Enzyme Kinetic and Spectroscopic Studies of Inhibitor and Effector Interactions with Indoleamine 2,3-Dioxygenase. 2. Evidence for the Existence of Another Binding Site in the Enzyme for Indole Derivative Effectors†

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ABSTRACT: To probe the active site of the heme protein indoleamine 2,3-dioxygenase, the effects of 3-indoleethanol (IET) (or tryptophol), one of the known indole derivative effectors, and indole (IND) on the catalytic (V_{max}, K_m) and spectroscopic properties (optical absorption and CD) of the enzyme 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 shown that, at millimolar concentrations, both IET and IND lower considerably the K_m value for D-Trp by ~25% and ~60%, respectively, at pH 7.0, while neither affects the K_m value for L-Trp. Interestingly, however, these effectors exert opposite effects with respect to each other on the V_{max} values for both D-Trp and L-Trp: IET enhances the V_{max} values by 40–60% while IND lowers them by 12–24%. These effects of IET and IND on the V_{max} values may be attributed to the shift in the ferric (inactive) enzyme ↔ ferrous (active) enzyme equilibrium either to the right (IET) or to the left (IND) caused by the binding of these effectors to the enzyme in the steady state of the catalytic reaction. Both effectors induce clearly detectable spectral changes, especially notable in CD spectra, upon binding (in a 1:1 molar ratio, $K_d = 10^{-4}$ to 2.5×10^{-3} M) to the ferrous enzyme and its complexes with O_2, CO, and NO, both in the presence and in the absence of L-Trp. Similar results are also obtained upon binding of IET and IND to the ferric enzyme adducts with the substrate L-Trp and the heme ligands cyanide and azide. Moreover, additions of IND noticeably enhance the affinities of the ferrous–CO and native ferric enzyme for L-Trp and the affinities of the ferric enzyme for cyanide and azide. These spectroscopic and equilibrium binding results provide clear evidence for the existence of another binding site near that for the substrate L-Trp and the heme iron for a possible natural cofactor or regulator for the enzyme.

Indoleamine 2,3-dioxygenase (Hayaishi et al., 1975) is one of the few heme-containing dioxygenases known to exist. The dioxygenase catalyzes 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. Although the same reaction is catalyzed by tryptophan 2,3-dioxygenase, another heme-containing dioxygenase (Feigelson & Brady, 1974), these two dioxygenases are distinct in several respects including their molecular and immunogenic properties, substrate specificities, biological sources, and tissue distribution (see the preceding paper for references). Protoheme IX is the sole prosthetic group for both dioxygenases (Hirata & Hayaishi, 1975; Ishimura et al., 1980).

Another notable distinction between these two dioxygenases is their cofactor requirement. Several nonspecific reductants such as ascorbic acid, borohydride, and hydrogen peroxide can serve as reductive activators for tryptophan 2,3-dioxygenase in vitro (Feigelson & Brady, 1974). These activators are required only to initiate the catalytic reaction by reducing the ferric (inactive) form, an autoxidation product that is probably a nonphysiological artifact in vitro (Feigelson & Brady, 1974), to the ferrous (active) form of tryptophan 2,3-dioxygenase. On the other hand, indoleamine 2,3-dioxygenase is highly autoxidizable, especially in the presence of O_2 and indoleamine substrates (Hirata et al., 1977). Thus, the dioxygenase requires superoxide anion ($O_2^{\cdot-}$) or a reductant such as ascorbic acid in either case in combination with methylene blue (or toluidine blue), an artificial dye, as cofactors in vitro (Yamamoto & Hayaishi, 1967). These cofactors are required not only to initiate but also to maintain the apparent maximal catalytic activity (Hirata & Hayaishi, 1975; Ohnishi et al., 1977). As candidates for the natural cofactor for indoleamine 2,3-dioxygenase, tetrahydrobiopterin and dihydroriboflavin mononucleotide ($FMNH_2$) have been suggested (Nishikimi, 1975; Ozaki et al., 1987). A recent study on the activation mechanism of this dioxygenase has also suggested the presence of a physiological cofactor or cofactor system instead of $O_2^{\cdot-}$ or in combination with $O_2^{\cdot-}$ (Sono, 1989). Thus, it is quite likely that such a cofactor molecule would bind to the enzyme at a specific site.

Tryptophan 2,3-dioxygenase has been shown to possess two binding sites for L-Trp, a catalytic and a regulatory site (Feigelson & Brady, 1974). L-Trp does not exert such a regulatory role for indoleamine 2,3-dioxygenase except that it exhibits substrate inhibition with $K_i = \sim 300K_m$ at pH 7.0 (Sono et al., 1980). However, some indole derivatives having substituents at the 3-position have been reported to serve as effectors (Eguchi et al., 1984), to enhance the L-Trp affinity for the enzyme (Uchida et al., 1985), or to bind to the L-Trp–ferrous enzyme–O_2 ternary complex of indoleamine 2,3-dioxygenase (Sono, 1986). Although these observations suggest the existence of a binding site(s) in the dioxygenase for these effectors other than that for L-Trp, studies on this

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[1] Abbreviations: Trp, tryptophan; $O_2^{\cdot-}$, superoxide anion radical; MCD, magnetic circular dichroism; Tris, tris(hydroxymethyl)aminoethane; FMN, flavin mononucleotide.

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subject have not been expanded any further.

To clarify the specific binding site(s) for the effector compounds, the changes in the catalytic (K_m, V_{max}), spectral, and substrate and heme ligand binding properties of indoleamine 2,3-dioxygenase caused by 3-indoleethanol (or tryptophol) and indole have been extensively investigated by enzyme kinetic assays and optical absorption, MCD, and CD spectroscopy. These two indole derivatives are selected as the representatives for those examined in previous studies by Eguch et al. (1984). The present studies not only confirmed the previous enzyme kinetic results but also, more significantly, provided clear evidence for the existence of another binding site in the enzyme for the effectors near the substrate binding site and the heme iron. This secondary binding site for some organic compounds might serve as a site for a yet unknown natural cofactor for the dioxygenase.

Experimental Procedures

Chemicals. L-Trp, D-Trp, and L-ascorbic acid were purchased from Sigma, and indole, 3-indoleethanol, and norharman were purchased from Aldrich. Methylene blue and glycerol were purchased from Fisher. All of these chemicals were of reagent grade and were used without further purification. Stock solutions of indole (0.5 M) and 3-indoleethanol (1 M) were prepared by using ethanol as solvent.

Preparations of Various Ferrous Derivatives of Indoleamine 2,3-Dioxygenase. The dioxygen complex of the ferrous enzyme was generated and examined at −30 °C in 65/35 (v/v) glycerol/0.1 M potassium phosphate buffer (pH 7.5) as described previously (Sono, 1986). For the preparations of the ferrous–CO and –NO adducts, refer to Sono et al. (1980) and Sono and Dawson (1984).

Other Materials and Procedures. Refer to Experimental Procedures in the preceding paper in this issue (Sono & Cady, 1989) for indoleamine 2,3-dioxygenase purification, enzyme kinetic experiments, titrations of the dioxygenase with effectors, heme ligands, and L-Trp, and spectroscopic measurements.

Results

Influences of the Effectors Indole and 3-Indoleethanol on the Enzyme Kinetic Parameters (K_m and V_{max}) for the Conversion of Trp to N-Formylkynurenine Catalyzed by Indoleamine 2,3-Dioxygenase. Detailed studies on enzyme kinetics with both L- and D-Trp and on the equilibrium binding of these two effectors were carried out in this work. Of these two effectors, only 3-indoleethanol has been examined in the past for its effects on the catalytic properties (K_m, V_{max}) of the dioxygenase purified from both rabbit small intestine and mouse epididymis by using L-Trp as the substrate (pH 6.6) (Eguchi et al., 1984). Double-reciprocal plots of the present results with L-Trp at pH 6.0 and with D-Trp at pH 7.0 are shown in Figure 1, parts A and B, respectively. While neither effector significantly changes the K_m value for L-Trp (x intercept, $-1/K_m = -20$ mM^{-1}; i.e., $K_m = 50$ μM), the V_{max} value ($1/y$ intercept) is considerably shifted in opposite directions upon additions of these effectors (2 mM). Essentially the same results were obtained with a 1 mM concentration of these effectors. 3-Indoleethanol enhances the apparent maximal enzyme activity by ~87%, while indole lowers it by ~13% for L-Trp under these conditions (Figure 1A). The present results for the effects of 3-indoleethanol on the K_m and V_{max} values are consistent with those previously reported by Eguchi et al. (1984) for L-Trp. The results with D-Trp are more complex. Both the K_m and V_{max} values are affected by these two compounds (Figure 1B); the K_m value is decreased by factors of 1.3 (1.7 to 1.3 mM) by 3-indoleethanol and 2.6 (1.7 to 0.66 mM) by indole at pH 7.0. The V_{max} value is increased by ~60% by the former, but decreased by ~12% by the latter. These results together with those obtained at different pH values are summarized in Table I. Essentially the same trends are seen for both L- and D-Trp at different pH values. It appears that for both of the isomeric Trps, these effectors exert opposite effects with respect to each other on the V_{max} values.

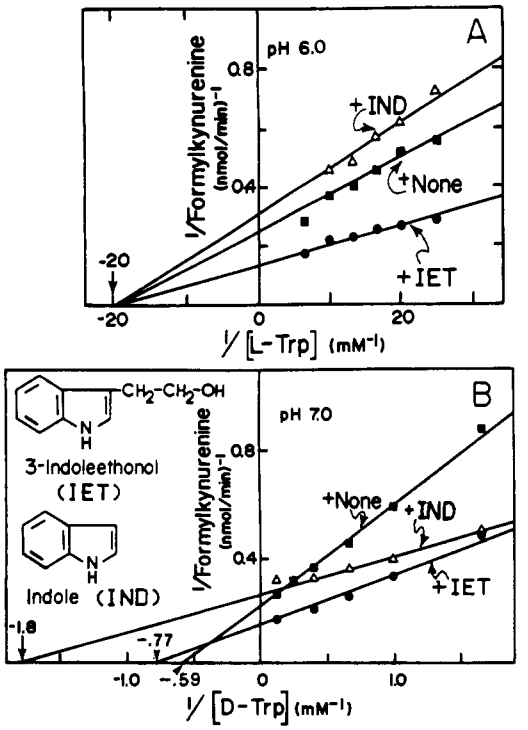

FIGURE 1: Double-reciprocal plots of the rates of the product (*N*-formylkynurenine) formation versus the concentration of the substrate L-Trp (A) and D-Trp (B) in the presence and absence of the effectors indole (IND) and 3-indoleethanol (IET). For L-Trp (A), the results were obtained at pH 6.0 without (■) and with 1 mM IND (△) and 2 mM IET (●) and for D-Trp (B) at pH 7.0 without (■) and with IND (△) and IET (●), both 2 mM. The structures of IET and IND are depicted in (B).

Table I: Effects of 3-Indoleethanol (IET) and Indole (IND) on the K_m and V_{max} Values for the Conversion of Trp to *N*-Formylkynurenine Catalyzed by Indoleamine 2,3-Dioxygenase[a]

substrate	effector (2 mM)	K_m (V_{max}) [mM (s^{-1})] at pH 6.0	7.0	8.0
L-Trp	none	0.05 (1.5)	0.013 (2.1)	[b]
	IET	0.05 (2.8)	0.013 (2.9)	[b]
	IND	0.05 (1.3)	0.013 (1.6)	[b]
D-Trp	none	7.4 (1.7)	1.7 (1.7)	0.83 (2.4)
	IET	5.1 (2.3)	1.3 (2.7)	0.45 (2.4)
	IND	2.5 (1.0)	0.56 (1.5)	0.22 (1.2)[c]

[a] All measurements were performed in 0.1 M potassium phosphate buffer at 25 °C. See Experimental Procedures for the K_m and V_{max} value determinations. [b] Not examined. [c] With 0.5 mM indole.

Interactions of 3-Indoleethanol and Indole with Various Derivatives of Indoleamine 2,3-Dioxygenase. Binding of these two effectors to the various forms of the enzyme including the catalytically active (ferrous, ferrous–O_2) and inactive (ferric) forms and exogenous ligand-bound unnatural forms (ferrous–CO and –NO, ferric–cyanide and –azide) was further studied by means of various spectroscopic methods (optical absorption, CD, and MCD). These effectors were found in this study to cause only minor MCD spectral changes in parallel with absorption spectral changes upon binding to the above-mentioned various dioxygenase derivatives. In addition, it appears that no particularly significant information relevant

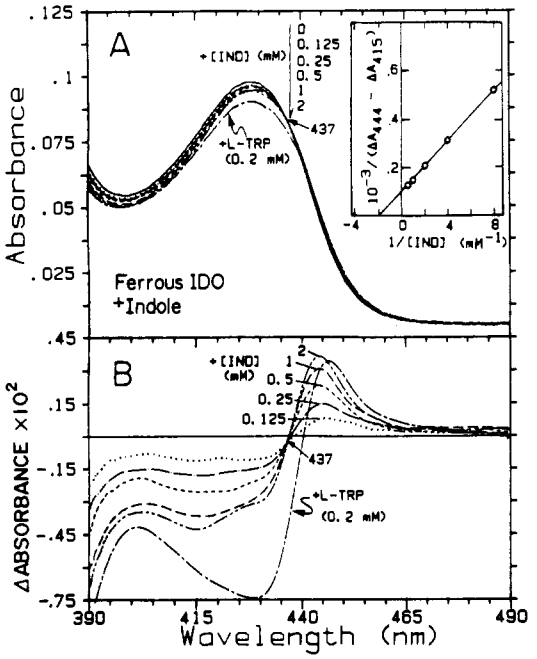

FIGURE 2: Absorption spectral titration of ferrous indoleamine 2,3-dioxygenase (IDO) with indole (IND) in the absence of L-Trp. The titration was carried out at pH 7.0. Changes in the absolute spectrum of the ferrous enzyme in the Soret region, upon incremental additions of 0–2 mM IND, are shown in (A). Difference spectra for partially IND bound minus IND free ferrous enzyme are plotted in (B) after smoothing. In both (A) and (B), the spectrum of the sample after an addition of 0.2 mM L-Trp (–·–) following the IND titration is overplotted. In the inset of (A), a double-reciprocal plot that was obtained from (B) is displayed for the peak (444 nm) to trough (415 nm) values of the difference spectra versus IND concentrations.

to the present study is obtainable from the MCD spectra other than that obtained from the absorption spectra. For this reason, MCD spectral properties of the new complexes of the enzyme with these effectors are not shown in the following sections. As for the effects of the substrate Trp, it has been shown in a previous study (Sono et al., 1980) that D-Trp exhibits about 2 orders of magnitude lower affinity than the L isomer for various derivatives of the ferric and ferrous enzyme. Thus, the detection of the effects of D-Trp within its solubility limit (≤60 mM at 25 °C) is practically impossible. Hence, only L-Trp is examined in the following spectroscopic experiments.

(*a*) *Ferrous Indoleamine 2,3-Dioxygenase.* Upon additions of either indole or 3-indoleethanol to the Trp-free ferrous enzyme, small but reproducible absorption spectral changes are observed, as examined in the Soret region (Figure 2). By taking advantage of the spectral change, a titration of the ferrous enzyme with indole in the absence of L-Trp was carried out as shown in Figure 2, where the absolute (A) and difference (B) spectra in the presence of various concentrations of indole (0–2 mM) are overplotted. An isosbestic point is seen at 437 nm during the spectral conversion. The double-reciprocal plot, shown in the inset from the data obtained in Figure 2B, yields a straight line with an x intercept of −2 [$=-1/K_d$ (mM^{-1})], indicating that indole binds to the ferrous enzyme in a 1:1 molar ratio with a K_d value of 0.5 mM at pH 7.0. Similar results with a K_d value of 0.1 mM and an isosbestic point at 436 nm were obtained with 3-indoleethanol (spectra and a plot not shown). It was also found that the L-Trp affinity for the ferrous enzyme [K_d = 13 μM at pH 7.0 without an effector (Sono et al., 1980)] does not change significantly by the addition of indole (2 mM) or 3-indoleethanol (4 mM).

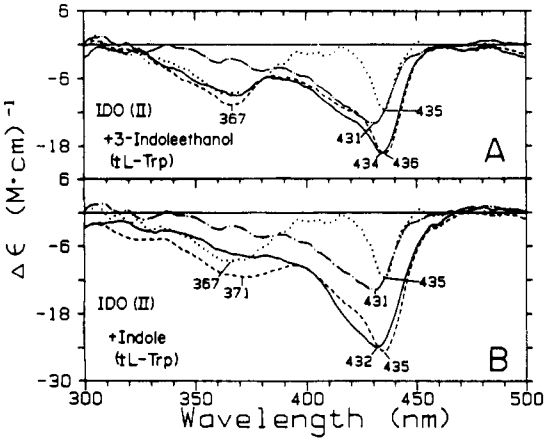

FIGURE 3: CD spectral changes in the Soret region upon the binding of 3-indoleethanol (IET) (A) and indole (IND) (B) to ferrous indoleamine 2,3-dioxygenase (IDO) in the presence and absence of L-Trp. IET (4 mM) and IND (2 mM) were added to the ferrous enzyme (dotted line → dashed line). A further addition of 0.2 mM L-Trp yielded a spectrum indicated by a solid line in both (A) and (B). The dot-dashed line represents the CD spectrum of the L-Trp–ferrous enzyme adduct in the absence of these effectors. The data in both (A) and (B) were obtained at pH 7.0 with 40–50 μM enzyme with a 0.2-cm cuvette for both cases.

In contrast to the relatively small absorption spectral changes caused by the binding of these effectors, the CD spectrum of the ferrous enzyme changes more drastically. Figure 3 compares the CD spectra of the free ferrous enzyme (dotted line), its L-Trp complex (dot-dashed line), its 3-indoleethanol (A) or indole (B) complex (both in dashed line), and its complex with both L-Trp and either one of these effectors (solid line). The first two derivatives have previously been studied with CD spectroscopy (Sono & Dawson, 1984). Note that upon binding of either effector a new trough appears around 435 nm (dashed line) that is considerably more intense than that of either the Trp-free or L-Trp-bound ferrous enzyme. Thus, the effector-bound ferrous enzyme is easily distinguishable from either the free ferrous enzyme or its L-Trp adduct. The binding of L-Trp to the effector–enzyme complex further causes a relatively small but detectable CD spectral change (dashed line → solid line) for both effectors. These effectors noticeably intensify the Soret CD trough around 430 nm both in the presence and in the absence of the substrate L-Trp. The magnitude of such effects seems to be greater for indole (Figure 3B) than for 3-indoleethanol (Figure 3A).

(*b*) *Oxygenated Indoleamine 2,3-Dioxygenase.* Previously, indole has been shown to cause a shift in the Soret absorption peak of the oxygenated enzyme only in the presence of L-Trp when examined at −30 °C in mixed solvents (Sono, 1986). Such effects of 3-indoleethanol (Figure 4A) and of indole (Figure 4B) have been examined in further detail in this study at −30 °C. An addition of 3-indoleethanol (20 mM) or indole (20 mM) to the Trp-free oxygenated enzyme only slightly lowers the Soret peak (415 nm) intensity (dotted line → dashed line). In the visible region, the α-peak (576 nm) intensity increases considerably upon addition of 3-indoleethanol (A), but little changes are observed with indole (B). CD spectral changes are, on the other hand, relatively large for both effectors, as shown in the insets. The Soret CD trough around 410 nm becomes more intense with a small blue shift (3-indoleethanol) (A) or red shift (indole) (B) of 1–2 nm for both cases upon the effector binding to the oxygenated enzyme (dotted line → dashed line). Absorption and CD spectra of the L-Trp-bound oxygenated enzyme appear to differ to some extent in the presence (solid line) and absence (dot-dashed line)

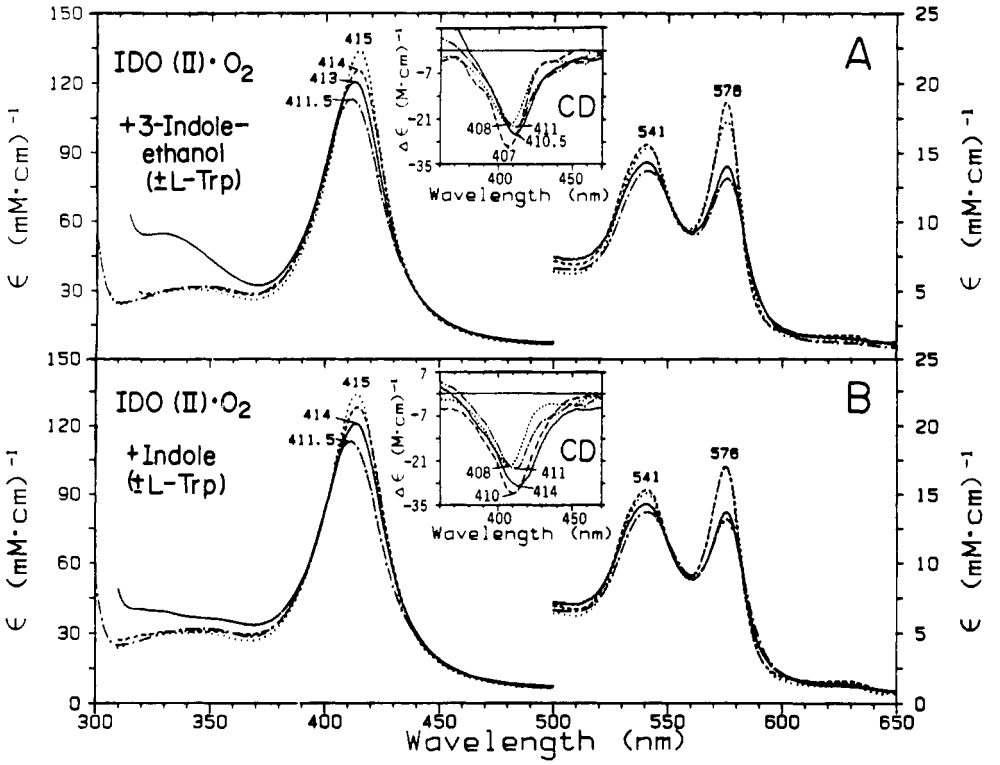

FIGURE 4: Effects of 3-indoleethanol (IET) (A) and indole (IND) (B) on the absorption and CD spectra (inset) of ferrous–O_2 indoleamine 2,3-dioxygenase (IDO) in the presence and absence of L-Trp. To the ferrous–O_2 IDO (dotted line) was first added 20 mM IET (A) or 20 mM IND (B) (both dashed lines), followed by an addition of 20 mM L-Trp for both (A) and (B) (solid line). The absorption and CD spectra of the L-Trp–ferrous enzyme–O_2 ternary complex (with 2 mM L-Trp) (dot-dashed line) were also overplotted in (A) and (B). All spectra were recorded at −30 °C. Both absorption and CD spectra of the ferrous–O_2 enzyme with and without L-Trp in the absence of the effectors are taken from Sono (1986). Somewhat larger values in the absorption spectra (shown by the solid line) below 380 nm are due to the product (*N*-formylkynurenine, λ_{max} = 321 nm) generated during the sample preparations. The high concentration (20 mM) of L-Trp added also partially contributes to the absorbance in this wavelength region (solid line). See Experimental Procedures for further details.

of either effector, although the differences are quite small in the visible region absorption spectra for both effectors and in the CD spectra for 3-indoleethanol (Figure 4A, inset). Nevertheless, it is clear that both indole derivatives bind to the oxygenated enzyme with and without L-Trp. Due to the technical difficulties associated with the mixed solvent system at subzero temperatures (−30 °C), e.g., a high viscosity (Sono, 1986), dissociation constants of the effector–oxygenated enzyme complexes were not determined in this study.

(*c*) *Ferrous–CO, Ferrous–NO, and L-Trp-Bound Ferric Forms of Indoleamine 2,3-Dioxygenase.* Although none of these enzyme derivatives are actual intermediates of the catalytic cycle of the dioxygenase (Sono et al., 1980), the first two ferrous enzyme derivatives are close analogues of the oxygenated enzyme (Sono et al., 1980; Sono, 1986). The ferric enzyme can be used to obtain useful information about the substrate binding site of the enzyme (Sono & Dawson, 1984). The binding of L-Trp to the first two ferrous enzyme derivatives in the absence of the effectors has already been studied (Sono et al., 1980; Sono & Dawson, 1984). The trends in spectral changes caused by either indole or 3-indoleethanol for the ferrous–CO and ferrous–NO enzymes are essentially the same as those observed with the oxygenated enzyme. The results for indole binding to the ferrous–CO enzyme and for 3-indoleethanol binding to the ferrous–NO enzyme are shown in Figure 5, parts A and B, respectively. An addition of indole (2 mM) or 3-indoleethanol (4 mM) to either of these enzyme derivatives causes only small absorption spectral changes with 0.5–1-nm red shifts in the major absorption bands both in the presence and in the absence of L-Trp. In contrast, a large CD spectral change, namely, an increase in the Soret trough intensity, is observed (see insets of Figure 5A,B) upon the binding of either effector to the L-Trp-free (dotted line → dashed line) and L-Trp-bound (dot-dashed line → solid line) ferrous enzyme complexes with CO (A) and NO (B).

To determine dissociation constants of the complexes of the ferrous–CO and –NO enzyme derivatives with these two effectors, CD spectral titrations were carried out. Results for the 3-indoleethanol titration of the ferrous–CO enzyme and for the indole titration of the ferrous–NO enzyme are displayed in Figure 6, parts A and B, respectively. For both cases, double-reciprocal plots, shown in the insets, yield a straight line. Hence, both effectors bind to these ferrous enzyme derivatives in a 1:1 molar ratio. K_d values of 1.54 mM (A) and 1.18 mM (B) are obtained from the double-reciprocal plots. It should be pointed out that neither enzyme derivative is completely saturated with these effectors at their highest concentrations used. The estimated percent saturations for these two cases in Figure 6 are ~72% and ~63%, respectively.

For the case of the native ferric enzyme, the apparent K_d values for its complexes with indole and 3-indoleethanol are estimated to be greater than 4 mM as judged from the small absorption, CD, and MCD spectral changes caused by these effectors. For this reason, further attempts to prepare and characterize a homogeneous complex of the Trp-free ferric enzyme have not been carried out in this work. Fortunately, however, the L-Trp complex of the native ferric enzyme undergoes a small but easily titratable spectral change upon binding of indole with isosbestic points observed at 412, 507, 536, and 590 nm (Figure 5C). Optical absorption and CD spectra of the L-Trp–ferric enzyme adduct in the presence and absence (dashed line) of indole (2 mM) (solid line) and 3-indoleethanol (4 mM) (dot-dashed line) are overplotted in Figure 5C. The K_d value for indole under these conditions

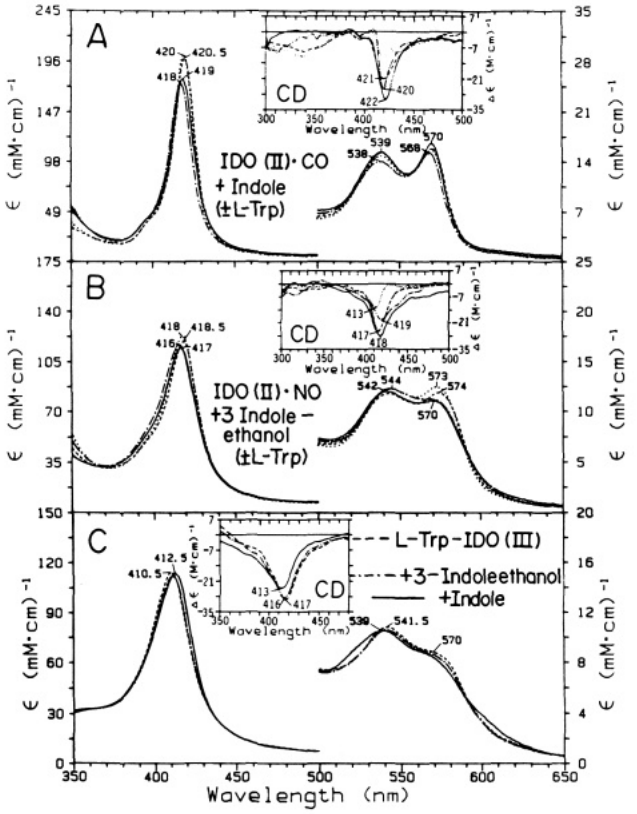

FIGURE 5: Effects of indole and 3-indoleethanol on the optical absorption and CD (inset) spectra of the ferrous–CO (A), ferrous–NO (B) and L-Trp-bound ferric (C) forms of indoleamine 2,3-dioxygenase (IDO). To the CO (A) and NO (B) complexes of the ferrous IDO (dotted line) were added 2 mM indole (A) and 4 mM 3-indoleethanol (B) (dashed line), followed by an addition of 10 mM L-Trp for both complexes (solid line) at pH 7.0. The spectra of the L-Trp complexes of the ferrous–CO and ferrous–NO enzyme in the absence of the effectors were also overplotted (dot-dashed line). For the ferric enzyme (C), 2 mM indole (solid line) or 4 mM 3-indoleethanol (dot-dashed line) was added to the L-Trp–enzyme complex (with 20 mM L-Trp) (dashed line) at pH 8.0. The enzyme concentrations were 40–50 μM. A 0.2-cm cuvette was used in all cases.

(pH 8.0, +20 mM L-Trp) is found to be ~60 μM. Thus, the indole adduct shown here can be considered to be homogeneous. Much smaller changes caused by 3-indoleethanol on both absorption and CD (inset) spectra do not allow for the determination of a K_d value for this compound, and the complex obtained here with 4 mM 3-indoleethanol is probably not homogeneous. Both effectors induce a slight red shift (1–2 nm) for the Soret peak and a blue shift (1.5–2.5 nm) for the visible region peak. As shown in the inset of Figure 5C, indole causes a blue shift (4 nm) and a decrease in the intensity of the Soret region CD trough.

Influence of the Effectors on the Affinities of the Substrate L-*Trp and Heme Ligands Cyanide, Azide, and Norharman for Indoleamine 2,3-Dioxygenase Derivatives.* It has already been shown in this study that neither indole nor 3-indoleethanol affects the L-Trp affinity of the ferrous enzyme. For some of the other enzyme derivatives, however, these effectors, especially indole, remarkably change the K_d values of the enzyme complexes with L-Trp. Such cases of the ferrous–CO and native ferric enzyme are shown in Figure 7, parts A and B, respectively. These findings have not been reported previously. Skatol (3-methylindole) was found in the past to have a similar effect on the L-Trp affinity for the ferrous–CO enzyme by Uchida et al. (1985). The effects of indole are similar in both cases for the ferrous–CO (A) and the native ferric enzyme (B) in that at <0.5 mM concentrations indole sharply

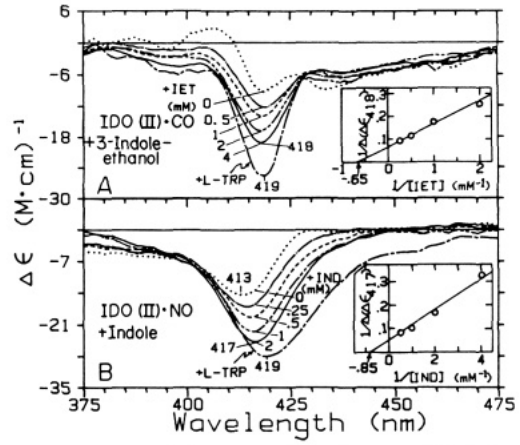

FIGURE 6: CD spectral titrations of the ferrous–CO (A) and ferrous–NO (B) forms of indoleamine 2,3-dioxygenase (IDO) with 3-indoleethanol (IET) and indole (IND), respectively. To the CO and NO complexes of the ferrous enzyme (dotted line) were added incremental concentrations of IET (0–4 mM) (A) and IND (0–2 mM) (B) as indicated in the figure. The dot-dashed line for both cases was obtained by an addition of 10 mM L-Trp after the titration with the effector. Double-reciprocal plots of the CD intensity change versus effector concentration are shown in the insets. All measurements were done at pH 7.0 with 40–50 μM enzyme concentrations and by using a 0.2-cm cuvette.

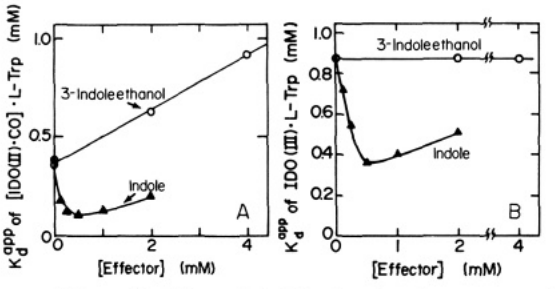

FIGURE 7: Effects of indole and 3-indoleethanol on the apparent L-Trp affinity (K_d^{app}) of the ferrous–CO (A) and native ferric (B) forms of indoleamine 2,3-dioxygenase (IDO). The data were obtained by spectrophotometric titrations in the presence of indole (▲) and 3-indoleethanol (○) at pH 7.0 (A) and pH 7.35 (B). See Experimental Procedures for details.

decreases the K_d value, i.e., increases the L-Trp affinity, by a factor of up to 3.3 for the ferrous–CO enzyme (K_d = 0.3 → 0.09 mM) (A) and 2.4 for the native ferric enzyme (K_d = 0.85 → 0.35 mM) (B). For an indole concentration range between 0.5 and 2 mM, the K_d value starts to gradually increase with an increase in the effector concentration for both cases. The initial phase of the observed effects of indole is a typical phenomenon for the positively cooperative binding of two different ligands to an enzyme, where one ligand enhances the affinity of the other in a reciprocal manner. A simple case of such a cooperative binding of L-Trp and indole would yield a hyperbolic-type decrease in the K_d value for L-Trp as a function of indole concentration, resulting in a limiting K_d value at an infinite indole concentration. The present results in Figure 7A,B apparently do not follow such a simple case and suggest that, at higher concentrations (>0.5 mM) of indole, a secondary binding of this effector is occurring that interferes with the binding of L-Trp to the enzyme. This point will be further discussed later. The effects of 3-indoleethanol are different for these two enzyme derivatives. The K_d value for the L-Trp complex of the ferrous–CO enzyme linearly increases with an increase in effector concentration (0–4 mM). As has already been demonstrated in Figure 5B, inset, in the preceding paper for the binding of 4-phenylimidazole and L-Trp to the ferric enzyme, this suggests that

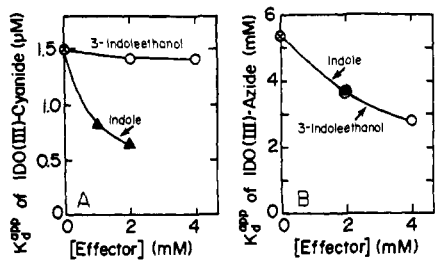

FIGURE 8: Effects of indole and 3-indoleethanol on cyanide (A) and azide (B) affinities of native ferric indoleamine 2,3-dioxygenase (IDO). The data were obtained at pH 7.0 for both (A) and (B) with indole (Δ) and 3-indoleethanol (O). The K_d^{app} values for the cyanide and azide complexes were determined by using a Hill plot and a double-reciprocal plot, respectively.

3-indoleethanol is competitive with L-Trp for the ferrous–CO enzyme. In the case of the native ferric enzyme, this effector exerts little influence (Figure 7B, open circle) on L-Trp binding.

Both indole and 3-indoleethanol were found to also influence the affinities of the ferric enzyme for heme ligands such as cyanide and azide in this study. Soret absorption bands of the ferric enzyme complexes with cyanide and azide undergo red shifts of 1–1.5 nm upon addition of these effectors (spectra not shown). Results for the cyanide and azide affinities of the ferric enzyme are displayed in Figure 8, parts A and B, respectively. It appears that indole (solid triangle) considerably enhances the affinities of the enzyme for both cyanide (A) and azide (B). The magnitude of the effect of 3-indoleethanol (open circle) is very small on the cyanide affinity (A), but is about the same on the azide affinity (B) as compared with that of indole. Neither the cyanide nor the azide complex of the native ferric enzyme appears to be saturated with either effector added to its near solubility limit concentration under the present experimental conditions.

The effects of indole and 3-indoleethanol on the affinity of norharman, an inhibitor and heme ligand (Sono & Cady, 1989), for the ferric and ferrous enzyme were also examined with and without the substrate L-Trp. Results are summarized in Table II. For the ferric dioxygenase, indole (2 mM) considerably increases the norharman affinity for the enzyme (K_d = 9.0 → 3.6 μM), while 3-indoleethanol has a much smaller effect (K_d = 9.0 → 7.7 mM). Identical results were obtained in the presence and absence of 0.5 mM L-Trp. For the ferrous enzyme in the absence of the substrate L-Trp, indole does not affect the norharman affinity of the enzyme, while 3-indoleethanol slightly increases the K_d value (K_d = 7.8 → 11.2 μM). As has been shown in the preceding paper, an addition of L-Trp considerably lowers the norharman affinity for the ferrous dioxygenase. This substrate effect is greatly decreased by indole (K_d = 26.7 → 16 μM). 3-Indoleethanol does not change the K_d value for norharman in the presence of L-Trp. These results indicate that the substrate L-Trp, the effectors 3-indoleethanol and indole, and the inhibitor (heme ligand) norharman can bind to the enzyme in both the ferric and ferrous states to form a quaternary complex, despite the relatively large size of these organic compounds that have an indole nucleus in common.

Discussion

The present study has provided clear evidence that a binding site exists in indoleamine 2,3-dioxygenase for some effectors, such as indole and 3-indoleethanol, other than that for the substrate L-Trp. To help discuss the present findings, the microscopic structural changes in the environment of the enzyme active site are schematically summarized in Figure 9. These changes are caused by the reduction and oxidation of

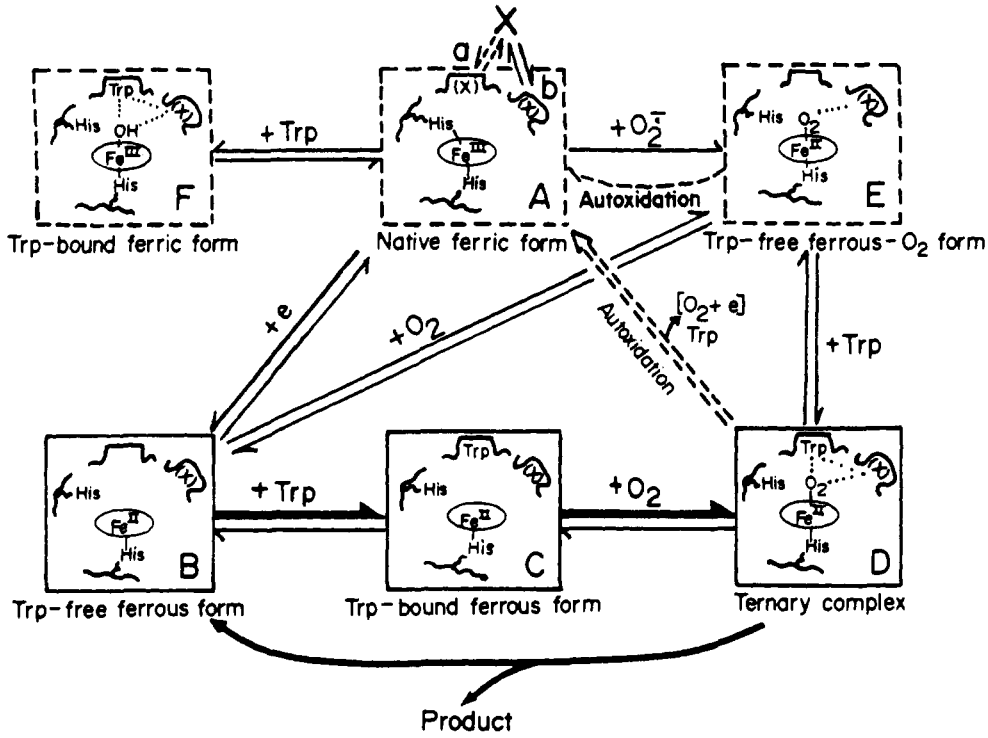

FIGURE 9: Schematic representation of the conversion of the native ferric indoleamine 2,3-dioxygenase (A) to various derivatives upon reduction of the enzyme and binding of Trp, O_2, $O_2^{\cdot-}$, and an effector (X). The axial ligands coordinated to the heme iron of the dioxygenase in various forms have been identified in previous studies (Shimizu et al., 1978; Sono & Dawson, 1984). The binding sites for the substrate L-Trp and for the effectors indole and 3-indoleethanol on the enzyme are indicated by a (substrate) and b (effector). The six enzyme derivatives, A–F, are the enzyme forms that are most likely involved under normal steady-state conditions of the catalytic reaction of the dioxygenase. Among them, the three ferrous derivatives, B–D, are the principal intermediates of the catalytic cycle (Sono et al., 1980). The dotted lines indicate direct or indirect steric and/or electronic interactions among the bound Trp, O_2, and an effector (X) at the enzyme active site. Trp represents L-Trp in this scheme, but it may also be applicable to D-Trp. See text for further details.

Table II: Effects of Substrate L-Trp and the Effectors Indole and 3-Indoleethanol on the Affinity of Ferric and Ferrous Indoleamine 2,3-Dioxygenase for Norharman[a]

substrate or effector[b]	K_d^{app} for norharman complex (μM)	
	ferric enzyme (pH 8.0)	ferrous enzyme (pH 7.0)
none	9.0	7.8
L-Trp	9.0	26.7
indole	3.6	7.9
3-indoleethanol	7.7	11.2
L-Trp + indole	3.6	16.0
L-Trp + 3-indoleethanol	7.7	26.5

[a] The data were obtained in 0.1 M potassium phosphate (pH 7.0) and 0.1 M Tris-HCl buffer (pH 8.0) at 25 °C. [b] The concentrations used were 0.5 mM L-Trp for the ferric and 0.1 mM L-Trp for the ferrous enzyme, 2 mM indole, and 4 mM 3-indoleethanol.

the heme iron and the binding of the substrates Trp and O_2, an activator $O_2^{\bullet-}$, and an effector (X).

Binding and Mechanism of Action of Indole and 3-Indoleethanol as Effectors. The effectors indole and 3-indoleethanol have been shown in this study to influence not only the catalytic but also the spectral and substrate binding and heme ligand binding properties of the dioxygenase. The effector titration experiments have provided a 1:1 molar ratio for the enzyme–effector complexes examined. These results not only confirm the presence of a single specific binding site in the enzyme for these indole derivatives but also suggest that the effector binding site is located in the vicinity of the heme iron and the substrate binding site. 3-Indoleethanol was reported to serve as the strongest effector among the several indole derivatives studied (Eguchi et al., 1984). Indole is the simplest structural analogue for 3-indoleethanol. The enzyme kinetic results (K_m) for L-Trp metabolism in the presence of either of these two effectors (Figure 1A) are consistent with the L-Trp titration results (K_d). This demonstrates that the L-Trp affinity of the ferrous enzyme does not change significantly upon the binding of either effector. The K_m value is considered to reflect a substrate binding equilibrium constant for the ferrous enzyme (B + L-Trp ↔ C in Figure 9) (Sono et al., 1980; Sono, 1986). On the other hand, the K_m value for D-Trp is markedly lowered with either 3-indoleethanol or indole (Table I). This indicates that these effectors considerably enhance the D-Trp affinity for the ferrous enzyme. Unfortunately, spectroscopic studies of the binding of D-Trp to any of the enzyme derivatives are practically impossible because of its extremely low affinity for the enzyme as described under Results. D-Trp probably binds to the same site as L-Trp in the enzyme but in a slightly different and sterically unfavorable manner as compared with the binding of L-Trp.

As for the mechanism of the enhancement by 3-indoleethanol and the decrease by indole of the apparent maximal enzyme activity (V_{max}) of the dioxygenase, the following two possibilities may be considered. The effectors change either (i) the rate of the breakdown of the ternary complex to the ferrous enzyme and the product (D → B + product, Figure 9) or (ii) the steady-state concentrations of the active (ferrous) forms of the enzyme, e.g., B–D in Figure 9. Since these effectors bind to the enzyme–Trp–O_2 ternary complex to affect its electronic structure (Figure 4), the first possibility above may be a reasonable cause. However, the second possibility cannot be ruled out in the present case. The apparent maximal enzyme activity was shown by Ozaki et al. (1987), using indoleamine 2,3-dioxygenase purified from mouse epididymis, to be enhanced by a factor of 3–4 by replacing the ascorbic acid–methylene blue cofactor system with the NADPH–FMN reductase–FMN system. In addition, a recent study on the roles of the cofactors has demonstrated that leuco-methylene blue (the two-electron-reduced dye) can reduce rabbit small intestinal indoleamine 2,3-dioxygenase only up to 40% under anaerobic conditions (Sono, 1989). A possible cause for the observed differences in the apparent maximal enzyme activity is the difference in the redox potential (for the two-electron reduction) of methylene blue (E_0' = 0.001 V) and FMN (E_0' = −0.219 V) [both at pH 7.0 and 30 °C (Dawson et al., 1986)]. The reduced form of FMN is obviously a stronger reductant than that of methylene blue. Thus, the apparent maximal enzyme activity with the ascorbic acid–methylene blue cofactor system may not account for the catalytic activity of the fully activated (reduced) enzyme. If this is the case, 3-indoleethanol may be able to enhance the apparent maximal enzyme activity by helping shift the ferric enzyme ↔ ferrous enzyme equilibrium (A + e ⇌ B in Figure 9) to the right in a manner similar to that observed with L-Trp in a previous study (Sono, 1989); this effector was shown in this study to have a higher affinity for the ferrous (K_d = 0.1 mM) than the ferric (K_d = >4 mM, Figure 5C) enzyme as does L-Trp (Sono et al., 1980). Indole appears to have comparable affinities (K_d = 0.05–0.5 mM, Figures 2 and 5C) for both oxidation states of the enzyme or even a little higher affinity for the ferric enzyme (Figure 7B) than for the ferrous enzyme (Figure 2). Thus, the main effects of 3-indoleethanol and indole may be attributed to the changes in the steady-state concentrations of the ferrous (active) forms of the dioxygenase caused by preferred binding of these effectors to either oxidation state of the enzyme.

The indole-concentration-dependent biphasic (cooperative and competitive) interactions between indole and L-Trp for their binding to the ferrous–CO (Figure 7A) and the native ferric enzyme (Figure 7B) can be interpreted as follows: indole can bind to both the effector and substrate binding sites (sites b and a of (A) in Figure 9, respectively) with different affinities. For example, indole may first occupy the effector binding site b at lower concentrations (<0.5 mM). The initial indole binding to site b is positively cooperative with the binding of substrate L-Trp to site a. After site b is occupied with indole, it starts to bind to site a, causing a competition with L-Trp. The reason for the uniform competition between 3-indoleethanol and L-Trp for the ferrous–CO enzyme is not clear at present. It appears that binding of the substrate L-Trp and the effectors to the enzyme is influenced not only by the oxidation and spin states of the enzyme but also by the type of the exogenous ligands (O_2, CO, NO, CN^-, N_3^-) bound to the heme iron.

Possible Cause for Substrate Inhibition. There has been no evidence for the binding of substrate L-Trp to the effector binding site. Titration binding experiments, using three different spectroscopic techniques (absorption, MCD, and CD), up to the solubility limit of L-Trp concentrations (e.g., ~60 mM at 25 °C in aqueous solution) have shown only uniform binding of this substrate with a 1:1 molar ratio to any of the following enzyme derivatives: the native and cyanide-bound ferric enzyme and the ferrous enzyme [up to 0.5 mM L-Trp (Sono et al., 1980)] and its complexes with CO and NO (results not shown). Hence, the substrate inhibition observed at relatively high concentrations of L-Trp [Yamamoto & Hayaishi, 1967; K_i = ~300K_m at pH 7.0 (Sono et al., 1980)] or 5-hydroxy-L-Trp (Hirata & Hayaishi, 1972) is likely caused by the formation of a low-spin ferric enzyme complex with L-Trp or 5-hydroxy-L-Trp at their higher concentrations (Sono et al., 1980; Sono & Dawson, 1984) rather than by a secondary

binding of these substrates to another site on the enzyme. The reduction of the enzyme to its active (ferrous) form(s) might be inhibited at higher L-Trp concentrations (>0.5 mM) in a manner similar to the case of 4-phenylimidazole described in the preceding paper.

Comparison of the Structure and Size of the Active Sites of Indoleamine 2,3-Dioxygenase and Tryptophan 2,3-Dioxygenase. Norharman and several side-chain-substituted indole derivatives including 3-indoleethanol have been reported to be competitive inhibitors for mammalian (mouse, rat) liver tryptophan 2,3-dioxygenase with respect to L-Trp (Friedman et al., 1961; Eguchi et al., 1984), while neither of these compounds competes with L-Trp for rabbit intestinal indoleamine 2,3-dioxygenase (Eguchi et al., 1984). These results suggest that, unlike the case of indoleamine 2,3-dioxygenase, the indole compounds occupy the L-Trp binding site(s), most likely the catalytic site, in tryptophan 2,3-dioxygenase. As pointed out in the preceding paper, indoleamine 2,3-dioxygenase appears to have a larger active site than tryptophan 2,3-dioxygenase.

Significance of the Effector Binding Site of Indoleamine 2,3-Dioxygenase. The catalytic and physiological significance of the newly confirmed binding site in indoleamine 2,3-dioxygenase for several indole derivatives seems to be quite different from that of the regulatory site for tryptophan 2,3-dioxygenase. Unlike the case for tryptophan 2,3-dioxygenase (Feigelson & Brady, 1974), L-Trp does not appear to bind to the effector binding site of indoleamine 2,3-dioxygenase, thus playing no regulatory role in the latter dioxygenase. Some indole derivatives simply enhance the V_{max} value by a factor of less than 2 for indoleamine 2,3-dioxygenase (Table I; Eguchi et al., 1984) without changing the normal Michaelis–Menten kinetics, i.e., a hyperbolic curve in a V versus [L-Trp] plot (not shown). In regard to this matter, one should be reminded that indoleamine 2,3-dioxygenase appears to have a rather specific cofactor requirement as mentioned in the introduction. The existence and the identity of the natural cofactor for indoleamine 2,3-dioxygenase have recently been intriguing subjects for investigations (Sono, 1989; Nishikimi, 1975; Ozaki et al., 1987). In this respect, one might be tempted to consider that the newly confirmed site in this dioxygenase is the binding site for the possible natural cofactor for the enzyme. Studies focusing on this subject are currently under way.

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