NADH Oxidase Activity of Indoleamine 2,3-Dioxygenase*

Received for publication, May 17, 2011, and in revised form, June 10, 2011. Published, JBC Papers in Press, June 20, 2011, DOI 10.1074/jbc.M111.262139

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The heme enzyme indoleamine 2,3-dioxygenase (IDO) was found to oxidize NADH under aerobic conditions in the absence of other enzymes or reactants. This reaction led to the formation of the dioxygen adduct of IDO and supported the oxidation of Trp to N-formylkynurenine. Formation of the dioxygen adduct and oxidation of Trp were accelerated by the addition of small amounts of hydrogen peroxide, and both processes were inhibited in the presence of either superoxide dismutase or catalase. Anaerobic reaction of IDO with NADH proceeded only in the presence of a mediator (e.g. methylene blue) and resulted in formation of the ferrous form of the enzyme. We propose that trace amounts of peroxide previously proposed to occur in NADH solutions as well as solid NADH activate IDO and lead to aerobic formation of superoxide and the reactive dioxygen adduct of the enzyme.

Indoleamine 2,3-dioxygenase (IDO; EC 1.13.11.52) is a monomeric heme enzyme (molecular weight, 45,608 (human)) that catalyzes the first and rate-limiting step of tryptophan degradation in non-hepatic mammalian tissues by inserting both atoms of dioxygen into the substrate tryptophan to produce N-formylkynurenine (N-FK) (1). In recent years, IDO has been implicated as a significant participant in a variety of normal and pathological processes that include prevention of allogeneic fetal rejection (2), antimicrobial defense mechanisms (3), and the escape of immune response by tumors (4). This increasing recognition of numerous biological roles for this enzyme has led to its identification as a target for development of therapeutic agents.

Initial chemical and enzymatic studies of IDO provided considerable insight into substrate specificity of the enzyme, reactivity of the enzyme with dioxygen, and spectroscopic properties of the enzyme. Subsequently, Suzuki and co-workers (5–8) noted that IDO is evolutionarily related to myoglobin and that some gastropod mollusks possess a myoglobin with a molecular weight comparable with that of IDO. Determination of three-dimensional structures of two inhibited forms of the enzyme by Sugimoto et al. (9) in 2006 enabled the development of structure- or computationally based mechanistic proposals for catalysis by IDO, but so far crystallographic characterization of an IDO-tryptophan complex has not been achieved. Notably, none of the mechanisms yet proposed for IDO catalytic turnover require an exogenous source of electrons as is the case forcytochromes P450 (10, 11).

Nevertheless, an electron source is required to maintain IDO in the active, ferrous form to overcome autoxidation to the ferric derivative that occurs during turnover (12–14). We (15) and others (16) have proposed that IDO is maintained in the active, reduced state in vivo by the action of cytochrome b5 and cytochrome b5 reductase as reported for hemoglobin (17, 18) and possibly myoglobin (19). Cytochrome P450 reductase and cytochrome b5 have also been used to develop assays for IDO activity (20), but evidence that cytochrome P450 reductase performs this function in vivo is currently lacking.

In the course of studying the catalytic activity of IDO in the presence of cytochrome b5 and cytochrome b5 reductase in vitro, we performed control experiments with components of this reaction mixture and found the previously unreported observation that addition of NADH to IDOFe3+ in the absence of any other reactant leads to formation of a form of the enzyme with an electronic absorption spectrum that is identical to that of the dioxygen complex (12, 13) (IDOFe2+-O2) that has been reported to be stabilized to autoxidation only at subfreezing temperature (14). The current report concerns the characterization of the reaction of human IDO and NADH and some implications of this reaction for improving our understanding of the chemical properties of IDO.

EXPERIMENTAL PROCEDURES

Proteins and Reagents—DNase I (D5025), hen egg white lysozyme (L6876), bovine blood superoxide dismutase (S8160), bovine liver catalase (C-100), α-NADH (N6879), β-NADH (N1161), β-NAD+ (N8410). 30% hydrogen peroxide solution (H1009), l- and d-tryptophan (T0254 and T9753, respectively), phenazine methosulfate (P13401), Cibacron blue 3GA agarose 3000-CL (C1535), phenylmethylsulfonyl fluoride (PMSF; P7626), and all analytical grade buffer salts (Trizma (Tris base), BisTris, MOPS, and MES) were from Sigma–Aldrich, and all except catalase were used without purification. The preservative thymol and other impurities were removed from catalase by gel filtration chromatography in 20 mm sodium phosphate buffer, pH 7.5 with a prepacked Superdex 200 column (60 cm) operated with a GE Healthcare Äkta purifier. Imidazole (03196) from Fisher Scientific was recrystallized from tolune after treatment with activated charcoal to remove impurities that absorb at 280 nm. δ-Aminolevulinic acid was from Chem–Impex International Inc. (01433; Wood Dale, IL), kanamycin sul-

* This work was supported in part by a grant from the Canadian Cancer Society.

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3 The abbreviations used are: IDO, indoleamine 2,3-dioxygenase; BisTris, bis(2-hydroxyethylamino)tris(hydroxymethyl)methane; TEV, tobacco etch virus; N-FK, N-formylkynurenine; NMNH, nicotinamide mononucleotide hydrate; SOD, superoxide dismutase.
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fate was from BioBasic Inc. (KB0286; Markham, Ontario, Canada), and isopropyl β-D-thiogalactopyranoside (TR-600) was from Molecula Inc. (Columbia, MD). Crotalus adamanteus phosphodiesterase I (202404) from US Biochemical Corp. was used without further purification. A plasmid for expression of a polyHis-tagged TEV protease (21) was kindly provided by Dr. David S. Waugh, and the enzyme was prepared as suggested.4 Where appropriate, reagent concentrations were determined spectrophotometrically on the basis of published molar absorptivities: β-NADH and nicotinamide mononucleotide hydride (NMNH), ε340 = 6,300 M⁻¹ cm⁻¹ (22, 23); catalase, ε405 = 120,000 M⁻¹ cm⁻¹ per monomer (24); tryptophan, ε290 = 5,470 M⁻¹ cm⁻¹ (25); H₂O₂, ε240 = 39.4 M⁻¹ cm⁻¹ (26); and N-formylkynurenine, ε321 = 3,750 M⁻¹ cm⁻¹ (27). SOD was quantified on the basis of the activity stated by the supplier for the specific lot number.

**IDO Expression**—A pET28 vector containing a gene encoding human IDO amplified from a permanent human cell line (28, 29) was modified to encode a TEV protease recognition sequence between the thrombin cleavage sequence and the IDO initiation codon. In addition, the codon for Cys-308 was mutated to encode a Ser residue to eliminate formation of the disulfide bond apparent in the crystal structure of the protein (9). The protein expressed from the resulting plasmid (pETevIDO) is regarded here as wild-type IDO despite the C308S substitution and the three amino acid residues (Gly-Glu-Gly) that remain at the N terminus of the enzyme after hydrolysis with TEV protease.

Cultures of *Escherichia coli* BL21(DE3) Rosetta::pETevIDO in LB broth (5.5 liters; 50 mg of kanamycin/liter) were grown in an Applikon fermentor (7 liters) at 37 °C until the culture reached an absorbance (600 nm) of 0.8–1. The culture was then lowered to 25 or 30 °C, and δ-aminolevulinic acid was added to a final concentration of 0.5 mM. Isopropyl β-D-thiogalactopyranoside was added to a concentration of 0.25 mM 30 min later to induce IDO expression. The culture was then grown overnight with limited O₂ flow (10% of the highest PO₂ achieved before inoculation and with maximum stirring) before harvesting the cells by centrifugation.

**IDO Purification**—Pelleted cells were resuspended in a minimum volume of 20 mM sodium phosphate buffer, pH 7.5, 0.5 M NaCl, and PMSF in ethanol was added to a concentration of 1 mM. This buffer (without PMSF) was used in all subsequent purification procedures unless specified and is referred to simply as phosphate buffer. DNase I and lysozyme (0.05 and 1 mg/g of cell paste, respectively) were added to the cell suspension, and the mixture was incubated on ice (1–2 h). Cell lysis was achieved by three cycles of freezing the cell suspension in liquid nitrogen and thawing. Alternatively, the cell suspension without lysozyme was diluted further for lysis with an EmulsiFlex-C5 high pressure homogenizer (Avestin, Inc., Ottawa, Ontario, Canada) operated at 10 °C. Following either procedure, cell debris were removed by centrifugation (45 min at 15,000 rpm; Sorvall SS34 rotor).

The supernatant fluid was collected, and a concentrated solution of imidazole prepared in phosphate buffer was added to a final concentration of 10 mM. The resulting solution was applied to a HiTrap Chelating HP column (5 ml; GE Healthcare catalog number 17-0409-03) equilibrated in phosphate buffer with imidazole (10 mM). The column was washed with 10 column volumes of phosphate buffer containing imidazole (40 mM) before eluting IDO with an imidazole gradient (40–175 mM in 100 ml) in phosphate buffer. IDO eluted at ~150 mM imidazole. Fractions with A₄₁₁/A₂₈₀ > 1.7 were pooled, concentrated, and exchanged into Tris–HCl buffer (50 mM Tris, pH 8.0, 4 mM EDTA, 100 mM NaCl) by repeated centrifugal ultrafiltration (30,000 molecular weight cutoff; Millipore). PolyHis-tagged TEV protease was added to the resulting IDO solution (0.2 mg TEV/ml of reaction mixture; 5-ml reaction volume), and the mixture was incubated overnight at ambient temperature. The reaction mixture was exchanged into sodium phosphate buffer lacking imidazole for re-elution over the immobilized metal affinity chromatography column described above with the same conditions except for the omission of imidazole for loading or washing and the use of a shallower imidazole gradient (0–22 mM in 56 ml). Most of the IDO eluted as a single peak at ~15 mM imidazole. Fractions with A₄₁₁/A₂₈₀ > 1.9 were pooled, concentrated, and exchanged into Tris–HCl buffer. The resulting IDO solution was diluted (4:1) with 80% glycerol prior to freezing (5 mg of IDO/sample) with liquid nitrogen for storage at ~86 °C.

Some IDO purified in this manner and exchanged into 10 mM Tris buffer, pH 7.5 was purified further by chromatography with a column (5 ml) of Cibacron blue 3GA agarose 3000-CL resin equilibrated in the same buffer. The enzyme was eluted with a sodium chloride gradient (20 mM NaCl/ml) or eluted isocratically with buffer containing either β-NADH or L-Trp (5 mM). The protein in the fractions constituting the principal peak was pooled, concentrated, and exchanged into appropriate buffer for storage or for experimentation. In all cases, IDO concentrations were determined from molar absorbility of the ferric form (ε₄₀₄.₅ = 172,000 M⁻¹ cm⁻¹ (30)).

**NMNH Preparation**—Reduced nicotinamide mononucleotide was prepared from β-NADH (2–10 mg) by hydrolysis with snake venom phosphodiesterase I (1–3 units) in 100–500 µl of 100 mM Tris buffer, pH 8.9 containing 100 mM NaCl and 14 mM MgCl₂ (31). This reaction mixture was incubated (2–3 h) on ice at room temperature in the dark. Reaction progress was monitored by the decrease in fluorescence resonance energy transfer (λex = 260 nm and λem = 460 nm, 5-nm slit bandwidths (32, 33)) with a Varian Cary Eclipse spectrofluorimeter.

**Conversion of IDOFe³⁺ to IDOFe²⁺**—The conversion of IDOFe³⁺ to IDOFe²⁺ upon addition of NADH and/or other reagents was monitored spectrophotometrically at 25 °C with Cary Model 3E, 4000, and 6000 spectrophotometers. Typically, reactions were initiated by addition of each reactant solution (10–20 µl) to IDOFe³⁺ (700 µl; 1–5 µM protein in the desired buffer(s)) in masked 1-ml quartz cuvettes. The solutions were mixed manually before acquiring spectra at regular time intervals or monitoring absorbance as a function of time at selected wavelengths. To monitor reactions under anaerobic conditions, IDOFe³⁺ solutions were prepared in a nitrogen-filled glove box (Vacuum Atmospheres; O₂ content <1 ppm;
Belle Technology oxygen meter Model O2M-1) and transferred into Thunberg cuvettes to permit mixing with reactants immediately prior to initiation of data acquisition with a spectrometer outside of the glove box.

**NADH-supported Oxidation of l-Trp by IDO**—Solutions of IDO and NADH (1 and 200 μM, respectively, in 20 mM MOPS buffer, pH 7.0) were prepared and incubated at 20 °C until the spectrum of the sample was fully converted to that of IDOFe2+−O2 before mixing with an equal volume of l-Trp solution (15–2000 μM in the same buffer) with an Olis RSM 1000 stopped-flow spectrophotometer (2-cm flow cell). Absorption spectra were collected at 1 kHz, and kinetic traces at selected wavelengths were extracted for analysis. Formation of N-FK was monitored continuously at 321 nm (34), and the reported rates were corrected for absorbance changes resulting from NADH oxidation and dilution as appropriate.

**Simulated Docking of NADH to IDO**—To evaluate the hypothetical feasibility of NADH binding to IDO docking simulations were undertaken. To begin, solvent water molecules were deleted from the coordinate set, Protein Data Bank code 2D0T (9), and the disordered region of the structure (residues 361–379) was reconstructed with Modeller 9v8 (35–38). The resulting structure was subjected to molecular dynamics simulations with GROMACS 4.0.5 (39–42)). The IDO model produced by these simulations was resolovated in a periodic cubic box and neutralized with Na+ counter-ions. After energy minimization using the steepest descent method, the system was subjected to 50 ps of isothermal (300 K) equilibration using the v-rescale thermostat and the particle mesh Ewald method (43) for electrostatic calculations. All residue positions were restrained except for those that were missing. Cutoff distances of electrostatic and van der Waals interactions were set to 0.9 and 1.4 nm, respectively. The energy-minimized structure was subsequently used in docking simulations with AutoDock 4.2 (44–47) following removal of exogenous ligands. NADH and Cibacron coordinates were obtained from the Hetero-compound Information Centre—Uppsala (HIC-Up) (48), and NMNH coordinates were generated using NADH as a template. All torsions were enabled in NADH, NMNH, and Cibacron blue but IDO was treated as a rigid body. Initially, a docking space encompassing the entire protein was implemented to simulate blind docking. The goal for this approach was to identify favorable regions of interactions rather than a single lowest energy solution. Subsequently, these favorable regions were used to focus on potential docking conformations solved with smaller docking volumes of ~60 nm³ and more exhaustive search parameters. Lamarckian genetic searches (100) were performed for each site with the minimum number of energy evaluations set to 25,000,000. Selected high scoring conformations identified by AutoDock 4.2 were refined further with the energy minimization procedure described above.

**RESULTS**

**IDO Purification**—Approximately 6 g of wet cell paste and 10 mg of purified IDO were obtained per liter of cell culture. The electronic absorption spectrum of the ferric protein after enzymatic removal of the polyHis tag and second passage through the immobilized metal affinity chromatography column exhibited maxima at 404.5, 505, and 635 nm (20 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl), consistent with the spectrum of a six-coordinate, high spin heme protein with water occupying the sixth axial coordination site as reported previously for rabbit IDO (49, 50). The purified enzyme exhibited molecular masses (Q-Star-XL Quadrupole TOF) of 48,760.3 and 45,643.9 atomic mass units before and after treatment with TEV protease, respectively, in excellent agreement with the expected masses (48,759.9 (without the N-terminal Met residue) and 45,643.6 atomic mass units). The X-band EPR spectrum (at 10 K; not shown) confirms the predominance of a high spin component (g = 5.9, 5.5, and 2.0) together with at least two less abundant low spin species (g = 2.89, 2.28, and 1.63 and g = 3.07 and 2.19). The A_{404.5}/A_{280} ratio (ambient temperature) was typically ~2, but it increased to ~2.15 following elution of the enzyme over a column of Cibacron blue 3GA agarose 3000-CL (Fig. 1). This column removes protein that appears to be aggregated based on the distinctive light scattering curve underlying the absorption spectra of early eluting fractions, and it removes IDO in late eluting fractions with an A_{404.5}/A_{280} ratio < 1. Therefore, this chromatographic treatment can be applied to recover protein of high spectroscopic quality from samples that may have deteriorated partially through prolonged storage or other treatments.

The spectrum of IDO reduced with sodium dithionite, IDOFe2+−, exhibited Soret and a/β bands at 429.5 and 559.0 nm, respectively, following removal of the reducing agent with a Sephadex G25 chromatography column in the glove box. Exposure of samples prepared in this manner to air resulted in the immediate and almost complete formation of IDOFe2+−O2 with maxima at 412.0, 541.5, and 577.0 nm. However, the observation of a weak absorbance band at ~640 nm indicates that some of the protein underwent rapid autoxidation in the process. Evidence of further protein oxidation was obtained from the decreasing absorbance at 577 nm that decayed biexponentially.
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with rate constants of 0.35 and 0.079 min⁻¹ to account for 77 and 23%, respectively, of the autoxidation of IDOFe²⁺-O₂.

Reaction of Ido with NADH—Aerobic reaction of freshly dissolved β-NADH with dilute solutions of IDOFe³⁺ resulted in increasing absorbance maxima at 577 and 544 nm that are characteristic of the α and β transitions in the spectrum of IDOFe²⁺-O₂ (14) (Fig. 2A). Well defined isosbestic points at 468.8, 526.5, 595.1, and ~410 nm argue against formation of a detectable amount of any third form of the enzyme. The rate of the reaction and the stability of the product were highly dependent on the concentration and history of the NADH solution (see below). Specifically, the initial rate and the extent of IDOFe²⁺-O₂ formation varied directly with NADH concentration (Fig. 2B). At lower [NADH], a relatively small fraction of the enzyme was maintained in the oxygenated state, presumably reflecting a balance between autoxidation (t₁/₂ ~36 s (51) to ~25 min (12)) and the conversion of IDOFe³⁺ to IDOFe²⁺-O₂ (or IDOFe³⁺-O₂) as the result of reaction with NADH. Probable by-products of autoxidation include O₂⁻ (12) and, through dismutation of this reactive oxygen species, H₂O₂ (52, 53), which can have deleterious effects on the protein (12, 54). At [NADH] >750 μM, near-quantitative conversion to IDOFe²⁺-O₂ could be achieved within minutes, but autoxidation of the product began more rapidly, and it continued at an accelerated rate. Following prolonged reaction, the intensities of the α and β bands diminished without restoration of the spectrum of the ferric enzyme. Instead, increased light scattering was observed that we attribute to protein damage incurred through continued exposure of Ido to the reactive oxygen by-products. At present, other reactions involving the reactive oxygen species and NADH cannot be ruled out. We note, however, that O₂⁻ has been reported not to react with NADH (10, 11, 55).

Role of Dioxygen—Anaerobic addition of NADH to IDOFe³⁺ resulted in little or no spectroscopic change even after prolonged incubation. Anaerobic saturation of the solution with carbon monoxide also failed to support reaction between NADH and IDOFe³⁺. However, addition of catalytic amounts of either phenazine methosulfate or methylene blue to a solution containing NADH and IDOFe³⁺ in the absence of dioxygen promoted formation of the deoxygenated, ferrous form of the enzyme, IDOFe²⁺ (Fig. 3A). With methylene blue, for example, the mediator was reduced to leucomethylene blue as can be seen from the disappearance of the absorbance maxima in the range of 580–700 nm. At the same time, the Soret and visible bands of Ido shifted to wavelengths corresponding to the ferrous protein at 429 and 559 nm, respectively. Once Ido was reduced, exposure to air led to rapid formation of the dioxygen adduct, accelerated consumption of NADH, and autoxidation of the protein to one or more ferric derivatives.

Effect of Scavengers—SOD inhibited conversion of IDOFe³⁺ to IDOFe²⁺-O₂ by NADH (Fig. 3, B and C). For example, 2.6 units of SOD activity/ml (~50 nm) in a reaction mixture containing Ido (3.7 μM) and β-NADH (500 μM) inhibited IDOFe²⁺-O₂ formation by 65% relative to the amount formed in the absence of SOD after 90 min (Fig. 3C), and IDOFe²⁺-O₂ formation was diminished by 78% in the presence of 26 units/ml SOD (Fig. 3C, inset). Interestingly, the IDOFe²⁺-O₂ formed when SOD was present reverted rapidly to the ferric state (exponential absorbance decay at 577 nm compared with the nearly linear decay observed without SOD (Fig. 2B)), and the autoxidation product under these conditions exhibited the spectrum of IDOFe³⁺ rather than the light scattering reported above. Presumably, dismutation of O₂⁻ that was produced interfered with IDOFe²⁺-O₂ formation, but the amount of H₂O₂ produced by this reaction was not sufficient to damage the enzyme within the 90-min monitoring period.

Catalytic amounts of catalase also decreased the rate and extent of IDOFe²⁺-O₂ formation (Fig. 4). Addition of increasing concentrations of catalase to Ido prior to addition of NADH resulted in decreasing efficiency of IDOFe²⁺-O₂ formation. At sufficiently high [catalase], IDOFe²⁺-O₂ formation was inhibited completely except for an initial and reversible reaction burst (Fig. 4A). Incubation of NADH with catalase prior to their addition to Ido also diminished conversion of IDOFe³⁺
to IDOFe\(^{2+}\)–O\(_2\), and the extent to which IDOFe\(^{2+}\)–O\(_2\) diminishes increased with the length of the incubation time (Fig. 4B). As observed above for SOD, any IDOFe\(^{2+}\)–O\(_2\) formed in the presence of catalase in time reverted to IDOFe\(^{3+}\) (not shown). In contrast, addition of small amounts of H\(_2\)O\(_2\) to IDOFe\(^{3+}\) solutions that had reached a low but stable level of IDOFe\(^{2+}\)–O\(_2\) formation by reaction with a low concentration of NADH resulted in a prompt increase in the rate of IDOFe\(^{2+}\)–O\(_2\) formation (Fig. 4C). This enhancement in IDOFe\(^{2+}\)–O\(_2\) formation increased with [H\(_2\)O\(_2\)], but the protein became more susceptible to autoxidation and to the protein damage that follows.

**pH Dependence**—The pH dependence of NADH-induced IDOFe\(^{2+}\)–O\(_2\) formation exhibited features that are consistent with the involvement of H\(_2\)O\(_2\) (Fig. 5A). Both the initial rate of reaction and the peak oxygenation level increased with pH to a maximum value at pH \~7.8 (Fig. 5B and C, respectively). In contrast, the rate of IDOFe\(^{2+}\)–O\(_2\) oxidation decreased with increasing pH (Fig. 5D) presumably because free O\(_2\) did not dismutate to H\(_2\)O\(_2\) and O\(_2\) as efficiently as it does at lower pH.

**FIGURE 3.** Role of dioxygen in reaction of indoleamine 2,3-dioxygenase with NADH. A, anaerobic reduction of IDOFe\(^{3+}\) (6 \(\mu\)M) with \(\beta\)-NADH (500 \(\mu\)M) mediated by methylene blue (4 \(\mu\)M) in 20 mM BisTris buffer, pH 7.0, 25 °C. The decrease in absorbance at 340 nm stems from dilution of the protein/NADH mixture by addition of methylene blue. The absorbance observed between 550 and 700 nm in the initial spectra corresponds to methylene blue. Comparison of the aerobic reaction of 3.7 \(\mu\)M IDO with 500 \(\mu\)M \(\beta\)-NADH in the absence (B) and presence (C) of 2.6 units of SOD activity/ml. The insets illustrate the time-dependent changes in absorbance at 503 and 577 nm. The inset in C exhibits two 577 nm profiles that correspond to reactions carried out with 2.6 (●) and 26 (×) units of SOD/ml of reaction.

**FIGURE 4.** Influence of H\(_2\)O\(_2\) on the aerobic reaction of \(\beta\)-NADH with indoleamine 2,3-dioxygenase.

Indoleamine 2,3-dioxygenase (4 \(\mu\)M protein in 500 \(\mu\)l of 20 mM BisTris buffer, pH 7.0) conversion to IDOFe\(^{2+}\)–O\(_2\) monitored at 577 nm following addition of bovine liver catalase to the final concentrations as shown followed by addition of freshly dissolved \(\beta\)-NADH to a final concentration of 800 \(\mu\)M (A) or treatment of a solution of \(\beta\)-NADH (incubated in 20 mM BisTris buffer, pH 7.0 in the dark and on ice for 4 days) with bovine liver catalase (500 \(pm\)) for the times indicated and prior to addition of the mixture to the IDO solution (final [NADH] = 205 \(\mu\)M) (B) is shown. C, enhancement of IDOFe\(^{2+}\)–O\(_2\) formation by reaction with freshly dissolved \(\beta\)-NADH (25 \(\mu\)M) followed by addition of H\(_2\)O\(_2\) to the concentrations indicated. Times shown have been adjusted to illustrate addition of peroxide after 8.4 min and starting at the same initial absorbance (Abs).
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![Graph A](image1.png)

**FIGURE 5.** Dependence of aerobic reaction of IDOFe³⁺ with NADH on pH. A, formation of IDOFe²⁺-O₂ at the pH indicated as monitored by the change in absorbance at 577 nm. B, extent of IDOFe²⁺-O₂ formation. Fitting these data to a three-state, two-deprotonation model yielded $pK_a$ values of 6.5(2) and 8.6(5). C, initial rate of IDOFe²⁺-O₂ formation. D, rate of IDOFe²⁺-O₂ oxidation. Fitting these data to a two-state single deprotonation model yielded a $pK_a$ of 6.8(1). Abs, absorbance; error bars denote the standard deviation of measurements performed in triplicate; numbers in parentheses indicate standard deviation of the least significant figure.

As a result, protein damage was less significant, and the availability of O₂ to react with IDOFe³⁺ increased the rate of IDOFe²⁺-O₂ formation. The rate and extent of IDOFe²⁺-O₂ formation increased with the age of the NADH solution. This enhanced reactivity of NADH solution (pH 7.0) stored in the dark at room temperature nearly doubled the yield of IDOFe²⁺-O₂ after 2 h of storage and continued to increase for more than 96 h (Fig. 6A). NADH solutions incubated on ice for prolonged periods or freshly prepared solutions heated for 5 min at 95°C also exhibited greater efficiency of IDOFe²⁺-O₂ production than did freshly prepared NADH solutions (not shown). We attribute the increased reactivity of stored or heated NADH solutions to spontaneous reaction of NADH with dissolved oxygen to form H₂O₂ and/or an NADH-peroxide adduct (57, 58). By comparison, hydrolysis of freshly dissolved NADH with phosphodiesterase I to produce NMNH significantly increased the rate and initial extent of IDOFe²⁺-O₂ formation (Fig. 6B). However, the reoxidation of the enzyme also occurred earlier and at a faster rate commensurate with the length of time that the NADH was subjected to phosphodiesterase hydrolysis. Thus, it appears that the nicotinamide mononucleotide is better able than NADH to react with IDO probably because it is not subject to the ring stacking interaction with the adenine moiety reported for NADH in solution (32). Moreover, in the absence of this stacking interaction, the nicotinamide group may react more rapidly with dioxygen to produce H₂O₂ (and/or NMNH-H₂O₂ complex).

![Graph B](image2.png)

**FIGURE 6.** Aerobic reaction of IDOFe³⁺ (5.6 μM) with NADH solution (200 μM in 20 mM BisTris buffer, pH 7.5, 25°C) that had been incubated in the dark in for 0, 2, 5, 8, 24, 28, and 96 h ($t_0$, respectively) at 23°C in presence (A) or absence (B) of snake venom phosphodiesterase I. An NADH solution (50 mM in 100 mM Tris buffer, pH 8.9 with 14 and 100 mM MgCl₂ and NaCl, respectively) was divided into two portions (0.5 ml) for addition of 0.5 units of phosphodiesterase concentrate (5 μl) to one stock solution. Samples (50 μl) from each solution were withdrawn at the times indicated and frozen immediately in liquid nitrogen until shortly before assaying the activity of each.

**FIGURE 7.** Phase 1 of NADH-supported IDO-catalyzed oxidation of Trp (1 and 200 μM of enzyme and β-NADH, respectively, in 20 mM MOPS buffer, pH 7.0, 20°C). A, N-formylkynurenine formed (based on absorbance changes monitored at 321 nm with a stopped-flow spectrometer) by reaction of NADH-generated IDOFe²⁺-O₂ and l-Trp (100 and 2000 μM). The amount of product replotted against $\exp(-k_1 t)$ for the formation of $N$-FK. Arrows indicate the direction of the absorbance changes. AU, absorbance units.

**l-Trp Oxidation by IDO Supported by NADH**—Reaction of NADH-generated IDOFe²⁺-O₂ with l-Trp resulted in the production of $N$-FK as indicated by the increasing absorbance at 321 nm (Fig. 7A). The substrate turnover was initially $\sim$3 s⁻¹, but this rate decreased monotonically to 0.02 s⁻¹ within 20 s. During this period (Phase 1), only $\sim$19 eq of $N$-FK accumulated despite the availability of NADH and substrates in high excess. After this point, the oxidation reaction continued at the base level (Phase 2) until the Trp concentration became limiting (see below). This behavior parallels that observed by Hayaishi and
co-workers (13) who identified enzyme oxidation as the reason for the premature termination of Trp oxidation reactions in which active enzyme was uncaged by flash photolysis of the IDOFe\textsuperscript{3+}-CO complex in oxygen-saturated buffer. N-FK production ceased because of the lack of an effective reducing agent that could maintain the enzyme active, thus leading to IDOFe\textsuperscript{3+}-Trp accumulation instead. The absorption changes in the visible region of the spectrum showing the formation of an IDOFe\textsuperscript{3+}-Trp-like species during Phase 1 (peaks at 538.5 and 568 nm, a shoulder at \( \sim \)593 nm, and isosbestic points at 584 and 524 nm; Fig. 7B) support this conclusion. Fitting the reaction profile to Equation 1 (13)

\[
[N-FK] = \frac{k_{\text{cat}}[E_o]}{k_{\text{ox}}}(1 - \exp(-k_{\text{ox}}t)) \quad \text{(Eq. 1)}
\]

yielded \( k_{\text{cat}} \) and \( k_{\text{ox}} \) values of 3.2(3) and 0.17(2) s\(^{-1}\) (numbers in parentheses indicate standard deviation of the least significant figure), respectively, for the rate constants for N-FK production and oxidation-induced IDO inactivation.

The initial value for the ternary complex concentration \( [E_o] \) used for this calculation was fixed at 1 \( \mu \text{M} \) based on the rapid formation of the ternary complex, which was complete within \( \sim 200 \) ms of mixing (isosbestic points at 572 and 505 nm; Fig. 7C), and on the assumption that all of the enzyme is in the form IDOFe\textsuperscript{2+}-O\(_2\) and active. The linear plot of N-FK produced versus \( \exp(-k_{\text{cat}}t) \) (Fig. 7A, inset) confirms that autoxidation of the heme iron was the primary cause for the loss of enzyme activity despite the presence of NADH. The \( k_{\text{cat}} \) obtained is in reasonable agreement with the value (2.0 s\(^{-1}\)) reported by Taniguchi et al. (13). The value for \( k_{\text{ox}} \) on the other hand, is in good agreement with the first order rate constant for the autooxidation of the enzyme measured from the change in absorbance at 577 nm (0.16 s\(^{-1}\)). In contrast, the autooxidation of IDOFe\textsuperscript{2+}-O\(_2\) in the absence of substrate and reducing agents was considerably slower (6 and \( 16 \times 10^{-3} \) s\(^{-1}\) determined in this work and estimated from the reported \( t_{1/2} \) value of 36 s\(^{-1}\) (51), respectively), indicating that IDO is more susceptible to oxidation during turnover and/or while involved in the ternary complex.

The inactivation of the enzyme by autoxidation is reversible. As Trp was consumed, the rate of N-FK production increased in the late stages of Phase 2, reflecting diminished enzyme autoxidation (Fig. 8A). Similarly, Trp consumption was slow when it was added to IDO prior to addition of NADH, but the rate of N-FK production accelerated as [Trp] fell below \( \sim 30 \) \( \mu \text{M} \) (Fig. 8B). Because this threshold concentration is substantially lower than the inhibition constant reported for substrate inhibition of IDO by Trp (\( k_{\text{si}} = 170 \) \( \mu \text{M} \)) (59)), substrate inhibition does not appear to contribute significantly to the inactivation of the enzyme under these conditions. With a \( K_m \) of 900 \( \mu \text{M} \) (60) for the binding of L-Trp by IDOFe\textsuperscript{3+}, only a small fraction of the enzyme is present as the low-spin, ferric complex. Consequently, the primary effect of Trp on the activity of IDO is to promote autoxidation of the heme iron as shown above. The fraction of reduced enzyme as a function of L- and D-Trp concentration is shown in Fig. 8, B and C, respectively. It can be seen that at low [L-Trp] (\( \ll K_m \)), a fraction of the enzyme remained in the reduced state as Trp oxidation proceeded, but increasing [L-Trp] resulted in an exponential increase in enzyme autoxidation and, as a result, diminished Trp consumption. Although similar results were obtained with D-Trp, complete conversion to the ferric enzyme occurred at a much higher [D-Trp], which is consistent with a greater \( K_m \) for this substrate.

**Simulated Docking of NADH to IDO**—Attempts to identify regions in the structure of IDO where Cibacron blue 3GA and possibly NADH and NMNH might bind were undertaken with the program AutoDock 4.2 (44–47). These simulations identified three positively charged regions on the surface of the protein (Fig. 9), each of which has a centrally located basic residue (e.g. Lys-116, Lys-186, and Arg-231 at the center of sites 1, 2, and 3, respectively) that could form electrostatically stabilized complexes with the (pyro)phosphate of NADH/NMNH or sulfonyl groups of Cibacron blue to stabilize binding. All three ligands clustered within two of the interaction sites (sites 1 and 2), but only a few complexes resulted for Cibacron blue binding.
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at the site that encompasses the heme cavity (site 3). NADH and NMNH, on the other hand, clustered well at this site. In at least one of these complexes, NADH is oriented in a position similar to that of phenylimidazole in the crystallographically defined structure (Protein Data Bank code 2D0T) (9). In this orientation, the nicotinamide ring is located above and orthogonal to the heme plane with ~4.7 Å separating the nicotinamide C4 atom and the heme iron, whereas the pyrophosphate group forms hydrogen bonds with Arg-231 and Ser-235. A similar arrangement has been observed in the crystal structure of cytochrome P450 nitric-oxide reductase (61) with bound nicotinic acid pyridine dinucleotide. In some complexes arising from these simulations, the adenine group of NADH occurred in the distal heme pocket rather than the nicotinamide ring, but steric restrictions evidently preclude formation of complexes in which both adenine and the nicotinamide ring occupied the distal heme pocket simultaneously. Simulated complexes with NMNH bound in the distal heme pocket exhibited significantly less structural variability presumably because the absence of the adenine facilitated more effective interaction of the NMNH with the active site.

DISCUSSION

The aerobic reaction of IDOFe3+/H11001 with NADH reported here affords a remarkably convenient means of generating IDOFe2+/H11001-O2 in good yield and in a relatively stable form at ambient temperature without use of mediators or enzymes. Although various mechanisms have been proposed for the oxidation of Trp by IDO (9, 62–64), all of these mechanisms include formation of an IDOFe2+/H11001-O2 intermediate, so this form of the enzyme is of considerable interest. Notably, the standard assay for measuring the activity of IDO (referred to at the time as intestinal tryptophan pyrrolase) that was developed by Yamamoto and Hayashi (65) requires both ascorbate and methylene blue in addition to D- or L-Trp and dioxygen. These authors also reported that neither glutathione nor cysteine could be substituted for ascorbate, although xanthine oxidase and hypoxanthine (a reaction that produces superoxide anion radical) could do so. Although toluidine blue could replace methylene blue, the same was not true for 2,3-dichlorophenolindophenol, potassium ferricyanide, phenazine methosulfate, FAD, FMN, ferrous sulfate, or cytochrome c. The ability of NADH to support catalysis of Trp oxidation by IDO provides a new means by which the reactivity of this enzyme can be investigated.

Although the reaction of IDO with NADH has not been studied previously, interaction and reactivation of NADH with related heme proteins has been reported. For example, metmyoglobin (to which IDO is evolutionarily related (5–8, 66)) was found not to react significantly with NADH in the absence of EDTA (67), flavins (67), or phenazine methosulfate (68), all of which (except EDTA) could mediate electron transfer between NADH and the protein. For this reason, it is likely that the small stimulatory effect on the reaction exhibited by EDTA results from the presence of trace amounts of contaminating iron and formation of the mediator Fe[EDTA]2+/H11001− (69). Although the thermal reduction of methemoglobin by NADH has apparently not been reported, NADH, NADP−, and NADPH do act as allosteric effectors of dioxygen binding to deoxyhemoglobin (70). Moreover, NADH has been shown to form a 1:1 complex with methemoglobin by sedimentation equilibrium analysis (71), and NADPH has been shown to form a complex with met- and deoxyhemoglobin by fluorescence quenching titrations (72).

On the other hand, NADH reacts with horseradish peroxidase to form the ferrous dioxygen adduct, compound III, as first observed by Yokota and Yamazaki (73, 74). Under certain conditions, the complex and unusual kinetics of this reaction are apparent from the oscillatory nature of compound III accumulation, decay, and reaccumulation (73, 75). As reviewed by Scheel and co-workers (76, 77), extensive modeling efforts have succeeded in accounting for many of the kinetic characteristics of this complex process. Other peroxidases (78, 79), including lactoperoxidase (78–80) and myeloperoxidase (81), have also been shown to exhibit similar oscillatory kinetics. At present, we have not observed oscillatory behavior of this type with IDO, but the extent of our exploration of reaction conditions so far is insufficient to rule out this possibility.

The generally accepted reaction mechanism for the reaction of peroxidases with NADH proposed by Halliwell and de Rycker (82) (adapted in Fig. 10 to illustrate the reaction with IDO) is initiated by H2O2 (exogenous or, from our work, as a contaminant from the slow reaction of NADH with dioxygen) reacting with the ferric enzyme to form a compound I-like intermediate. This compound I then abstracts an electron from NADH to yield compound II and NADH− cation radical. Upon deprotonation (pKα = 1.43 (83)), NAD+ can reduce (a) compound II to the ferric enzyme, (b) the ferric enzyme to the ferrous enzyme, and/or (c) dissolved dioxygen to superoxide anion radical. IDOFe2+/H18528 and IDOFe3+/H18528 then bind dioxygen or superoxide anion, respectively, to produce IDOFe2+/H18528-O2 (compound III). Excess superoxide dismutes spontaneously to H2O2 and water (particularly under acidic conditions (56)) or enzymatically by reaction with the oxygenated peroxidase (52). Because reaction a consumes H2O2 to oxidize NADH to NAD+, the reaction stops when H2O2 is depleted. In contrast, the continuous oxidation of NADH and the accumulation of compound

FIGURE 10. Reactions involved upon aerobic exposure of IDOFe3+/H11001 to NADH in the presence of trace amounts of hydrogen peroxide.
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III observed in some cases indicate that reactions b and c dominate. Importantly, the latter two reactions allow for the regeneration of the catalytic H$_2$O$_2$ through dismutation of superoxide anion radical.

NADH had no effect on IDOFe$^{3+}$ under anaerobic conditions unless a mediator (e.g. phenazine methosulfate or methylene blue) was present. Thus, dioxygen acts as more than a ligand in the generation of IDOFe$^{2+}$-O$_2$. This requirement for dioxygen and the inhibition of IDOFe$^{2+}$-O$_2$ formation by either catalase or SOD suggest that IDO reacts with trace amounts of H$_2$O$_2$ in NADH solutions in a manner similar to a peroxidase to initiate the reactions that lead to IDOFe$^{2+}$-O$_2$ formation. Involvement of peroxide in the reaction of IDO with NADH is also consistent with the observation that reaction with aged solutions of NADH resulted in the accelerated formation of IDOFe$^{2+}$-O$_2$ and with the variations in the rate of this reaction observed with fresh solutions of NADH prepared from different lots of crystalline NADH (data not shown). Bernofsky (58) and Bernofsky and Wanda (84) proposed nearly 20 years ago that a peroxide adduct of NADH forms slowly in aerobic solutions of NADH and that this adduct forms to varying extent even in the solid state. The hygroscopic nature of solid NADH and the storage of solid NADH under conditions of undefined humidity could explain the variation in behavior observed for freshly prepared solutions of NADH in the results we report here.

The reactivity of IDO with hydrogen peroxide has not been studied in the same depth as has been achieved with several other heme enzymes and proteins. Although Shimizu et al. (27) noted in passing that IDO exhibits a peroxidase activity, no further consideration of the interaction of IDO with peroxide was reported until Poljak et al. (54) demonstrated that exposure of IDO to hydrogen peroxide ($\geq 10 \mu M$) decreases the dioxygenase activity of the enzyme and oxidizes up to five of the eight Cys residues to sulfenic or sulfonic acid to result in changes to the structure of the enzyme. Following the detection of ferryl heme intermediates by resonance Raman spectroscopy during turnover of IDO (63, 85, 86), Lu and Yeh (87) used this same technique to observe formation of a relatively stable compound II-like intermediate as the result of reaction of IDOFe$^{3+}$ with hydrogen peroxide. This result is fully consistent with the peroxidatic mechanism proposed here for formation of IDOFe$^{2+}$-O$_2$ in the presence of NADH with low [H$_2$O$_2$] (\(\leq 2 \mu M\)).

The docking simulations conducted in this study suggest that binding of NADH at the active site of IDO is possible, and the ability to elute IDO bound to Cibacron blue affinity resin with either NADH or L-Trp is consistent with such interaction (88, 89). Nevertheless, we were unable to detect any change in the electronic absorption spectrum of IDOFe$^{3+}$ or in the fluorescence emission spectrum of NADH upon mixing these two components anaerobically, so no simple means of quantifying or even verifying the affinity of the distal side of the heme cavity for NADH is available. Conversion of IDOFe$^{3+}$ to IDOFe$^{2+}$-O$_2$ by a peroxidatic mechanism of the type described here does not require high affinity binding. Instead, rapid electron transfer first from NADH to a ferryl adduct of IDO and then from NAD$^+$ to either IDOFe$^{3+}$ or dioxygen is the minimal requirement for reaction because of the reactivity of the radicals involved. In view of the high reactivity of these species, it is reasonable to assume that they are generated in proximity to the active site of the protein.

The poor efficiency of NADH relative to NMNH in the formation of IDOFe$^{2+}$-O$_2$ may be related to the equilibrium of unfolded and folded (formed by stacking of the adenine and nicotinamide groups) conformations that NADH exhibits in solution (32, 90, 91). Presumably, the folded form interacts less efficiently with the active site of IDO and/or inhibits reaction of the nicotinamide with dioxygen. The greater propensity of NMNH to oxidize relative to NADH could result in more efficient formation of superoxide anion radicals that in turn accelerate all subsequent reactions (i.e. dismutation to H$_2$O$_2$ and IDOFe$^{2+}$-O$_2$ formation). It is thus noteworthy that whereas in solution NADH exists in an equilibrium of folded and unfolded conformations to the best of our knowledge NADH oxidoreductases bind only the unfolded form (92–94). Many of the docking simulation results also show NADH bound to IDO in this unfolded, more reactive state.

Nearly 50 years ago, Nishizuka and Hayaishi (95) reported evidence that tryptophan metabolism leads to formation of vitamin ribonucleotide, a key intermediate in the biosynthesis of NAD. This proposal explained the observation in several species, including humans, that adequate amounts of dietary Trp replace the dietary requirement for the vitamin niacin (96). The current work raises the intriguing possibility of another metabolic relationship between NAD and Trp metabolism in which the reduced pyridine nucleotide activates the first and rate-limiting reaction in the metabolic pathway that leads to its own formation.

Acknowledgments—We thank Dr. Marcia R. Mauk for developing the original IDO purification protocol, Dr. David S. Waugh for providing the polyHis-tagged TEV protease expression system, Drs. Susanne Ludwiczek and Tomoko Misono for expressing and purifying the TEV protease used, and Dr. Wei Chen for analysis of recombinant IDO by electrospray mass spectrometry. The Cary spectrophotometers and Applied Biosystems Q-Star mass spectrometers were obtained through grants from the Canadian Foundation for Innovation to the University of British Columbia Laboratory of Molecular Biophysics and the Centre for Blood Research.

REFERENCES

1. Hayaishi, O., Hirata, F., Ohnishi, T., Henry, J. P., Rosenthal, I., and Katoh, A. (1977) J. Biol. Chem. 252, 3548–3550
2. Mann, D. H., Zhou, M., Attwood, J. T., Bondarev, I., Conway, S. I., Marshall, B., Brown, C., and Mellor, A. L. (1998) Science 281, 1191–1193
3. Mackenzie, C. R., Heseler, K., Müller, A., and Diabouner, W. (2007) Curr. Drug Metab. 8, 237–244
4. Mann, D. H., and Mellor, A. L. (2007) J. Clin. Invest. 117, 1147–1154
5. Suzuki, T. (1994) J. Protein Chem. 13, 9–13
6. Suzuki, T., Kawamichi, H., and Imai, K. (2001) Biochem. Pharmacol. 61, 117–128
7. Suzuki, T., and Takagi, T. (1992) J. Mol. Biol. 228, 698–700
8. Suzuki, T., Yokouchi, K., Kawamichi, H., Yamamoto, Y., Uda, K., and Yuasa, H. I. (2003) Gene 308, 89–94
9. Sugimoto, H., Oda, S., Otsuki, T., Hino, T., Yoshida, T., and Shiro, Y. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 2611–2616
10. Schenkmann, I. B., and Jansson, I. (1999) Drug Metab. Rev. 31, 351–364
NADH Oxidase Activity of Indoleamine 2,3-Dioxygenase

11. Zhang, H., Myshkin, E., and Waskell, L. (2005) Biochem. Biophys. Res. Commun. 338, 499–506
12. Hirata, F., Ohnishi, T., and Hayashi, O. (1977) J. Biol. Chem. 252, 4637–4642
13. Taniguchi, T., Sonoda, M., Hirata, F., Hayashi, O., Tamura, M., Hayashi, K., Iizuka, T., and Ishimura, Y. (1979) J. Biol. Chem. 254, 3288–3294
14. Sono, M. (1986) Biochemistry 25, 6089–6097
15. Vottero, E., Mitchell, D. A., Page, M. J., MacGillivray, R. T., Sadowski, I. J., Roberge, M., and Mauk, A. G. (2006) FEBS Lett. 580, 2265–2268
16. Maghazal, G. J., Thomas, S. R., Hunt, N. H., and Stocker, R. (2008) J. Biol. Chem. 283, 12014–12025
17. Hultquist, D. E., and Passon, P. G. (1971) Nat. New Biol. 229, 252–254
18. Hultquist, D. E., Sannes, L. J., and Schafer, D. A. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 301–308
19. Livingston, D. J., McLachlan, S. J., La Mar, G. N., and Brown, W. D. (1985) J. Biol. Chem. 260, 15699–15707
20. Pearson, J. T., Sui, S., Meininger, D. P., Wienkens, L. C., and Rock, D. A. Biochemistry 49, 2647–2656
21. Luca, L. J., Baty, R. T., and Doudna, J. A. (2001) BioTechniques 30, 544–550
22. McComber, R. B., Bond, L. W., Burnett, R. W., Keech, R. C., and Bowers, G. N., Jr. (1976) Clin. Chem. 22, 141–150
23. Berger, F., Lau, C., Dahlmann, M., and Ziegler, M. (2005) J. Biol. Chem. 280, 36334–36341
24. Vlastis, J., Jakopitsch, C., Schwanninger, M., Holubar, P., and Obinger, C. (2007) FEBS Lett. 581, 320–324
25. Marmorstein, R. Q., Joachimiak, A., Sprinzl, M., and Sigler, P. B. (1987) J. Biol. Chem. 262, 4922–4927
26. Nelson, D. P., and Kiesow, L. A. (1972) Annu. Rev. Biochem. 41, 474–478
27. Shimizu, T., Nomiyama, S., Hirata, F., and Hayashi, O. (1978) J. Biol. Chem. 253, 4700–4706
28. Edgell, C. J., McDonald, C. C., and Graham, J. B. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 3734–3737
29. Vottero, E. R. (2007) New Inhibitors of Human Indoleamine 2,3-Dioxygenase, Ph.D. dissertation, University of British Columbia, Vancouver, Canada
30. Papadopoulou, N. D., Mewies, M., McLean, K. J., Seward, H. E., Svishtunenko, D. A., Munro, A. W., and Raven, E. L. (2005) Biochemistry 44, 14318–14328
31. Sarma, R. H., and Kaplan, N. O. (1970) Biochemistry 9, 539–548
32. Hull, R. V., Conger, P. S., 3rd, and Hoobler, R. J. (2001) Biophys. Chem. 90, 9–16
33. Weber, G. (1957) Nature 180, 1409
34. Ishimura, Y., Nozaki, M., and Hayashi, O. (1970) J. Biol. Chem. 245, 3593–3602
35. Eswar, N., Webb, B., Marti-Renom, M. A., Madhusudhan, M., Eramian, D., Shen, M. y., Pieper, U., and Sali, A. (2006) Current Protocols in Bioinformatics 15, 5.6.1–5.6.30, John Wiley and Sons, Inc., New York
36. Fiser, A., Do, R. K., and Sali, A. (2000) Protein Sci. 9, 1753–1773
37. Martí-Renom, M. A., Stuart, A. C., Fiser, A., Sánchez, R., Melo, F., and Sali, A. (2000) Annu. Rev. Biophys. Biomol. Struct. 29, 291–325
38. Sali, A., and Blundell, T. L. (1993) J. Mol. Biol. 234, 779–815
39. Berendsen, H. J. C., van der Spoel, D., and van Drunen, R. (1995) Comput. Phys. Commun. 91, 43–56
40. Hess, B., Kutzner, C., van der Spoel, D., and Lindahl, E. (2008) J. Chem. Theory Comput. 4, 435–447
41. Lindahl, E., Hess, B., and van der Spoel, D. (2001) J. Mol. Model. 7, 306–317
42. Van Der Spoel, D., Lindahl, E., Hess, B., Groenhof, G., Mark, A. E., and Berendsen, H. J. C. (2005) J. Comput. Chem. 26, 1701–1718
43. Essmann, U., Perera, L., Berkowitz, M. L., Darden, T., Lee, H., and Pedersen, L. G. (1995) J. Chem. Phys. 103, 8577–8593
44. Goodsell, D. S., and Olson, A. J. (1990) Proteins 8, 195–202
45. Morris, G. M., Goodsell, D. S., Huey, R., and Olson, A. J. (1996) J. Comput. Aided Mol. Des. 10, 293–304
46. Morris, G. M., Goodsell, D. S., Halliday, R. S., Huey, R., Hart, W. E., Belew, R. K., and Olson, A. J. (1998) J. Comput. Chem. 19, 1639–1662
47. Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., and Olson, A. J. (2009) J. Comput. Chem. 30, 2785–2791
NADH Oxidase Activity of Indoleamine 2,3-Dioxygenase

85. Yanagisawa, S., Yotsuya, K., Hashiwaki, Y., Horitani, M., Sugimoto, H., Shiro, Y., Appelman, E. H., and Ogura, T. (2010) *Chem. Lett.* **39**, 36–37.
86. Yanagisawa, S., Horitani, M., Sugimoto, H., Shiro, Y., Okada, N., and Ogura, T. (2011) *Faraday Discuss.* **148**, 239–247.
87. Lu, C., and Yeh, S. R. (2011) *J. Biol. Chem.* **286**, 21220–21230.
88. Stellwagen, E. (1977) *Acc. Chem. Res.* **10**, 92–98.
89. Stellwagen, E. (1990) *Methods Enzymol.* **182**, 343–357.
90. Velick, S. F. (1961) in *A Symposium on Light and Life* (McElroy, W. D., and Glass, B. eds) p. 108, Johns Hopkins Press, Baltimore.
91. McDonald, G., Brown, B., Hollis, D., and Walter, C. (1972) *Biochemistry* **11**, 1920–1930.
92. Bellamacina, C. R. (1996) *FASEB J.* **10**, 1257–1269.
93. van den Heuvel, R. H. H., Westphal, A. H., Heck, A. J. R., Walsh, M. A., Rovida, S., van Berkel, W. J. H., and Mattevi, A. (2004) *J. Biol. Chem.* **279**, 12860–12867.
94. Irimia, A., Madern, D., Zaccai, G., and Vellieux, F. M. D. (2004) *EMBO J.* **23**, 1234–1244.
95. Nishizuka, Y., and Hayaishi, O. (1963) *J. Biol. Chem.* **238**, 483–485.
96. Krehl, W. A., Teply, L. J., Sarma, P. S., and Elvehjem, C. A. (1945) *Science* **101**, 489–490.