Enzyme Kinetic and Spectroscopic Studies of Inhibitor and Effector Interactions with Indoleamine 2,3-Dioxygenase. 1. Norharman and 4-Phenylimidazole Binding to the Enzyme as Inhibitors and Heme Ligands†

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ABSTRACT: The effects of norharman, one of the few known inhibitors of the heme protein indoleamine 2,3-dioxygenase, and of 4-phenylimidazole (4-PheImid), a heme ligand, on the catalytic (V_{max}, K_m) and spectroscopic properties (optical absorption, CD, and magnetic CD) of the rabbit small intestinal dioxygenase were investigated. Assays were performed with the substrate L- or D-tryptophan (Trp) and an ascorbic acid–methylene blue cofactor system at 25 °C. This study has revealed that both norharman and 4-PheImid exhibit noncompetitive inhibition with respect to L-Trp and D-Trp. The binding of norharman to the enzyme results in the formation of a low-spin complex in both the ferric and ferrous enzyme with comparable dissociation constants (K_d = ~10 μM at pH 7.0) that are about 10 times smaller than the observed K_i value. L-Trp exerts no effect for the ferric enzyme and slight negative cooperative effects for the ferrous enzyme on norharman binding. Close spectral similarities are observed between the adducts of the enzyme with norharman and 4-PheImid in the respective oxidation states. This, together with competition experiments using cyanide, demonstrates that norharman binds directly to the heme iron of the enzyme as a nitrogen donor ligand. Thus, norharman competes with O_2 for the heme iron of the ferrous (active) enzyme, resulting in the observed inhibition. L-Trp and 4-PheImid appear to compete for the heme binding site in the ferric enzyme and display slight negative cooperativity on binding to the ferrous enzyme. The observed K_i value for 4-PheImid is comparable with the K_d value (~10 μM at pH 7.0) of its adduct with the ferric enzyme, but is about 40 times smaller than the K_d value for the corresponding ferrous complex. These results suggest that the inhibition of the dioxygenase activity by 4-PheImid is due to its binding to the heme iron of the ferric enzyme, preventing the reductive activation of the dioxygenase by the ascorbic acid–methylene blue cofactor system.

Indoleamine 2,3-dioxygenase (Hayaishi et al., 1975) and tryptophan 2,3-dioxygenase (Feigelson & Brady, 1974) both catalyze the oxidative cleavage of the pyrrole ring of the indole nucleus of Trp[1] upon the insertion of two oxygen atoms of molecular oxygen to yield *N*-formylkynurenine. Protoheme IX is the sole prosthetic group for both dioxygenases (Feigelson & Brady, 1974; Hirata & Hayaishi, 1975).[2] However, these two dioxygenases are distinct in several respects. Indoleamine 2,3-dioxygenase is a monomeric glycoprotein with M_r ~ 41 000 (Shimizu et al., 1978) having a wide substrate specificity for several indoleamines including L-Trp and D-Trp (Hayaishi et al., 1975; Shimizu et al., 1978). Tryptophan 2,3-dioxygenase is a tetrameric ($\alpha_2\beta_2$) polypeptide with M_r ~ 167 000 metabolizing exclusively L-Trp (Feigelson & Brady, 1974). The immunogenic properties (Watanabe et al., 1981), biological sources, and tissue distribution (Hayaishi et al., 1975; Feigelson & Brady, 1974; Yoshida et al., 1980; Cook et al., 1980; Yamazaki et al., 1985) of the two dioxygenases are also different.

A previous study by Eguchi et al. (1984) on organic inhibitors of these two dioxygenases has revealed that norharman, a β-carboline derivative that possesses an indole nucleus, serves as an inhibitor for both dioxygenases. Interestingly, norharman is reported to be an uncompetitive inhibitor for indoleamine 2,3-dioxygenase and a competitive inhibitor for tryptophan 2,3-dioxygenase with respect to the substrate L-Trp. This suggests that the active-site structures of the two dioxygenases differ somewhat. However, due to the lack of detailed studies of the interactions between norharman and these enzymes, the actual binding site for this inhibitor in either enzyme remains unclear. Expansion of such inhibitor studies might likely provide useful clues toward an understanding of the mechanism of activation and catalytic reaction and of the active-site structure of these enzymes.

To clarify the specific binding site(s) for the inhibitor norharman, effects of this compound on the catalytic (K_m, V_{max}), spectral, and substrate (L- and D-Trp) and heme ligand (cyanide) binding properties of rabbit small intestinal indoleamine 2,3-dioxygenase have been extensively investigated in the present study by employing enzyme kinetic assays and optical absorption, MCD, and CD spectroscopy. Parallel experiments with 4-phenylimidazole, a heme ligand that has been found in this study to bind to the dioxygenase in a manner similar to that of norharman, have also been carried out for comparison. The present studies have clarified that the heme iron of indoleamine 2,3-dioxygenase is the binding site for the inhibitor norharman. In addition, both norharman and 4-phenylimidazole have been found to behave as noncompetitive inhibitors with respect to the substrates L-Trp and D-Trp. However, the actual mechanisms of the inhibition of the dioxygenase activity by these two nitrogen donor heme ligands

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[1] Abbreviations: Trp, tryptophan; MCD, magnetic circular dichroism; Tris, tris(hydroxymethyl)aminomethane.

[2] Copper was later shown to be nonessential for the catalytic activity of tryptophan 2,3-dioxygenase (Ishimura et al., 1980).

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appear to differ. These findings in relation to the reductive activation of indoleamine 2,3-dioxygenase by an ascorbic acid–methylene blue cofactor system have been discussed.

Experimental Procedures

Chemicals. L-Trp, D-Trp, and L-ascorbic acid were purchased from Sigma, and norharman, harman hydrochloride, and 4-phenylimidazole were purchased from Aldrich. Methylene blue was purchased from Fisher. All of these chemicals were of reagent grade and were used without further purification.

Enzymes. Indoleamine 2,3-dioxygenase was purified from rabbit small intestine by the method of Shimizu et al. (1978), except that the final isoelectrofocusing step was omitted and step 6 (Sephadex G-100 chromatography) was repeated two to four times instead. The purified native ferric enzyme exhibited an A_{405}/A_{280}[3] value of 1.7–1.8 in 20 mM potassium phosphate buffer at pH 6.0 and 25 °C and was 60–70% pure as judged by sodium dodecyl sulfate gel electrophoresis (Sono & Dawson, 1984). The amount of enzyme was expressed in terms of its heme content on the basis of absorbance at 405 nm[3] [ϵ = 159 mM^{-1} cm^{-1} at pH 6.0 and 25 °C (Sono & Dawson, 1984)]. Bovine liver catalase was a product of Sigma.

Enzyme Kinetic Experiments. The assays of indoleamine 2,3-dioxygenase were performed at 25 °C. The reaction mixture (1.0 mL) consisted of 0.1 M potassium phosphate buffer (pH 6–8), 25 μM methylene blue, 200 μg of catalase, 10 mM ascorbic acid, 50 nM dioxygenase, and various concentrations of L- or D-Trp and the inhibitor compounds as described previously for a standard assay (Sono et al., 1980). The rate of product formation ($\Delta\epsilon_{321}$ = 3.75 mM^{-1} cm^{-1} for *N*-formylkynurenine) was determined from the slope of the initial linear absorbance increase at 321 nm as a function of time.

Titrations of Indoleamine 2,3-Dioxygenase with Norharman, 4-Phenylimidazole, and Cyanide. Spectrophotometric titrations of the ferric and ferrous forms of indoleamine 2,3-dioxygenase with the inhibitor norharman and the heme ligands 4-phenylimidazole and cyanide (ferric form only) were performed either under air in an open cuvette (ferric derivatives) or under nitrogen in a rubber-septum-stoppered cuvette in the presence of excess sodium dithionite (ferrous derivatives) (Sono et al., 1980). Microliter volumes of the following concentrated titrant stock solutions were added to the enzyme solution in a stepwise manner by using an airtight microsyringe: 80 mM norharman in 0.1 N HCl and 1 M 4-phenylimidazole in ethanol, which were further diluted with H_2O and ethanol, respectively, when necessary. All titration experiments were performed in 0.1 M potassium phosphate buffer (pH 5.5–8.0) and in 0.1 M Tris-HCl buffer (pH 7.5–8.0) at 25 °C. Analysis of titration results for K_d value determinations was carried out by using either double-reciprocal plots or Hill plots (Sono et al., 1980). All data were confirmed to be reproducible within 10%.

Spectroscopic Measurements. Optical absorption spectra and MCD/CD spectra were recorded on a Varian Cary 219 spectrophotometer and a Jasco J-40 spectropolarimeter, respectively, both equipped with a circulator for temperature control (±1 °C). All experiments were carried out in 0.1 M potassium phosphate buffer at 25 °C unless otherwise indicated. The detailed methods and instrumental conditions for the data acquisition, storage, and manipulations are described elsewhere (Sono et al., 1982).

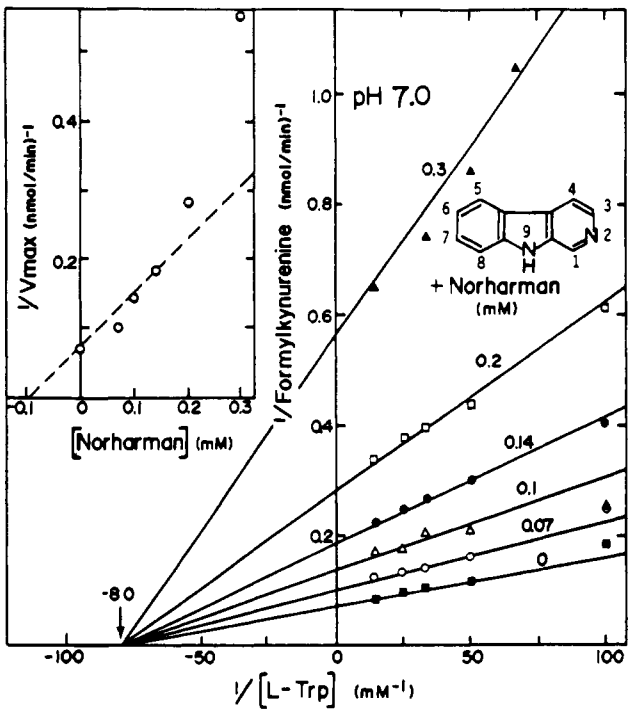

FIGURE 1: Double-reciprocal plot of the rate of the product (*N*-formylkynurenine) formation versus the substrate L-Trp concentration in the presence and absence of varying concentrations of norharman. Assays were carried out at pH 7.0. The concentrations of norharman (0–0.3 mM) used for each plot are indicated in the figure. A structure of norharman is shown in the figure depicting its numbering system. In the inset, a replot of the $1/V_{max}$ value versus norharman concentration is shown. See Experimental Procedures and Results for further details.

Results

Enzyme Kinetics of Norharman and 4-Phenylimidazole Inhibition of the Conversion of Trp to N-Formylkynurenine Catalyzed by Indoleamine 2,3-Dioxygenase. Although enzyme kinetic results using norharman as an inhibitor with L-Trp as a substrate have been previously reported by Eguchi et al. (1984), the experiments were repeated in this study. The present examination confirmed their findings except that a different type of inhibition (i.e., noncompetitive rather than uncompetitive)[4] was observed in this study (see below and Discussion). In addition, in the course of another study on ligand binding properties of the dioxygenase, 4-phenylimidazole, a known heme ligand, has been found to behave in a manner very similar to that of norharman in terms of spectral properties and dissociation constants of their complexes with the enzyme (see the following section). Hence, a parallel enzyme kinetic study with 4-phenylimidazole was also performed for comparison. Double-reciprocal plots for the rates of the product *N*-formylkynurenine formation from L-Trp and D-Trp in the presence of varying concentrations of norharman (0–0.3 mM) at pH 7.0 for L-Trp and of 4-phenylimidazole (0–4 μM) at pH 8.0 for D-Trp under air are shown in Figures 1 and 2, respectively. The results with both norharman and

[3] Careful reexamination of the absorption spectrum of the native ferric indoleamine 2,3-dioxygenase, using known heme proteins as controls (e.g., ferric sperm whale myoglobin, λ_{max} = 409 nm at pH 6.0), has repeatedly shown that its Soret peak position is 405 nm in 0.02–0.1 M potassium phosphate buffer at pH 5.5–8 and 25 °C rather than 406 nm as reported and mentioned in previous studies.

[4] Noncompetitive and uncompetitive inhibition mechanisms are distinguished depending on whether the inhibitor binds to both the substrate-free and -bound enzyme (noncompetitive) or only to the enzyme–substrate complex (uncompetitive).

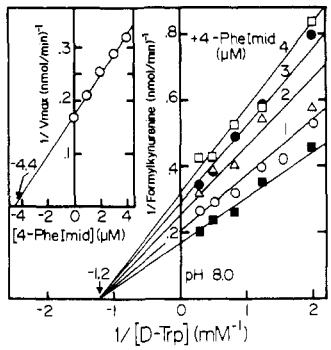

FIGURE 2: Double-reciprocal plot of the rate of the product (*N*-formylkynurenine) formation versus the substrate D-Trp concentration in the presence and absence of varying concentrations of 4-phenylimidazole (4-PheImid). Assays were performed at pH 8.0. The concentrations of 4-PheImid (0–4 μM) used for each plot are indicated in the figure. In the inset, a replot of the $1/V_{max}$ value versus the 4-PheImid concentration is shown.

4-phenylimidazole are indicative of a noncompetitive inhibition mechanism and yield straight lines with different slopes having a common crossover point on the x axis. The observed inhibition pattern implies that norharman and 4-phenylimidazole bind to the dioxygenase *both* in the presence *and* in the absence of the substrate L-Trp.

To determine the K_i values, the results are replotted in the insets ($1/V_{max}$ versus [inhibitor]). For the case of norharman (Figure 1), the data in the replot deviate upward from a straight line (cf. dashed line) at higher norharman concentrations, although all points are supposed to lie on a straight line for typical noncompetitive inhibition. This behavior of the inhibitor norharman will be discussed later. Because of the deviation, the accurate K_i value cannot be determined, but it is estimated to be about 0.1 mM from the data at norharman concentrations below 0.14 mM (dashed straight line). Similar noncompetitive-type double-reciprocal plots have also been obtained with D-Trp (K_m = 0.83 mM, K_i = ~0.05 mM at pH 8.0; plots not shown).

The magnitude of the inhibition by norharman becomes considerably smaller by a factor of over 2 when the assay is carried out in O_2-saturated buffer as compared with the results obtained in air-saturated buffer. Quantitative analysis of the effects of O_2 concentration has not been attempted in this study because the ascorbic acid–methylene blue cofactor system itself consumes O_2 to a significant extent (Hirata & Hayaishi, 1975).

For the case of 4-phenylimidazole, a replot of the double-reciprocal plots yields an expected linear correlation for $1/V_{max}$ versus [4-phenylimidazole] (Figure 2, inset). The K_i value is determined from the replot to be 4.4 μM at pH 8.0. The K_i value for D-Trp at pH 7.0 is 8.0 μM. Comparable K_i values are obtained with L-Trp under the same conditions (plots not shown).

Titrations of Ferric and Ferrous Indoleamine 2,3-Dioxygenase with Norharman and 4-Phenylimidazole. Norharman is found in this study to cause large spectral changes upon binding to both the ferric and ferrous dioxygenase. Consistent with the noncompetitive inhibition type enzyme kinetic results described above, norharman can bind to the enzyme both in the presence and in the absence of substrate L-Trp. Typical results of spectrophotometric titrations of the native ferric and L-Trp-bound ferrous enzyme with norharman in the Soret region are shown in Figure 3, parts A and B, respectively. The native ferric enzyme undergoes a red shift in its Soret peak (405 nm) upon complex formation concomitant with a decrease in intensity with an isosbestic point at 411.5 nm (Figure 3A). Similar spectral changes are also seen for the ferric enzyme in the presence of L-Trp except that the changes are much smaller (not shown). This is because both the peak positions and the intensities of the spectra of the L-Trp complex and the norharman complex of the ferric enzyme are similar in the Soret region. For the ferrous dioxygenase, on the other hand, the binding of norharman causes a blue shift in the Soret peak (~428 nm) for both the free and Trp-bound (Figure 3B) enzyme to form an intense peak at 421 nm.

Double-reciprocal plots for the absorbance change versus norharman concentration yield a straight line (insets in Figure 3A,B) for all cases examined. This indicates that norharman forms a complex with the dioxygenase in a 1:1 molar ratio both in the presence and in the absence of L-Trp. This stoichiometry is further confirmed with Hill plots of the data (not shown), which yield straight lines with a slope of unity for all cases

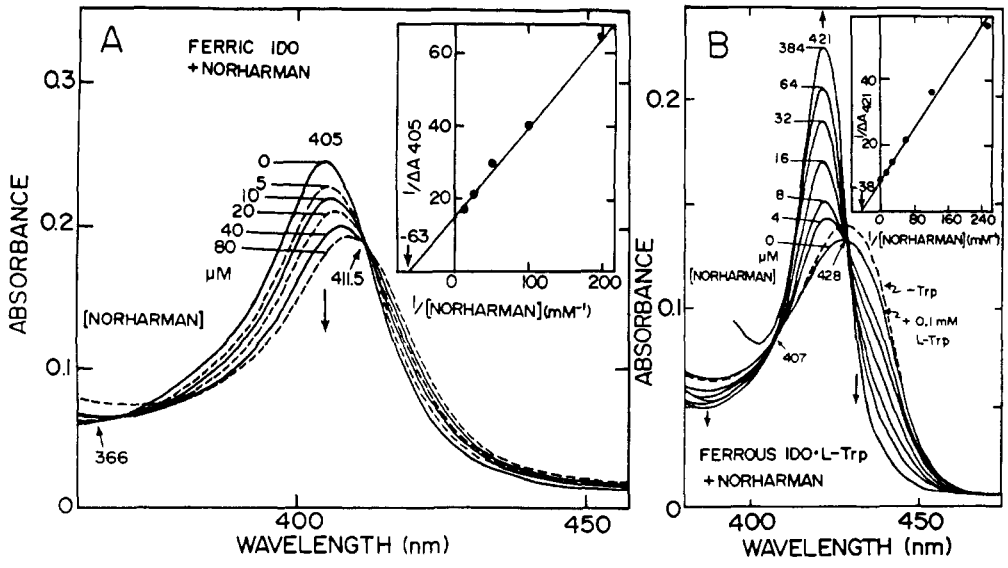

FIGURE 3: Absorption spectral titrations of ferric (A) and ferrous (B) indoleamine 2,3-dioxygenase (IDO) in the Soret region with norharman in the presence (ferrous enzyme) and absence (ferric enzyme) of L-Trp (0.1 mM). Both titrations were done at pH 7.0 by using a 0.2-cm cuvette. The increments in the norharman concentration for respective spectra are indicated in the figure. The directions of the absorbance changes upon complex formation are indicated with vertical arrows. Double-reciprocal plots for the titrations are shown in the inset. Note that at >40 μM norharman concentrations, its absorbance below 410 nm becomes considerably large.

Table I: Apparent Dissociation Constants (K_d^{app}) of the Ferric and Ferrous Indoleamine 2,3-Dioxygenase (IDO) Complexes with Norharman and 4-Phenylimidazole (4-PheImid) at Various pH Values[a]

oxidation state of IDO	ligand	K_d^{app} value (μM) at pH 6.0	7.0	8.0
ferric	norharman	87.1	15.8	9.1
	4-PheImid	25	10	5.0
ferrous	norharman	39.8	7.8	5.8[b]
	4-PheImid	1000	430	350[c]

[a] All data were determined in 0.1 M potassium phosphate buffer. [b] At pH 7.5. [c] At pH 7.7.

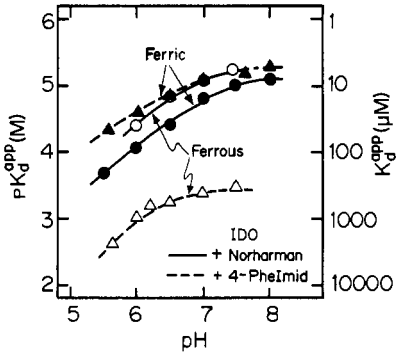

FIGURE 4: Effects of pH on the apparent dissociation constants (K_d^{app}) of the norharman and of 4-phenylimidazole (4-PheImid) adducts of ferric and ferrous indoleamine 2,3-dioxygenase (IDO). The measurements were performed at various pH values in 0.1 M potassium phosphate buffer (pH 5.5–7.7) and in 0.1 M Tris-HCl buffer (pH 8.0). The results shown are for the ferric enzyme complexes with norharman (●) and 4-PheImid (▲) and for the ferrous enzyme complexes with norharman (○) and 4-PheImid (△). Titrations of ferrous IDO with norharman and 4-PheImid at pH 8.0 in either of the above buffers were unsuccessful due to the incomplete formation of the complexes.

[see Sono et al. (1980)]. The apparent K_d values for the ferric (−Trp) and ferrous (+0.1 mM L-Trp) enzyme (Figure 3A,B), as determined from the x intercepts of the insets ($-1/K_d$), are 16 and 26 μM, respectively. Spectrophotometric titrations of both the ferric and ferrous dioxygenase with 4-phenylimidazole were also carried out. Dissociation constants of the enzyme adducts of both norharman and 4-phenylimidazole at various pH values are summarized in Table I.

Effects of pH, the Substrate L-*Trp, and the Heme Ligand Cyanide on the Norharman and 4-Phenylimidazole Affinities of Indoleamine 2,3-Dioxygenase.* To further probe the norharman binding site in indoleamine 2,3-dioxygenase, effects of pH (Figure 4) and L-Trp (Figure 5) on the affinities of the compound for the ferric and ferrous enzyme were examined. Within an accessible pH range for the dioxygenase (pH 5.5–8.0), the apparent affinity, pK_d^{app}, increases (i.e., the K_d^{app} value decreases) with an increase in the pH for both the ferric and ferrous enzyme with a slope of near unity below pH 6.5. Above pH 7.5, the pK_d^{app} value appears to approach a constant value. An inflection point is seen around pH 7.0. Although the pH range and the number of the data points are quite limited, the results are indicative of the involvement of a single ionizable group with a pK_a value of about 7.0 for the binding of norharman to both the ferric and ferrous enzyme [cf. Sono et al. (1986)]. Note that the ferrous enzyme has slightly higher affinity for norharman than the ferric enzyme by a factor of ~1.6 throughout the pH range examined.

Similar pH profiles for the affinity of 4-phenylimidazole for the respective cases of the ferric and ferrous enzyme are observed (dashed lines in Figure 4). However, the 4-phenylimidazole affinity of the ferric enzyme, which is nearly

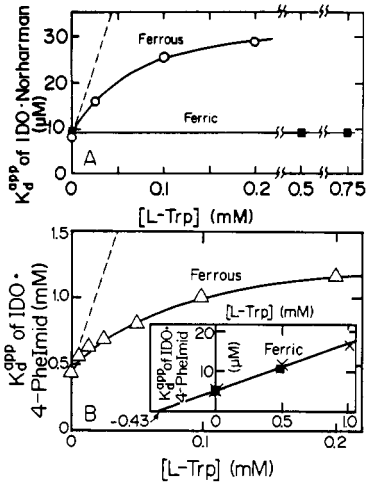

FIGURE 5: Effects of L-Trp on the apparent dissociation constants (K_d^{app}) of the norharman (A) and 4-phenylimidazole (4-PheImid) (B) adducts of ferric and ferrous indoleamine 2,3-dioxygenase. The data for the ferrous enzyme were obtained in 0.1 M potassium phosphate buffer, pH 7.0, for norharman (○) in (A) and 4-PheImid (△) in (B). For the ferric enzyme, 0.1 M Tris-HCl buffer, pH 8.0, for both norharman and 4-PheImid [■ for (A) and (B)] and 0.1 M potassium phosphate, pH 8.0, for 4-PheImid (×) were used. The dashed lines in both (A) and (B) are drawn on the basis of the assumption that L-Trp and norharman (A) or 4-PheImid (B) are competitive for the ferrous enzyme. In both cases, the dashed straight line has the x intercept of $-K_d$ (=−13 μM) (not shown), which is the negative value of the dissociation constant of the L-Trp–ferrous enzyme complex at pH 7.0 (Sono et al., 1980).

comparable to the norharman affinity of the ferric enzyme, is considerably higher than that of the ferrous enzyme by factors of 40–70. In addition, an inflection point for the 4-phenylimidazole binding is shifted to a lower pH value by about 1 pH unit than that for norharman for both oxidation states of the enzyme.

L-Trp exerts virtually no effect on the norharman affinity of the ferric enzyme (Figure 5A, solid square), at least up to 0.75 mM; the K_d value of the L-Trp–ferric enzyme complex at pH 8.0 is 0.51 mM (Sono et al., 1980). Because of the close spectral similarity between the norharman-free and -bound L-Trp–ferric enzyme adducts, spectrophotometric titrations at higher L-Trp concentrations were not attempted. On the other hand, the apparent K_d value of the norharman–ferrous enzyme complex (K_d = 7.8 μM at pH 7.0 without L-Trp; Table I) increases with an increase in L-Trp concentration (0–0.2 mM) in an apparent hyperbolic mode as shown by open circles in Figure 5A. This is typical for the negatively cooperative binding of two ligands to an enzyme. Hence, norharman and L-Trp are not competitive for the ferrous enzyme, but they mutually lower the other's affinity for the enzyme. This can be clearly distinguished from the competitive binding of norharman and L-Trp, which would yield a straight line as indicated by a dashed line in Figure 5A [refer to Sono et al. (1982)]. The upper limit of the K_d value for norharman at an infinite concentration of L-Trp can be estimated from the plot (Figure 5A, open circle) to be 30–35 μM. Thus, the norharman affinity is about 4 times lower for the L-Trp-bound ferrous enzyme than for the substrate-free ferrous enzyme.

The effects of L-Trp on the 4-phenylimidazole affinity of the ferrous enzyme (Figure 5B, open triangles) are very similar to those observed with norharman (Figure 5A, open circles). Thus, 4-phenylimidazole can bind to the ferrous enzyme both in the presence and in the absence of the substrate L-Trp. In contrast, 4-phenylimidazole and L-Trp are found to compete for the ferric enzyme as shown in the inset of Figure 5B. This

Table II: Optical Absorption Spectral Parameters for the Ferric and Ferrous Indoleamine 2,3-Dioxygenase Complexes with Norharman and with Some Nitrogen Donor Ligands[a]

enzyme complex[b]	absorption bands, λ_{nm} [ϵ (mM^{-1} cm^{-1})]			
	δ	Soret	β	α
ferric enzyme				
+NHM	357 (34.5)	410.5 (125)	530 (11.8)	~556[c] (9.6)
+NHM + L-Trp (10 mM)	357 (34.2)	411 (123)	531 (11.6)	~556[c] (9.8)
+Imid[d]	357 (27.6)	411 (127)	534 (11.6)	~560[c] (9.7)
+4-PheImid	358 (28.3)	412.5 (130)	533 (12.5)	~560[c] (10.1)
+pyridine[d,e]	358 (34.0)	419 (111)	538 (11.3)	~570[c] (8.8)
ferrous enzyme				
+NHM	*f*	421 (175)	527 (18.6)	556 (31.2)
+NHM + L-Trp (1 mM)	*f*	421 (170)	527 (18.3)	556 (30.1)
+NHM + L-Trp (10 mM)	*f*	421 (159)	527 (16.7)	556 (27.8)
+Imid[d]	*f*	424 (155)	529 (13.1)	558 (25.3)
+4-PheImid	*f*	423.5 (193)	529 (14.8)	557 (29.4)
+4-PheImid + L-Trp (1 mM)	*f*	423.5 (185)	529 (14.6)	557 (27.6)

[a] All data were obtained in 0.1 M potassium phosphate buffer at pH 8.0 for the ferric and at pH 7.0 for the ferrous enzyme adducts at 25 °C. [b] Abbreviations and concentrations of the ligands used are (NHM) norharman, 0.35 mM (–L-Trp, ferrous enzyme), 0.45 mM (±L-Trp, ferric enzyme), and 0.60 mM (+L-Trp, ferrous enzyme); (Imid) imidazole, 1 M; (4-PheImid) 4-phenylimidazole, 8 mM. [c] Shoulder. [d] Except for the α, β, and δ bands for the ferric complexes, the data have been previously reported (Sono & Dawson, 1984). [e] At pH 7.0 and 4 °C. [f] Not determined.

is clearly demonstrated by a straight line with an x intercept of –0.43 mM, which nearly coincides with the $-K_d$ value (–0.51 mM; Sono et al., 1980) [refer to Sono et al. (1982) for the interpretation of the plot].

The effects of cyanide, a typical heme ligand, on the binding of norharman to the dioxygenase were also examined. Since any effect should be reciprocal (Weber, 1975), the ferric enzyme was titrated with cyanide in the presence of various concentrations (0–60 μM) of norharman. A plot of the apparent K_d value of the ferric enzyme–cyanide complex as a function of norharman concentration (0, 20, 40, and 60 μM) yields a straight line (plot not shown). The x intercept is –20 (μM) at pH 7.0, which almost coincides with the $-K_d$ value for the ferric enzyme–norharman adduct (K_d = 16 μM; see Figure 3A). The results clearly indicate that norharman and cyanide are competitive for the ferric enzyme. This, in turn, demonstrates that norharman binds directly to the heme iron of the ferric dioxygenase.

Analogous but only qualitative competition experiments with the ferrous dioxygenase for the binding of norharman and heme ligands, such as CO and NO, were also performed. The affinities of CO and NO for the ferrous enzyme are too high to quantitatively examine the displacement of these heme ligands by norharman or vice versa. Nevertheless, the small changes observed in the absorption spectra of the ferrous–CO [λ_{max} = 420 nm (Sono et al., 1980)] and ferrous–NO enzyme [λ_{max} = 418.5 nm (Sono & Dawson, 1984)] upon additions of norharman up to its near solubility limit (<1 mM at pH 7.0) appear to be dependent on the concentration of norharman. The observed changes are indicative of the partial formation (<10%) of the norharman–ferrous enzyme adduct (λ_{max} = 421 nm). Thus, these results are consistent with the competitive binding of norharman and a heme ligand to the ferrous heme iron of the dioxygenase.

Characterization of the Norharman and 4-Phenylimidazole Adducts of Ferric and Ferrous Indoleamine 2,3-Dioxygenase by Optical Absorption, MCD, and CD Spectroscopy. Spectroscopic properties of the norharman and 4-phenylimidazole complexes of the enzyme including their visible region features (500–700 nm) were examined. Absorption spectral parameters (peak positions and extinction coefficients) of the resulting complexes in the presence and absence of L-Trp are summarized in Table II. Data for the imidazole and pyridine complexes are also included for comparison. The absorption spectra of the norharman-saturated ferric indoleamine 2,3-dioxygenase in both the presence and absence of L-Trp (Figure 6, bottom) are nearly identical. These spectra are also very similar to those of the imidazole (Table II) and 4-phenylimidazole (Figure 6, bottom, dotted line) of the ferric enzyme. A close similarity is also seen in the MCD spectra of the ferric complexes with norharman and 4-phenylimidazole (Figure 6, top). The visible region features are characteristic of a bis(imidazole)-type low-spin ferric heme protein such as ferricytochrome b_5 (Vickery et al., 1976). Thus, norharman binds to the heme iron through one of its two nitrogen atoms, most likely through the one at the 2-position (see Figure 1). In support of this interpretation, harman, in which a methyl group is bound to the carbon adjacent to this nitrogen, hardly binds to the heme iron (this work). This is probably due to steric

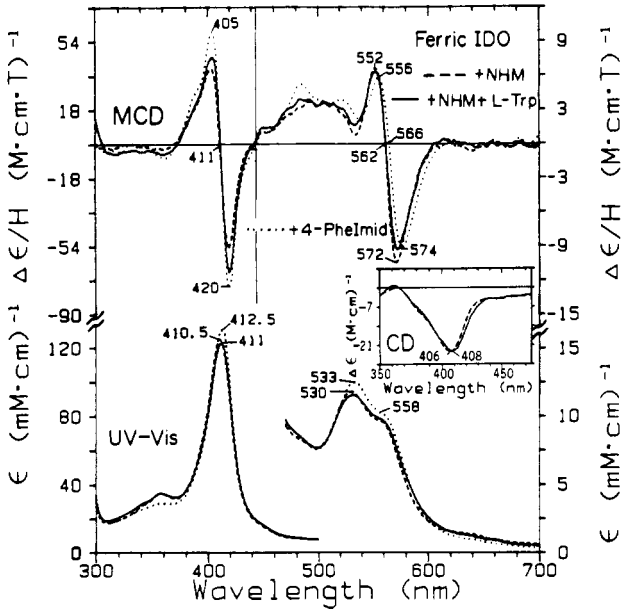

FIGURE 6: Optical absorption (UV–vis), MCD, and CD spectra of ferric indoleamine 2,3-dioxygenase (IDO) complexes with norharman in the presence and absence of L-Trp and with 4-phenylimidazole (4-PheImid) in the absence of L-Trp. The complexes were prepared with 40–50 μM enzyme and ~450 μM norharman in the presence (solid line) and absence (dashed line) of 10 mM L-Trp at pH 8.0. Optical absorption and MCD spectra of the 4-phenylimidazole (8 mM) complex of the ferric enzyme at pH 8.0 in the absence of L-Trp are overplotted (dotted line). In the inset, CD spectra of the norharman complexes of the enzyme with and without 10 mM L-Trp are shown. The cuvette path length was 0.2 cm for all cases except that a 1.0-cm cuvette was used for the recording of the visible region (440–700 nm) MCD spectra.

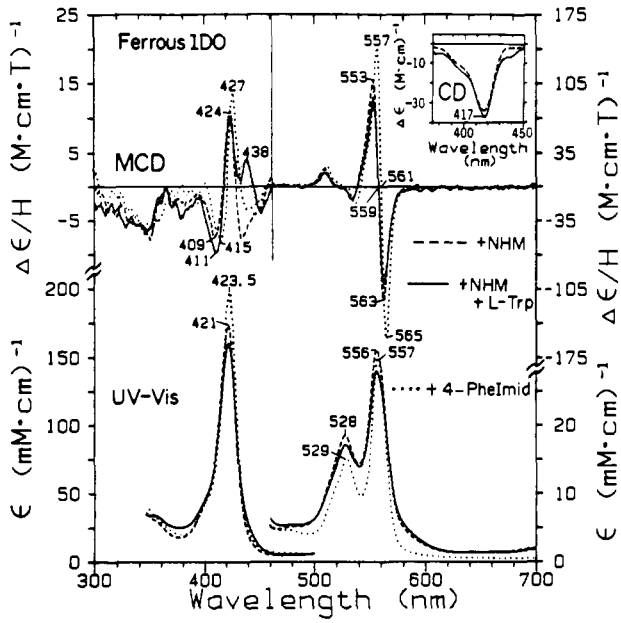

FIGURE 7: Optical absorption, MCD, and CD spectra of ferrous indoleamine 2,3-dioxygenase (IDO) complexes with norharman in the presence (solid line) and absence (dashed line) of L-Trp (10 mM) and with 4-phenylimidazole (4-PheImid) in the absence of L-Trp (dotted line). The spectra were obtained at pH 7.0 in the presence of 0.35 mM (without Trp) and 0.60 mM (+10 mM L-Trp) norharman and in the presence of 8 mM 4-PheImid (without Trp). Spectra below 350 nm are not shown here because the reductant sodium dithionite absorbs in this region (λ_{max}, 315 nm). The inset displays the CD spectra of the norharman complexes of the enzyme with (solid line) and without 10 mM L-Trp (dashed line). The measurements were performed in a 0.2-cm cuvette under nitrogen in the presence of a slight excess of sodium dithionite.

hindrance by the methyl group. Harman was previously reported not to inhibit the catalytic reaction of indoleamine 2,3-dioxygenase (Eguchi et al., 1984). The nitrogen atom in the indole moiety at the 9-position of norharman (see Figure 1) is unlikely to coordinate to the heme iron because of its sterically unfavorable position. In fact, none of the 3-substituted indole derivatives examined in this study or in other previous studies (Eguchi et al., 1984) appears to bind to the heme iron of the dioxygenase. The pyridine complex of the ferric enzyme exhibits absorption (Table II) and MCD spectra [a visible region trough at 579 nm; see Sono and Dawson (1984) for the Soret region feature] that are somewhat different from those of the norharman-ligated ferric dioxygenase. Despite the close similarity in the Soret region absorption spectra (vide supra), the ferric enzyme complexes with norharman (Figure 6) and L-Trp (Sono & Dawson, 1984) exhibit distinctly different absorption and MCD spectral features in the visible region. CD spectra of the norharman–ferric enzyme adduct in the presence and absence of bound L-Trp differ only slightly (Figure 6, inset). The 4-phenylimidazole adduct of the ferric enzyme exhibits a negative-sign Soret CD spectrum with a trough at 409 nm ($\Delta\epsilon = -31$ M^{-1} cm^{-1}) (not shown).

The ferrous enzyme complexes with norharman and 4-phenylimidazole also exhibit very similar absorption (Table II) and MCD spectra with respect to each other as shown in Figure 7. Both of these complexes have relatively intense Soret and visible region absorption peaks (Figure 7, bottom). Noticeable in their MCD spectra is an intense derivative-shaped feature in the visible region whose crossover point roughly corresponds to the position of the absorption α-band. The 4-phenylimidazole adduct has somewhat more intense features than the norharman adduct in its Soret absorption peak and in the visible region MCD peak and trough. Small differences are also seen between the spectra of the norharman–ferrous enzyme adduct with (solid line) and without (dashed line) L-Trp. An additional MCD peak at 438 nm is observed for the complex in the presence of L-Trp (10 mM) (Figure 7, top). This is attributable to the partial formation of the norharman-free L-Trp–ferrous enzyme complex, which has a very intense MCD peak near this wavelength [$\Delta\epsilon/H$ = 90 M^{-1} cm^{-1} T^{-1} at 434 nm (Sono & Dawson, 1984)]. Since norharman and L-Trp exhibit negative cooperativity in their binding to the ferrous enzyme (vide supra), this extra band is likely caused by the addition of a high concentration of L-Trp (10 mM) to the norharman–ferrous enzyme complex. The ratio (n) of the free ligand concentration to the K_d value for one ligand in the presence of infinite concentrations of the other ligand at pH 7.0 (Figure 5A) is considerably greater for L-Trp (n = 10 mM/$\sim$4 × 0.013 mM = $\sim$192) than for norharman (n = 600 μM/$\sim$4 × 7.8 μM = $\sim$19.2). Under these conditions, the estimated fractional saturations [$Y = n/(n + 1)$][5] of the ferrous enzyme with norharman and L-Trp are $\sim$95% and $\sim$99.5%, respectively. Thus, the norharman saturation decreases from 98.5% (n = 600/7.8 = 76.8) to 95% (n = 19.2) upon binding of L-Trp (10 mM). The small difference observed in the CD spectra of the norharman adducts of the ferrous enzyme with and without L-Trp (inset of Figure 7) can also be attributed to the same cause. Such small absorption spectral differences are almost negligible when less than 1 mM ($19 \leq n$) L-Trp is added (Table II). Hence, the ferrous enzyme adducts with norharman appear to have nearly identical spectral properties in the L-Trp-free and -bound forms. The effects of L-Trp on the spectral properties of the 4-phenylimidazole adducts of the ferrous dioxygenase (Table II) (Soret CD trough, $\Delta\epsilon_{418} = -26$ M^{-1} cm^{-1} with and without 1 mM L-Trp) are also similar to the results of norharman. Higher concentrations of L-Trp ($\geq$ 1 mM) cause partial dissociation of the bound 4-phenylimidazole (Table II).

Discussion

Nature of Norharman as an Inhibitor. The present spectroscopic binding experiments have clearly demonstrated that the heme iron of indoleamine 2,3-dioxygenase is the binding site for the inhibitor norharman. It has also been shown that norharman can bind to the enzyme not only in the presence but also in the absence of L-Trp. This is consistent with a noncompetitive-type inhibition mechanism. The different conclusion drawn previously by Eguchi et al. (1984) most likely arose from difficulties in interpreting the somewhat scattered data in their radioisotope enzyme assays using L-[*ring*-2-^{14}C]Trp as a labeled substrate (Ohnishi et al., 1977). The indirect and qualitative experimental results in this work support the assumption that norharman is a competitive inhibitor with respect to O_2. The considerably large value for K_i ($\sim$0.1 mM at pH 7.0; Figure 1A) as compared with the K_d value (0.01–0.03 mM at pH 7.0; Figure 5A) for the complex of the ferrous enzyme with norharman is thus attributable to the competitive binding of norharman and O_2 ($=\sim$0.25 mM under air) to the enzyme heme iron. The apparent lack of effects of norharman concentration on the K_m values for L-Trp in the inhibition experiment results shown in Figure 1A, although slightly inconsistent with the negative cooperativity between L-Trp and norharman in their binding to the ferrous enzyme (Figure 5B), may also be explained by the same reasoning. The "effective" concentrations of norharman under air for its complex formation with the ferrous enzyme are only

[5] The fractional saturation with ligand: $Y = [L]_f/([L]_f + K_d) = n/(n + 1)$, where $[L]_f$ is the concentration of free ligand and $n = [L]_f/K_d$.

about $^1/_{10}$ the actual values (K_d versus K_i).

The somewhat unusual behavior of norharman as a noncompetitive inhibitor at its higher concentrations (see Figure 1, inset) may be interpreted, at least in part, as follows. The binding of norharman to the enzyme heme iron converts its spin state from predominantly high spin (Uchida et al., 1983; Sono & Dawson, 1984) to low spin. This spin-state change most likely shifts the redox potential of the enzyme to a lower value. The redox potential of a heme protein is often sensitive to its spin state; e.g., values for the ferric/ferrous couples of both horseradish peroxidase (Harburg, 1957) and sperm whale myoglobin (Brunori et al., 1971) are noticeably lower in the alkaline (high pH) low-spin form than in the acidic high-spin form. This probable redox potential change would inhibit the reductive activation of the enzyme by the ascorbic acid–methylene blue cofactor system (Sono, 1989a). Such an effect of norharman might become more significant at higher norharman concentrations (i.e., at higher saturation levels of the enzyme with this compound), causing an apparent increase in the inhibition by norharman to a greater extent than expected for a simple noncompetitive-type inhibition.

An additional explanation for the unexpected effect of norharman as a noncompetitive inhibitor is that the K_i values for the reactions E + I $\rightleftharpoons$ EI and ES + I $\rightleftharpoons$ ESI, where E, I, and S represent the enzyme dioxygenase, the inhibitor norharman, and the substrate L-Trp, respectively, might not be identical. In fact, the spectrophotometric titration data presented in Figure 5 suggest that this might be the case.

Cause of the Noncompetitive Inhibition by 4-Phenylimidazole. The inhibitory effects of 4-phenylimidazole on the conversion of Trp to *N*-formylkynurenine catalyzed by indoleamine 2,3-dioxygenase seem to arise from a somewhat different cause from that of the above case of norharman. The affinity of 4-phenylimidazole for the ferric enzyme is more than 40 times higher than that for the ferrous enzyme. The observed K_i value for the noncompetitive inhibition (with respect to Trp) by 4-phenylimidazole at pH 7.0 is 8.0 μM, which is nearly identical with the K_d value of its complex with the ferric (K_d = 10 μM) rather than with the ferrous enzyme (K_d = 430 μM). Hence, it appears that the binding of 4-phenylimidazole to the ferric rather than the ferrous enzyme is directly reflected in the inhibitory effects of this ligand.

These observations can be interpreted in terms of the influence of the binding of this ligand on the redox potential of the dioxygenase. A previous study by Brunori et al. (1971) demonstrated that the redox potential of sperm whale myoglobin (ϵ_3' = 0.05 V at pH 8.0 and 30 °C) drops considerably by ~140 mV upon addition of saturating amounts of imidazole. In analogy to the present case of the 4-phenylimidazole binding to indoleamine 2,3-dioxygenase, the imidazole affinity of sperm whale myoglobin is about 188 times higher in its ferric state than in its ferrous state [K_d values of 8 mM versus ~1.5 M at pH 8.0 (Brunori et al., 1971)]. Thus, for indoleamine 2,3-dioxygenase, 4-phenylimidazole most likely shifts the ferric form $\leftrightarrow$ ferrous form equilibrium to the left, resulting in a lower redox potential. This would cause inhibition of the reductive activation of the dioxygenase by the ascorbic acid–methylene blue cofactor system (Sono, 1989a). In a sense, 4-phenylimidazole may be considered to be competing with an electron for the ferric enzyme.

It should be noted that the level of 4-phenylimidazole concentration (1–10 μM) which causes the effective inhibition of the dioxygenase activity is too low to form a complex with the ferrous enzyme (K_d = ~400 μM). Under these conditions little competition will occur between O_2 and 4-phenylimidazole for the ferrous enzyme. Therefore, even though the apparent inhibition modes for norharman and 4-phenylimidazole are similar (i.e., noncompetitive with respect to the substrates L-Trp and D-Trp), the actual mechanisms of the inhibition of the dioxygenase activity by these two compounds are distinctly different. Norharman competes with O_2 for the ferrous (active) enzyme, while 4-phenylimidazole competes with an electron (or a reducing equivalent) for the ferric (inactive) enzyme. It should also be mentioned that, although a millimolar concentration level of L-Trp can compete with 4-phenylimidazole for the ferric enzyme (Figure 5B, inset), such high concentrations of L-Trp will cause substrate inhibition (Yamamoto & Hayaishi, 1967; Sono et al., 1980).

Binding Mode and pH Profiles of Norharman and 4-Phenylimidazole. The relatively high affinity of norharman as a heme ligand for the enzyme (K_d = 6–8 μM at pH 8.0; Figure 4) is most probably due to its hydrophobic interactions with the enzyme active site in addition to its ligation to the heme iron. In fact, 4-phenylimidazole exhibits about 3 orders of magnitude higher affinity for the ferric enzyme (K_d = 5 μM) than imidazole (K_d = ~5 mM)[6] at pH 8.0. The apparent pK_a value of about 7 detected for the norharman binding to both the ferric and ferrous enzymes (Figure 4) might likely reflect the pK_a value of this compound for the nitrogen base at the 2-position [cf. pK_a = 7.37 for harman (Perrin, 1965)] rather than that of an ionizable residue in the enzyme. This interpretation is supported by the similar results with 4-phenylimidazole [pK_a = 6.0 at 25 °C (Perrin, 1965)]. The pH profile for 4-phenylimidazole that is shifted to a lower value by about 1 pH unit (Figure 3) from that for norharman is attributed to the difference in the pK_a values in their enzyme-free forms. The results shown in Figure 4 are consistent with the fact that within the pH range (pH 5.5–8.0) examined only the neutral (unprotonated) forms of norharman and 4-phenylimidazole can coordinate to the heme iron of the dioxygenase.

The slightly negative cooperativity in the binding of norharman and the substrate L-Trp to ferrous indoleamine 2,3-dioxygenase (Figure 5) suggests a significant steric interaction between these compounds within the enzyme active site. For other smaller ferric heme ligands, such as cyanide, azide, and fluoride, a strong positive cooperativity was observed between L-Trp and these ligands (Sono & Hayaishi, 1980). Preliminary experiments in this study have suggested that, unlike the case of indoleamine 2,3-dioxygenase, the heme iron of *Pseudomonas* tryptophan 2,3-dioxygenase might not be the binding site for norharman,[6] a competitive inhibitor with respect to L-Trp for the latter dioxygenase (Eguchi et al., 1984). 4-Phenylimidazole seems to bind only to the heme iron of ferric tryptophan 2,3-dioxygenase (K_d = ~5 mM at pH 8.0) both in the presence and in the absence of L-Trp.[6] Hence, indoleamine 2,3-dioxygenase appears to have a somewhat different, larger, active-site (heme pocket) structure than tryptophan 2,3-dioxygenase. This might reflect, at least in part, their different cofactor requirements (Sono, 1989a), as will be further discussed in the following paper (Sono, 1989b).

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[6] M. Sono, unpublished results.

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