rH-iNOS was expressed in SF9 cells using a recombinant baculovirus system. Time course studies indicated that total iNOS activity in S-100 lysates, as measured by conversion of L-[^3H]Arg to L-[^3H]citrulline, increased from 24 to 40 h postinfection and then declined (Fig. 1A). This activity was inhibited by NMA and was not detected with each inhibitor. As reported previously (25), aminoguanidine also inhibited rH-iNOS and native human iNOS much less potently than murine iNOS (IC50 10.4 ± 2.1 and 21.2 ± 1.3 µM), Nω-nitro-L-Arg (2.1 ± 0.1 and 1.8 ± 0.1 µM), and aminoguanidine (101.2 ± 4.8 and 86.5 ± 6.3 µM) were similar for partially purified rH-iNOS and native human iNOS (respectively), indicating that the active sites of both enzymes interacted similarly with each inhibitor. As reported previously (25), aminoguanidine also inhibited rH-iNOS and native human iNOS much less potently than murine iNOS (IC50 6.6 ± 1.0 µM). We tested the effect of EGTA on the recombinant and native enzymes at concentrations ranging from 0.03 to 2 mM. Both rH-iNOS and native human iNOS were inhibited up to 50–70% by EGTA, while native murine iNOS was inhibited <10% at 2 mM EGTA. With each enzyme preparation, inhibition by EGTA was completely reversed by the addition of excess calcium (data not shown).

Comparison of Recombinant and Native Human iNOS—The IC50 values for Nω-methyl-L-Arg (10.4 ± 2.1 and 21.2 ± 1.3 µM), Nω-nitro-L-Arg (2.1 ± 0.1 and 1.8 ± 0.1 µM), and aminoguanidine (101.2 ± 4.8 and 86.5 ± 6.3 µM) were similar for partially purified rH-iNOS and native human iNOS (respectively), indicating that the active sites of both enzymes interacted similarly with each inhibitor. As reported previously (25), aminoguanidine also inhibited rH-iNOS and native human iNOS much less potently than murine iNOS (IC50 6.6 ± 1.0 µM). We tested the effect of EGTA on the recombinant and native enzymes at concentrations ranging from 0.03 to 2 mM. Both rH-iNOS and native human iNOS were inhibited up to 50–70% by EGTA, while native murine iNOS was inhibited <10% at 2 mM EGTA. With each enzyme preparation, inhibition by EGTA was completely reversed by the addition of excess calcium (data not shown).

Purification of rH-iNOS by Immunoaffinity Chromatography—ADP-purified rH-iNOS preparations eluted and maintained in 5 mM NADPH were unstable, losing up to 50% of their activity following a 2-h incubation on ice. These preparations could be stabilized for up to 120 min at 37 °C by dilution into assay buffer containing only 250 µM NADPH or by removal of NADPH using sulfopropyl, DEAE, or gel filtration chromatography (data not shown). To isolate stable, concentrated rH-iNOS for spectroscopic studies without prior exposure to
NADPH, a novel immunoaffinity purification protocol was developed using a polyclonal antiserum generated against peptide A. The critical aspect of the column construction was that during the cross-linking process, the antigen-combining sites were protected from chemical modification by preincubation with acetylated peptide B. After removal of the protective peptide, the column specifically bound 2.7 nmol of peptide B/ml of resin, as quantified by RP-HPLC. In contrast, an unrelated peptide (residues 111–123 of the human interleukin-1β precursor) was not bound.

After passage of the filtered S-100 crude Sf9 lysate over the immunoaffinity column and extensive washing, the enzyme was eluted by incubation of the column with a 25 μM solution of peptide B (Fig. 2). Similar results were obtained with higher concentrations of the peptide (50–500 μM). As shown in Table I, three 90-min incubations resulted in the elution of 21% of the applied activity with an additional 4% recovered after an overnight incubation. Recoveries from other purification runs ranged from 16 to 32%. Coomassie Blue-stained SDS-PAGE analysis showed a 130-kDa band in all of the specifically eluted fractions (Fig. 2, inset).

The peptide was removed, and the enzyme was concentrated approximately 200-fold by DEAE chromatography and isocratic elution with 0.2 M NaCl. This step provided highly concentrated enzyme solutions (0.75–3 mg/ml), with 60–90% recovery of activity. Peptide B eluted with 0.5 M NaCl or 0.1 M sodium citrate, pH 3.0. Immunoaffinity-purified enzyme was stable for greater than 4 h at room temperature and greater than 18 h on ice. In contrast, 50% of the activity was lost following a 2-h incubation with 5 mM NADPH; the concentration used to elute the enzyme from ADP-Sepharose.

The specific activities of several immunoaffinity-purified rH-iNOS preparations were determined using two methods to measure protein concentration. By the method of Bradford, using bovine serum albumin as a standard, the specific activities ranged from 94 to 199 nmol/min/mg at 37 °C with an average of 131 ± 41 (S.D., n = 6). These values were in good agreement with those obtained by amino acid analysis, which ranged from 114 to 260 nmol/min/mg at 37 °C with an average of 164 ± 57 (S.D., n = 5). The Km determined for L-Arg was 2.30 ± 0.25 μM. In addition, two preparations of rH-iNOS with specific activities that differed by 3.3-fold gave indistinguishable IC50 values for N′-methyl-L-Arg: 2.24 ± 0.22 and 2.38 ± 0.17 μM, using 1 μM L-Arg as substrate. In summary, rH-iNOS prepared by the immunoaffinity purification protocol was suitable for direct use in all subsequent enzyme analyses.

**Physical Characterization of Purified rH-iNOS by Mass Spectrometry, Peptide Mapping, and Microsequencing—**rH-iNOS separated by RP-HPLC yielded three major peaks (Fig. 3A). Each peak was analyzed by MALDI-TOF MS. Peak 1 afforded abundant molecular ions at m/z 615.4, corresponding to the protomeric molecular mass of the monomeric form of CHAPS (data not shown). Peak 2 yielded singly (m/z 16,723) and doubly (m/z 8,362) protonated species (Fig. 3B) in excellent agreement with the predicted molecular mass for *D. melanogaster* CaM, 16,721.5 atomic mass units (42). Analysis of peak 3 showed a spectrum (Fig. 3C) with an abundance of singly charged molecular ions at m/z 131,673.4. This value is within 0.4% of the predicted mass of the primary sequence of human iNOS (131,160 atomic mass units) and is well within the experimental accuracy of the method for proteins with this mass range (0.1–0.5%). In addition, aliquots of peaks 2 and 3 were digested with trypsin, separated by RP-HPLC, analyzed by LC-ESI MS, and compared against a computer-generated tryptic digest of insect CaM or human iNOS. All of the predicted peptides for *D. melanogaster* CaM and 90% of the predicted peptides for iNOS were identified by this method (data not shown). The amino terminus of the protein could not be identified by LC-ESI MS or Edman sequencing, strongly suggesting that it is blocked.

**Cofactor Analysis of Purified rH-iNOS—**The stoichiometry of CaM versus rH-iNOS was calculated following RP-HPLC separation as described under “Experimental Procedures.” The stoichiometry was calculated to be 1.15 mol of CaM/mol of rH-iNOS monomer (range, 1.12–1.19 ± 0.04 (S.D., n = 4)). Following removal of excess BH4 by DEAE chromatography, trytin analysis revealed that the reisolated enzyme was essentially saturated, with 11.6 pmol of BH4 per 12.3 pmol of iNOS monomer using the Bradford assay to measure protein. Heme content of the enzyme was determined by two methods, by the porphyrin hemochromogen method and/or from the extinction coefficient of the CO-ferrous adduct of the enzyme. Both methods gave similar values. The values based on Bradford protein determinations ranged from 0.26 to 0.49 mol of heme/mol of rH-iNOS monomer, with an average of 0.34 (n = 5). The values,

![Fig. 2. Immunoaffinity purification of rH-iNOS. Enzyme activity from representative purification steps is expressed as total L-[3H]Arg to L-[3H]citrulline conversion/min. Inset, Coomassie Blue-stained SDS-PAGE of rH-iNOS during steps of the immunoaffinity purification. Lane A, molecular weight markers; lane B, crude S-100 lysate; lane C, column flow-through; lane D, column wash; lanes E and F, elutions 1 and 2; lane G, elution from overnight incubation. Molecular weight markers are myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), and ovalbumin (45,000).](image)

| Table I | Immunoaffinity purification of rH-iNOS | | | | |
| --- | --- | --- | --- | --- |
| Volume | Total protein | Specific activity at 37 °C | Yield | Purification |
| ml | mg | nmol citrulline/min/mg | % | -fold |
| Crude lysate | 900 | 13,575 | 0.13 | 100 | 1 |
| Immunocolumn | 150 | 5.3 | 68.6 | 21 | 528 |
| DEAE | 0.75 | 2.3 | 105.3 | 14 | 810 |

*a The data do not include an overnight elution, which yielded an additional 4% of enzyme activity.*
based on amino acid analysis as an alternative method of protein determination, were 0.28–0.64 mol of heme/mol of rH-iNOS monomer, with an average of 0.41 (n = 5).

Optical Spectral Characterization—Solutions at concentrations of 1 mg/ml or greater exhibited a yellowish color, consistent with the enzyme having heme and flavin cofactors. The rH-iNOS showed visible absorbance maxima at 278 and 396 nm, with an absorbance shoulder at 460 nm (data not shown). The CO spectrum upon reduction with dithionite yielded an absorbance maximum of 446 nm indicative of a cytochrome P450-type heme. Titration with up to 2 mM imidazole produced a type I spectral shift to 430 nm and a trough at 395 nm (data not shown), which was reversed by the addition of 2 mM L-Arg. Inhibition Studies with ADT—Cyclic isothioureas have been reported to be potent inhibitors of rH-iNOS (46, 47). However, mechanistic studies on this class of compounds have been limited due to insufficient quantities of pure human iNOS. Kinetic analysis demonstrated that ADT potently inhibited rH-iNOS with an IC$_{50}$ of 24.5 ± 4.5 nM. The inhibition was not time-dependent and was competitive versus L-Arg (Fig. 4). The calculated $K_i$ for ADT is 22.6 ± 1.9 nM. The IC$_{50}$ values for recombinant human n-iNOS and recombinant human ec-cNOS were 17.6 ± 2.0 and 78.4 ± 4.6 nM, respectively.

Optical difference spectrophotometry allows for the direct analysis of the interactions of ADT with the P$_{450}$-like heme of rH-iNOS (48). The heme of NOS has been shown to exist in two states: high spin and low spin. As isolated, rH-iNOS exists predominately in the high spin state. The addition of ADT to purified rH-iNOS resulted in a spectral perturbation of only the enzyme in the low spin state (Fig. 5, inset). The group of peaks in C is a series of ions that can be attributed to multiply protonated forms of $m/z$ 131,673.4 rH-iNOS, aufs, absorbance units at full scale.

**FIG. 3.** RP-HPLC and MALDI-TOF MS analysis of the immunopurified rH-iNOS. A, separation of CaM and rH-iNOS by RP-HPLC. B and C, MALDI-TOF MS analysis of CaM and rH-iNOS, respectively. The peaks in B with $m/z$ values of 8,362.0 and 18,723.0 represent the doubly and singly protonated forms of CaM, respectively. The group of peaks in C is a series of ions that can be attributed to multiply protonated forms of $m/z$ 131,673.4 rH-iNOS.

**FIG. 4.** ADT is a competitive inhibitor versus L-Arg. Dixon analysis of the steady kinetic parameter of L-Arg as a function of ADT concentration shows a typical competitive pattern. The substrate concentrations were 0.5 μM (circle), 1.5 μM (square), and 4.5 μM (triangle). The replot of the slopes versus L-Arg concentration yielded a $K_i$ of 22.6 nM.

**FIG. 5.** Optical spectroscopic titration of rH-iNOS with ADT. The addition of varying concentrations of ADT to the imidazole-induced low spin ferric form of the enzyme caused a spectral change of the Soret, characterized by an increase at 396 nm and a decrease at 430 nm in the difference spectrum. Inset, the plot of ADT concentration added against the magnitude of the spectral change between 395 and 430 nm. The calculated $K_d$ for ADT after correction for the presence of imidazole was 17.7 nM.
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We next examined the effects of ADT on the CO-ferrous adduct of the enzyme, to further investigate the interactions of ADT with the heme site. The addition of 30 μM ADT to the enzyme dramatically affected the CO-ferrous absorbance, by depleting the absorbance at 446 nm and increasing the absorbance between 395 and 430 nm (Fig. 6A). 1 μM L-Arg alone did not perturb the absorbance of the CO-ferrous adduct. However, L-Arg (1 mM) partially reversed the ADT-induced spectral change with recovery of the P405 absorbance (Fig. 6B).

DISCUSSION

The rH-iNOS described in these studies faithfully represents its native counterpart from stimulated human hepatocytes in each comparative analysis performed: (i) specific activity as estimated by immunoblot analysis (Fig. 1), (ii) each comparative analysis performed: (i) specific activity as its native counterpart from stimulated human hepatocytes in ADT with the heme site. The addition of 30 μM ADT; dotted line, CO-ferrous adduct 10 min after the addition of 30 μM ADT followed by the addition of L-Arg to a final concentration of 1 mM.

We previously identified CaM as a tightly bound component of the native iNOS from murine macrophages (18). The amount of CaM bound to rH-iNOS purified in the absence of exogenous calcium, as determined in four enzyme preparations, showed a stoichiometry of 1:1.5:1. This study confirms that rH-iNOS also binds CaM tightly, and it is the first to demonstrate stoichiometric binding of CaM to the inducible NOS isoform. These results demonstrate that rH-iNOS is suitable for performing detailed spectral and mechanistic studies of inhibitors directed against the native human enzyme.

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The availability of milligram quantities of purified rH-iNOS has allowed us to begin to determine the mechanism of inhibition of rH-iNOS by ADT, a representative of the cyclic isothiourea class of inhibitors, using kinetic and spectrophotometric methods. This class of compounds contains some of the most potent inhibitors of human iNOS described to date (46, 47). A closely related analog of ADT, the 6-methyl derivative, inhibits murine iNOS with an IC50 of 3.6 nM (46); however, detailed mechanistic studies with this or related compounds have not been reported for murine or human iNOS. Understanding how members of this class of inhibitors interact with rH-iNOS will provide crucial information for the design of potent and selective inhibitors. In the present study, detailed kinetic and spectrophotometric analyses indicate that ADT is a competitive inhibitor versus L-Arg. This suggests that ADT can occupy the L-Arg binding site, probably at the guanidinium recognition subsite. However, the binding of ADT is 150-fold stronger than that of L-Arg, despite the lack of an amino acid moiety. It is conceivable that additional interactions are present between the rH-iNOS and parts of the dihydrothiazine ring structure that give rise to the increase in binding energy (~3 kcal). The presence of a sulfur at the 2-position may allow for ligation to the ferric heme, but the optical spectrophotometric data do not support this view. ADT binding to the enzyme is characterized by a type I difference spectrum. Direct interactions of the sulfur with the heme would produce a type II or a modified type II difference spectrum, as observed for thioctirulin with rat brain cNOS (51), or a split Soret absorbance as observed for isobornylercapton with cytochrome P450-cam (51).

ADT also perturbs the spectral absorbance of the CO-ferrous adduct of rH-iNOS, whereas L-Arg does not. CO is known to bind at the sixth axial ligand site of the ferrous heme, similar to that of imidazole binding to the ferric heme (4, 5, 52). This is also the site of dioxygen binding and presumably the site of the first hydroxylation of L-Arg to form N-hydroxyarginine (53). The loss of the P405 absorbance indicates that CO may be displaced from the heme. It is conceivable that the binding of ADT to the subsite occupied by the guanidinium of L-Arg places it significantly close to the dioxygen site, such that it can displace CO from the sixth axial ligand site. This interpretation is consistent with the capacity of L-Arg to partially reverse the spectral changes induced by ADT, presumably by allowing CO to rebind to the enzyme. Although speculative, the effect of ADT on the CO-ferrous enzyme may indicate that the additional binding energies observed for ADT may be from its interactions with the dioxygen binding site. Further studies are necessary to substantiate this hypothesis.

In summary, we have presented a novel procedure to generate and purify milligram quantities of stable rH-iNOS that is structurally and catalytically similar to the native enzyme. Using this recombinant enzyme, we performed detailed mechanistic studies with a member of the cyclic isothiourea class of inhibitors, ADT. Kinetic and optical spectroscopic analyses of rH-iNOS with ADT have provided additional insights into the interaction of this class of inhibitors with the active site of the enzyme, demonstrating the utility of rH-iNOS expressed and purified by this methodology to characterize the interaction of inhibitors against this potentially key therapeutic target.

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