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Catalytic activity of human indoleamine 2,3-dioxygenase (*h*IDO1) at low oxygen

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ABSTRACT

A cytokine-inducible extrahepatic human indoleamine 2,3-dioxygenase (*h*IDO1) catalyzes the first step of the kynurenine pathway. Immunosuppressive activity of *h*IDO1 in tumor cells weakens host T-cell immunity, contributing to the progression of cancer. Here we report on enzyme kinetics and catalytic mechanism of *h*IDO1, studied at varied levels of dioxygen (O_2) and L-tryptophan (L-Trp). Using a cytochrome b_5-based activating system, we measured the initial rates of O_2 decay with a Clark-type oxygen electrode at physiologically-relevant levels of both substrates. Kinetics was also studied in the presence of two substrate analogs: 1-methyl-L-tryptophan and norharmane. Quantitative analysis supports a steady-state rather than a rapid equilibrium kinetic mechanism, where the rates of individual pathways, leading to a ternary complex, are significantly different, and the overall rate of catalysis depends on contributions of both routes. One path, where O_2 binds to ferrous *h*IDO1 first, is faster than the second route, which starts with the binding of L-Trp. However, L-Trp complexation with free ferrous *h*IDO1 is more rapid than that of O_2. As the level of L-Trp increases, the slower route becomes a significant contributor to the overall rate, resulting in observed substrate inhibition.

Introduction

In the human body, L-tryptophan (L-Trp) is one of the nine essential dietary amino acids [1]. It functions as a building block of proteins and as a precursor of niacin, an intermediate in the biosynthesis of NAD^+ [2], and serotonin, a mood-modulating neurotransmitter and physiological regulator [3]. Up to 90% of dietary L-Trp is catabolized via the kynurenine pathway [4,5]. The kynurenine pathway starts with a dioxygenation reaction of L-Trp that is catalyzed by indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) [6,7]. Both IDO and TDO contain a type-*b* heme and use dioxygen (O_2) to open the five-membered ring of L-Trp to form N-formyl-L-kynurenine (NFK), as shown in Scheme 1 [6]. Excessive exhaustion of L-Trp via the kynurenine pathway significantly hinders T-cell proliferation, differentiation, effector function, and viability, resulting in a suppressed immune response [8]. Catabolites of L-Trp are capable of promoting immunosuppression and tumor tolerance during cancer [9], formation of cataracts [10,11], HIV-related neurological damage, and ischemic brain injury [12].

IDO is induced extrahepatically throughout the body [6]. The strongest inducer of IDO is a proinflammatory cytokine interferon-γ (IFN-γ) [13]. IDO-initiated L-Trp degradation accelerates during conditions that cause cellular activation of the immune response such as malignancy, inflammation, autoimmune disorder, and pregnancy [12]. The induction of IDO by IFN-γ is greatly diminished in a hypoxic environment, where the O_2 concentration is between 5% and 10% of air-saturation level [14–16]. During hypoxia, cells stimulated with IFN-γ demonstrate reduced levels of IDO compared to normoxic conditions. IDO antimicrobial and immunoregulatory functions are also significantly impaired [14–16].

Even under normoxic conditions, the physiological levels of O_2 are quite low (between 14% and 24% of air-saturation level or between ~35 μM and ~65 μM O_2) [17]. The steady-state kinetic data for IDO are known primarily in solutions saturated with air [18–28]. Here, we report on enzyme kinetic studies of human IDO isoform-1 (*h*IDO1)[3] (EC 1.13.11.52) at physiologically-relevant levels of O_2 as a function of both substrates – O_2 and L-Trp. This

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[3] *Abbreviations used:* *h*IDO1, human indoleamine 2,3-dioxygenase isoform-1; TDO, tryptophan 2,3-dioxygenase; L-Trp, L-tryptophan; 1-Me-L-Trp, 1-methyl-L-tryptophan; NFK, N-formyl-L-kynurenine; O_2, dioxygen; $O_2^{\cdot-}$, superoxide anion; H_2O_2, hydrogen peroxide; NHM, norharmane (β-carboline); IFN-γ, interferon-γ; NAD^+, nicotinamide adenine dinucleotide; β-NADH, β-nicotinamide adenine dinucleotide hydride; Cu,Zn-SOD, copper-zinc superoxide dismutase; DNase I, deoxyribonuclease I; IPTG, isopropyl β-D-1-thiogalactopyranoside; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LRF MALDI-TOF, linear-reflectron matrix-assisted laser desorption/ionization time-of-flight mass spectrometer.

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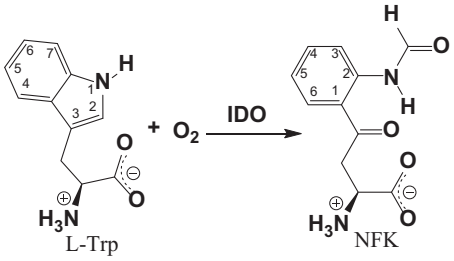

Scheme 1. Reaction catalyzed by IDO.

information is vital for understanding enzyme-substrate interactions in *h*IDO1 and for designing inhibitors with enhanced therapeutic responses [29–31]. Using a new assay methodology, we quantify the effects of O_2 concentration on the initial rates of *h*IDO1 catalysis. We analyze the data within a mechanistic model that allows for initial complexation of either O_2 or L-Trp with a free ferrous form of *h*IDO1 – $^{Fe(II)}$*h*IDO1 [6,23]. This model also considers that both resulting substrate-bound species – $^{Fe(III)}$*h*IDO1·O_2^- and $^{Fe(II)}$*h*IDO1·L-Trp [32], lead to a ternary complex – *h*IDO1·O_2·L-Trp.

The ternary complex has been previously characterized in human and rabbit IDO by the stepwise mixing of substrates with the ferrous enzyme either manually, at a low temperature, or on a stopped-flow, at room temperature [24–28,33,34]. Here we analyze the kinetics of the formation of the ternary complex. We observe that under steady-state conditions, the ternary complex forms via two separate pathways. Even though the O_2-first/L-Trp-second addition route is faster than the L-Trp-first/O_2-second path [23,35], the organic substrate binds to the free ferrous *h*IDO1 at a higher rate than O_2 [35]. At low physiologically-relevant concentrations of both substrates, the slower L-Trp-initiated pathway is a significant contributor to the overall catalytic rate, resulting in pronounced substrate inhibition of catalysis. Such kinetic control of *h*IDO1 activity could be operational *in vivo* where, depending on tissue oxygenation [17], O_2 supply may be limited relative to L-Trp level [36,37].

Materials and methods

Reagents

L-tryptophan (Cat. # T0254), β-NADH (Cat. # N1161), Cu,Zn-SOD (Cat. # S8160), catalase (Cat. # C100), lysozyme (Cat. # L6876), DNase I (Cat. # D5025), PMSF (Cat. # P7626), Trizma (Cat. # T1503), MOPS (Cat. # M3183), imidazole (Cat. # 56750), EDTA (Cat. # ED4SS), kanamycin sulfate (Cat. # K1377), 1-methyl-L-tryptophan (Cat. # 447439), norharmane (Cat. # N6252) and norharmane hydrochloride (Cat. # N6377) were from Sigma–Aldrich; agar (Cat. # N833) and yeast extract (Cat. # J850) were from Amresco; tryptone (Cat. # 95039) was from Fluka; sodium chloride (Cat. # SX0425), monobasic sodium phosphate (Cat. # SX0710), dibasic sodium phosphate (Cat. # SX0715) were from EMD; δ-aminolevulinic acid hydrochloride (Cat. # 01433) and IPTG (Cat. # 00194) were from Chem-Impex International, Inc. in Wood Dale, IL; N_2 (pre-purified grade) and O_2 (USP grade) were from Norco, Inc. in Boise, ID. The pETevIDO plasmid for *h*IDO1 expression and the plasmids for cytochrome b_5 and cytochrome b_5 reductase were kindly provided by Prof. A. Grant Mauk (University of British Columbia).

Enzyme preparation

*h*IDO1 (EC 1.13.11.52) was overexpressed in One Shot BL21 Star (DE3) chemically competent *Escherichia coli* cells (Invitrogen) using a published procedure [38]. Greater than 95% homogeneity of *h*IDO1 was confirmed by the SDS–PAGE after the second Ni-affinity column and dialysis into 50 mM Tris·HCl pH 8.0 buffer, containing 100 mM NaCl and 4 mM EDTA. The purified enzyme exhibited an $[M+H]^+$ peak at m/z = 45,586 on a Microflex LRF MALDI-TOF mass spectrometer (Bruker), which is in good agreement with the expected value of m/z = 45,643. Cytochrome b_5 and cytochrome b_5 reductase, overexpressed in One Shot BL21 Star (DE3) cells, were purified as described elsewhere [39,40]. The homogeneity of these proteins was confirmed by 10% SDS–PAGE analysis. Protein concentrations were determined spectrophotometrically. All enzyme stocks were stored at −75 °C.

Measurement of initial rates of hIDO1 catalysis by monitoring O_2 consumption

Initial rates of *h*IDO1 catalysis of L-Trp dioxygenation were measured at 25.0 °C using a Clark-type oxygen electrode consisting of a digital ammeter (Biological Oxygen Monitor, model 5300A, Yellow Springs Instruments (YSI)) and a polarographic oxygen probe (5331A, YSI). Highly reproducible O_2 depletion traces (Fig. S1) [41] were recorded in a reaction chamber thermostated on a modified bath assembly (5301B, YSI) interfaced to a recirculating water bath (DC10, Thermo Scientific; K20, Haake). A VWR 10″ × 10″ professional stirrer was used to maintain the stirring rate at 700 rpm. O_2 and N_2 gases were metered using a Riteflow flow meter (PMR1-010976, Bel-Art Scienceware). This experimental setup allowed for precise control of temperature, stirring rate, and oxygen level. The oxygen probe was calibrated daily to the dissolved oxygen in air-saturated water (resistivity ⩾18.2 MΩ × cm) at normal pressure. Due to the elevation of Missoula, the 100% air-saturation level of pure water at 25.0 °C corresponds to $[O_2]$ = 230 μM, which is lower than $[O_2]$ = 258 μM at sea level [42]. Solubility of O_2 was calculated using atmospheric pressure measured with a mercury manometer interfaced to a high-vacuum line (Chemglass). Atmospheric pressure at sea level was taken as 760 mmHg [42].

A typical reaction mixture (3.000 mL) contained 20 mM MOPS buffer at pH 7.0, 150 μM β-NADH, 1.0 μM cytochrome b_5, 140 nM cytochrome b_5 reductase, 54 nM Cu,Zn-SOD, 12 nM catalase, and varying concentrations of L-Trp [43]. The solution was equilibrated against air or a mixture of pure O_2 and N_2, saturated with water vapor at atmospheric pressure by passing through a fritted gas washing bottle (Chemglass, Cat. # CG-1114-13). During inhibition studies, the reaction mixture was also supplemented with 3–15 μL of inhibitor stock solution to the final inhibitor concentration of 1–5 μM. Inhibitor stocks (100 mL of 1.00 mM solution of either 1-methyl-L-tryptophan or norharmane hydrochloride) were prepared in 100 mM potassium phosphate buffer at pH 7.0. The reaction was initiated by injecting a 5-μL aliquot of *h*IDO1 stock (~120 μM) into a reaction chamber to an expected final concentration of ~200 nM, using a gas-tight syringe (Hamilton). The exact concentrations of *h*IDO1 stock solutions were measured spectrophotometrically on an AVIV Model 14 Spectrophotometer (Aviv Biomedical), or a NanoDrop 2000 (Thermo Scientific) using molar absorptivity $\varepsilon_{\lambda=404nm}$ = 172,000 M^{-1} cm^{-1} [44]. Initial rates of O_2 consumption were measured in the range of the steepest $[O_2]$ decline (12 s long), normally 2–4 s after injecting the ferric *h*IDO1. All measurements were performed in triplicate or greater. Readouts of the oxygen probe were transmitted to a PC workstation with a 1-Hz sampling rate. The slopes of the oxygen consumption traces were determined in Excel 2010 (Microsoft) and their absolute values were expressed in μM/s. These initial velocities of O_2 consumption were converted to specific activity of *h*IDO1 by dividing by the exact final micromolar enzyme concentration – $[hIDO1]_T$. All nonlinear