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Spectroscopic and Equilibrium Studies of Ligand and Organic Substrate Binding to Indolamine 2,3-Dioxygenase[†]

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ABSTRACT: The binding of a number of ligands to the heme protein indolamine 2,3-dioxygenase has been examined with UV–visible absorption and with natural and magnetic circular dichroism spectroscopy. Relatively large ligands (e.g., norharman) which do not readily form complexes with myoglobin and horseradish peroxidase (HRP) can bind to the dioxygenase. Except for only a few cases (e.g., 4-phenylimidazole) for the ferric dioxygenase, a direct competition for the enzyme rarely occurs between the substrate L-tryptophan (Trp) and the ligands examined. L-Trp and small heme ligands (CN^-, N_3^-, F^-) markedly enhance the affinity of each other for the ferric enzyme in a reciprocal manner, exhibiting positive cooperativity. For the ferrous enzyme, L-Trp exerts negative cooperativity with some ligands such as imidazoles, alkyl isocyanides, and CO binding to the enzyme. This likely reflects the proximity of the Trp binding site to the heme iron. Other indolamine substrates also exert similar but smaller cooperative effects on the binding of azide or ethyl isocyanide. The pH dependence of the ligand affinity of the dioxygenase is similar to that of myoglobin rather than that of HRP. These results suggest that indolamine 2,3-dioxygenase has the active-site heme pocket whose environmental structure is similar to, but whose size is considerably larger than, that of myoglobin, a typical O_2-binding heme protein. Although the L-Trp affinity of the ferric cyanide and ferrous CO enzyme varies only slightly between pH 5.5 and 9.5, the unligated ferric and ferrous enzymes have considerably higher affinity for L-Trp at alkaline pH than at acidic pH. L-Trp binding to the ferrous dioxygenase is affected by an ionizable residue with a pK_a value of 7.3.

Despite the spectral similarities among the three protoheme IX containing monomeric proteins indolamine 2,3-dioxygenase, myoglobin, and horseradish peroxidase (HRP)[1] in their various oxidation states and ligand-bound forms (Sono & Dawson, 1984), the functions of these proteins are different from each other. In particular, although indolamine 2,3-dioxygenase and myoglobin bind O_2 reversibly in their normal functions, only the former can catalyze the insertion of molecular oxygen into the organic substrate L-tryptophan (Trp) to yield the product *N*-formylkynurenine (Hayaishi et al., 1975; Hayaishi, 1976). The existence of a binding site for Trp at the active site in the dioxygenase might be a prerequisite for its catalytic capability. Beyond this point, however, little information is available about either the conformational or the electronic structural difference of their active sites, which is directly related to their functional distinctions.

Ligand binding properties of heme proteins have often been shown to provide a useful clue to the understanding of the active-site structure which can be correlated to the functionality of the proteins. Typical examples are (a) myoglobin (O_2 binding) and HRP (H_2O_2 activation) and (b) cytochrome P-450_{cam} (O_2 activation) and chloroperoxidase (H_2O_2 and Cl^-

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[1] Abbreviations: Trp, tryptophan; CD, circular dichroism; MCD, magnetic circular dichroism; HRP, horseradish peroxidase; $O_2^{\bullet -}$, superoxide anion radical.

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activation); within each pair of the proteins, the two proteins exhibit very similar spectroscopic properties, but they have considerably different ligand binding characteristics. These pairs of heme proteins have histidine imidazole (a) and cysteine thiolate (b) (Dawson & Sono, 1987) endogenous axial ligands coordinated to the iron atom of the protoheme IX prosthetic group. A protonated (neutral) form as opposed to a deprotonated form of weakly anionic ligands ($pK_a > 3$) can exclusively enter the heme pocket of the native ferric form of HRP (Dunford & Alberty, 1967; Ellis & Dunford, 1967) and chloroperoxidase (Sono et al., 1986), while no such strict charge restriction for a ligand is required by myoglobin (Antonini & Brunori, 1971) and cytochrome P-450$_{cam}$ (Sono et al., 1982).

Although extensive studies have been done on the catalytic (Hayaishi et al., 1975; Hirata et al., 1977; Ozaki et al., 1987; Sono, 1989a), molecular (Shimizu et al., 1978), and spectroscopic properties (Sono et al., 1980; Uchida et al., 1983a,b; Sono & Dawson, 1984; Sono, 1986) of indolamine 2,3-dioxygenase, its ligand binding characteristics have not been examined in detail. In this study, a number of heme ligands with various donor atoms (C, F, N, O, P, S) that are known to bind to the heme iron of myoglobin or HRP were extensively examined for their complex formation with indolamine 2,3-dioxygenase in the presence and absence of the organic substrate L-Trp or other known indole substrates. The spectroscopic (UV-visible, and natural and magnetic CD) properties and the dissociation constants of the resulting complexes were studied between pH 5.5 and 9.5 and then compared with those of myoglobin and HRP. This study revealed that the dioxygenase has a considerably larger heme pocket size than myoglobin and HRP and that the ligand binding properties of the dioxygenase are very similar to those of myoglobin but not to those of HRP. In addition, the pH profile of the L-Trp affinity of the unligated ferrous dioxygenase suggested the existence of an ionizable residue in the enzyme with a pK_a value of 7.3 that affects the L-Trp binding to the catalytic site of the enzyme.

EXPERIMENTAL PROCEDURES

Enzymes. Indolamine 2,3-dioxygenase was purified from rabbit small intestine to 60–70% homogeneity [A_{405}/A_{280} = 1.7–1.8, ϵ_{405} = 159 mM^{-1} cm^{-1} at pH 6.0 and 25 °C (Sono & Dawson, 1984; Sono & Cady, 1989)] 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. Bovine liver catalase was purchased from Sigma.

Chemicals. L-Ascorbic acid and the following indole derivatives were purchased from Sigma: L-Trp, D-Trp, 5-hydroxy-L-Trp, 5-hydroxy-D-Trp, tryptamine hydrochloride, serotonin creatine sulfate, α-methyl-DL-Trp, and indoleacetic acid. The following chemicals were obtained from the companies indicated: 4-phenylimidazole, 4,5-diphenylimidazole, 3-phenylpyridine, 4-phenylpyridine, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), and 1-propanethiol from Aldrich; methylene blue from Fisher; *N*-phenylimidazole from Trans World Chemicals; and dimethylphenylphosphine and bis(hydroxymethyl)methylphosphine from Alfa Products. All of these chemicals were of reagent grade and were used without further purification. Ethyl isocyanide was a generous gift from Professor Isao Morishima (Kyoto University, Kyoto, Japan).

Titrations of Indolamine 2,3-Dioxygenase with Various Ligands and with L-Trp. General titration methods and analysis have been described in previous papers (Sono et al., 1980; Sono & Cady, 1989). Stock solutions of Trp and other indole derivatives (20–50 mM) were prepared in water, and those of water-soluble ligands were made in 0.1 M potassium phosphate buffer. When necessary, the pH values of the ligand stock solutions were adjusted to a desired value by using NaOH or phosphoric acid (85%). Poorly water soluble ligands such as alkyl isocyanides, phenylimidazoles, phenylpyridines, phosphines (stored and used under N_2), and 1-propanethiol were added to the enzyme from ethanol stock solutions (0.01–1 M). Titration experiments were performed in 0.1 M potassium phosphate buffer (pH 5.5–8.5) or 0.1 M glycine–NaOH buffer (pH 9–10) at 25 °C. No detectable effects of the ionic strength (μ) of phosphate buffer on dissociation constants (K_d) were observed between pH 6.0 and 8.0 for $0.1 \leq \mu \leq 0.2$.

pH Titrations for the Spectral Change of Indolamine 2,3-Dioxygenase. The pH dependence of the absorption spectrum of the ferric dioxygenase in the presence of L-Trp or D-Trp was examined in 0.1 N NaCl at 25 °C by using 0.1–1 N HCl or NaOH to change the pH. At pH >8.5 in the absence of Trp, the ferric dioxygenase was not stable for equilibrium measurements (see Results). For this case, the absorption spectrum of the enzyme at various pH values (pH 8.85–10.0) was recorded for separate samples within 2 min after a concentrated enzyme stock solution (~300 μM in 20 mM potassium phosphate buffer, pH 6.0) was diluted in 0.1 M glycine–NaOH buffer whose pH value had been preadjusted to desired values.

Enzyme Kinetic Experiments. The pH dependence of the K_m value for L-Trp for its conversion to *N*-formylkynurenine catalyzed by indolamine 2,3-dioxygenase was examined in 0.1 M potassium phosphate buffer at 25 °C by using an ascorbic acid–methylene blue cofactor system in the presence of catalase as described previously (Shimizu et al., 1978).

Spectroscopic Measurements. Spectrophotometric measurements were performed on either a Union Giken SM-401 spectrophotometer (Kyoto University) or a Cary 219 spectrophotometer (University of South Carolina), each of which was equipped with a circulator for temperature control (25 ± 1 °C unless otherwise stated). MCD/CD spectra were recorded on a JASCO J-40 spectropolarimeter as previously described (Sono et al., 1982).

RESULTS

Spectroscopic Properties and Dissociation Constants of the Indolamine 2,3-Dioxygenase–Ligand Complexes. Indolamine 2,3-dioxygenase binds a number of different types of ligands such as cyanide, alkyl isocyanides, fluoride, azide, imidazoles, pyridines, formate, benzhydroxamate, and phosphines. Optical absorption spectral parameters (peak positions and millimolar extinction coefficients) and apparent dissociation constants (K_d^{app}) of the various ligand complexes of the ferric and ferrous dioxygenase have been reported previously (Sono et al., 1980; Sono & Dawson, 1984; Sono, 1986; Sono & Cady, 1989). Additional data are listed in Tables I (ferric derivatives) and II (ferrous derivatives). It should be mentioned that not all the ligands that form complexes with myoglobin bind readily to the dioxygenase, e.g., thiocyanate and nitrite. Except for a few cases (e.g., fluoride complex), L-Trp causes small changes in the optical absorption and MCD spectra but considerably large CD spectral changes (Sono & Dawson, 1984). The binding of formate, acetate (both high-spin complexes), and benzhydroxamate (low-spin complex, Figure 1A) to the ferric enzyme and the binding of 4-phenylpyridine, phosphines (e.g., dimethylphenylphosphine, Figure 1B), and alkyl isocyanides (e.g., *n*-butyl isocyanide) to both the ferric and ferrous enzymes have not been previously reported.

Table I: Absorption Spectral Parameters and Dissociation Constants (K_d) of the Ferric and Ferrous Indolamine 2,3-Dioxygenase Complexes with Ligands and L-Trp[a]

exogenous ligand	±L-Trp	absorption band, λ (ϵ_{mM}) [nm (mM^{-1} cm^{-1})]				K_d (mM) at pH 6.0[b]	
		δ	Soret	β	α	ligand	L-Trp
none	–		405 (159)	500 (8.8)[c]	570 (4.4)	–	–
				534 (7.5)	632 (3.7)[c]		
	+	354 (32.0)	410.5 (114)	541.5 (10.9)	572 (8.9)[d]	–	30[e]
formate	–		403.5 (160)	499 (8.7)[c]	568 (4.9)		
				532 (7.0)	626 (5.1)[c]	280	–
fluoride	–		402.5 (147)[f]	488 (9.1)[c]	580 (6.3)		
				528 (7.0)	608 (7.5)[c]	200	–
	+		401 (132)[f]	474 (9.8)[c]	574 (7.9)		
				541 (7.4)	600 (10.5)[c]	0.04	<0.01
benzhydroxamate	–	354 (29.0)	411.5 (121)	539 (10.7)	570 (9.4)	160	–
cyanide	–	359 (32.1)	418 (115)[b]	540 (11.7)		0.011	–
	+	359 (32.1)	417 (107)[b]	540 (11.7)		<0.0005	0.016
azide	–	357 (33.0)	414.5 (119)[b]	540 (10.2)	572 (8.0)[d]	5.3	–
	+	357 (33.0)	414 (121)	540 (11.3)	572 (8.7)[d]	0.38	0.9
N-phenylimidazole	–	*g*	411.5 (127)	530 (11.6)	560 (9.5)[d]	0.24	–
4-phenylpyridine	–	360 (34.1)	410 (123)	530 (11.4)	560 (9.4)[d]	0.5	–
n-butyl isocyanide[h]	–	354 (~31)	427 (131)	533 (~14)	560 (~13)	5.0	–
dimethylphenylphosphine	–	369 (39)	426 (73)	546 (10.7)		0.006	–
bis(hydroxymethyl)methylphosphine[i]	–	*g*	425 (*g*)	~542 (*g*)		*g*	–
1-propanethiol	–	358 (35)	423.5 (93.3)	542 (10.0)	574 (8.3)	~0.8	–
nitric oxide	–	355 (38.6)	418.5 (146)[f]	534 (13.9)	568 (19.9)	*g*	–

[a] The spectral data of the complexes were obtained at pH 6–8 in the presence of sufficiently high concentrations (>20K_d, i.e., >95% saturation) of the ligands or L-Trp. [b] Previously reported spectral data (Sono et al., 1980; Sono & Dawson, 1984) have been slightly revised. [c] Not α and β bands but charge-transfer high-spin marker bands. [d] Shoulders. [e] Sono et al. (1980). [f] Sono and Dawson (1984). [g] Not determined. [h] Spectral data for the fully ligand saturated complex were calculated on the basis of 64% ligand saturation. [i] The complex is highly autoreducible.

Table II: Absorption Spectral Parameters and Dissociation Constants (K_d) of the Ferrous Indolamine 2,3-Dioxygenase Complexes with Ligands and L-Trp[a]

exogenous ligand	±L-Trp	absorption bands, λ (ϵ_{mM}) [nm (mM^{-1} cm^{-1})]			K_d (μM) at pH 7.0 for ligand[b]
		Soret	β	α	
ethyl isocyanide	–	428 (161)	530 (13.6)	558 (18.7)	12.6
n-butyl isocyanide	–	427.5 (177)	530 (16.7)	559.5 (23.4)	0.29
	+	428.5 (179)	531 (17.0)	560 (22.5)	*c*
benzyl isocyanide	–	426.7 (166)	529.5 (13.5)	559 (17.7)	~0.1
cyanide	–[d]	427 (182)	530.5 (17.3)	561 (26.1)	*c*
	+[e]	427 (177)	531 (17.3)	561 (28.7)	*c*
4-phenylpyridine	–	420 (161)	527 (18.1)	556 (34.2)	40
dimethylphenylphosphine	–	457 (79.0)	552 (15.4)	576 (6.5)	10
bis(hydroxymethyl)methylphosphine	–	450 (99)	546 (17.3)	~572 (7.6)	~1000

[a] The spectral data of the complexes were obtained at pH 6–8 in the presence of sufficiently high concentrations (>20K_d, i.e., >95% saturation) of the ligand or L-Trp. [b] K_d values for L-Trp were not determined. [c] Not determined. [d] Sono and Dawson (1984). Previously reported spectral data at 4 °C have been slightly revised. [e] Examined at 4 °C in the presence of 0.8 mM L-Trp.

Among these ligands, benzhydroxamate (pK_a = 8.8), which has been reported to bind to ferric HRP to form a high-spin complex (Schonbaum, 1973), does not compete either with L-Trp or with the heme ligand cyanide for the dioxygenase. This suggests that this ligand neither occupies the substrate binding site nor directly coordinates to the heme iron. As shown in Figure 1A, the absorption spectra of the benzhydroxamate complex of the ferric dioxygenase with (dashed line) and without L-Trp (solid line) are very similar to that of the L-Trp–ferric enzyme adduct (dotted line). These three complexes exhibit similar MCD spectra that are characteristic of a hydroxide adduct of a histidine imidazole ligated low-spin ferric heme (Figure 1A, inset) (Vickery et al., 1976; Sono & Dawson, 1984). The apparent affinity (K_d^{app} for 50% overall saturation) of benzhydroxamate for the ferric enzyme[2] increases with an increase in pH; K_d^{app} values were ~160, ~12, and 1 mM at pH values 6, 7, and 8, respectively. Unlike formate, acetate appears to bind to the ferric enzyme at a site other than the heme iron, causing only a small spectral change (not shown); the cyanide affinity of the ferric enzyme (K_d = 11 μM at pH 6.0 without acetate) is considerably increased by an addition of 0.5 (K_d = 7.6 μM) or 5 M acetate (K_d = 2.6 μM).

The binding of imidazole to the ferric dioxygenase is biphasic, similar to the case of benzhydroxamate (footnote 2). Furthermore, the binding of imidazole and L-Trp to the ferric enzyme seems to be negatively cooperative or competitive. This was judged from the partial conversion of the ferric enzyme–imidazole complex (with 0.2 M imidazole, pH 8.0, K_d = ~5 mM) to the L-Trp–ferric enzyme adduct (K_d = ~0.5 mM at pH 8.0 without imidazole) upon an addition of 10 mM L-Trp as observed with optical absorption, MCD, and CD spectroscopy (spectra not shown). Note that 4-phenylimidazole and L-Trp are competitive for the ferric dioxygenase (Sono & Cady, 1989). Further details for the imidazole binding were not examined in this work. An imidazole complex of the ferrous enzyme is unstable at room temperature below pH 7.5; its absorption spectral intensity gradually decreases with time. Pyridine (0.58 M at pH 7.0) forms a

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[2] The binding of benzhydroxamate to the ferric enzyme is not uniform but biphasic, even though one set of isosbestic points is seen throughout a titration. Addition of L-Trp (0.5 mM at pH 8.0) changes the benzhydroxamate binding mode to a uniform pattern.

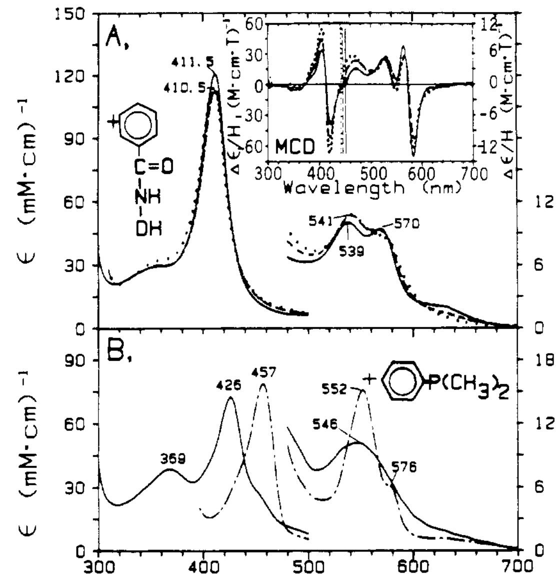

FIGURE 1: Optical absorption spectra of the benzhydroxamate (A) and dimethylphenylphosphine (B) complexes of ferric (A and B) and ferrous (B) indolamine 2,3-dioxygenase (IDO). (A) The native ferric dioxygenase (~45 μM) + 10 mM benzhydroxamate (—), further addition of 20 mM L-Trp (- - -), and the native ferric enzyme + 20 mM L-Trp (···), all at pH 8.0 with a 0.2-cm cuvette. MCD spectra of these complexes are shown in the inset. (B) The ferric dioxygenase (0.85 μM) + 0.26 mM dimethylphenylphosphine (—), followed by reduction with excess sodium dithionite to generate the ferrous enzyme–phosphine adduct (-·-), at pH 7.0 with a 1-cm cuvette. The spectrum of the ferrous complex below 400 nm is not shown here because of a large absorbance by dithionite (λ_{max} = 315 nm).

complex with the ferric enzyme (Sono & Dawson, 1984), but the ferrous enzyme–pyridine complex is unstable as judged from its conversion to a pyridine hemochromogen-type species (λ_{max} = 418, 525, and 556 nm) [see Antonini and Brunori (1971)].

4-Phenylimidazole, *N*-phenylimidazole, and 4-phenylpyridine readily form stable complexes with both the ferric and ferrous enzymes in a uniform binding fashion (cf. imidazole, see above). 4-Phenylimidazole has about 10 and 200 times higher affinity for the ferric dioxygenase than *N*-phenylimidazole and imidazole, respectively, at pH 6.0 (Table I; Sono & Cady, 1989). 3-Phenylpyridine is a poorer ligand than 4-phenylpyridine for both oxidation states of the enzyme. 4-Phenylpyridine complexes of the ferric and ferrous enzymes exhibit absorption spectra similar to those of the corresponding norharman complexes (Sono & Cady, 1989). Metyrapone, a ligand that tightly binds to cytochrome P-450 (Peterson et al., 1971), and 4,5-diphenylimidazole do not readily bind to either the ferric or ferrous dioxygenase.

Dimethylphenylphosphine binds to both the ferric (Figure 1B, solid line) and ferrous dioxygenases (dot-dashed line) without competing with L-Trp with the similar K_d^{app} value of ~10 μM for both oxidation states. The ferric enzyme complex is slightly autoreducible. Noticeable in the spectral features of the dioxygenase–dimethylphenylphosphine complex is an extremely low Soret peak intensity for both the ferric (ϵ_{426} = 73 mM^{-1} cm^{-1}) and the ferrous enzymes (ϵ_{457} = 79 mM^{-1} cm^{-1})[3] as compared with the corresponding values of other ligand complexes (see Tables I and II). Ferric HRP forms a stable low-spin complex with dimethylphenylphosphine [λ_{max} ($\epsilon_{mM^{-1}cm^{-1}}$) = 427 (~72), 536 (~10)] (this work).

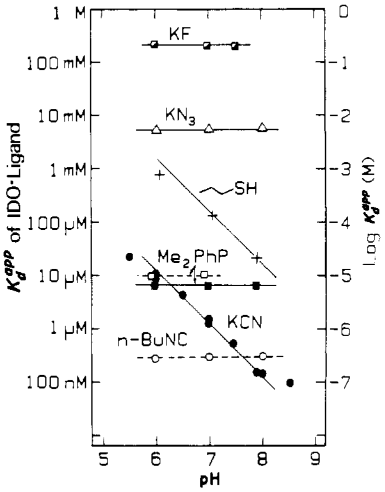

FIGURE 2: pH dependence of the apparent dissociation constants (K_d^{app}) of ferric and ferrous indolamine 2,3-dioxygenase (IDO) complexes with various ligands. Results (data points) for the ferric and ferrous enzymes are connected with solid lines and dashed lines, respectively. The ligands used are indicated in the figure. Me$_2$PhP, dimethylphenylphosphine; *n*-BuNC, *n*-butyl isocyanide.

Ethyl isocyanide has considerably lower affinity (K_d = 12.6 μM) than *n*-butyl isocyanide (K_d = 0.29 μM) for the ferrous dioxygenase (Table II). Since the cyanide complex was not stable below pH 8.0 at room temperature (see the case of the imidazole complex described above), the experiments were performed at pH 8.0 and ~4 °C. For the cyanide–ferrous dioxygenase adduct, the binding of L-Trp is more clearly detectable with CD [λ_{nm} ($\Delta\epsilon_{M^{-1}cm^{-1}}$) = 420 (6.0), 558 (−6.5) without Trp and 435 (−20), 563 (−10) with 0.8 mM L-Trp] than with optical absorption (Table II) or MCD spectroscopy (not shown). Effects of L-Trp on the absorption, MCD, and CD spectra of the ferrous enzyme–*n*-butyl isocyanide complex (not shown) are relatively small. The ferric dioxygenase binds *n*-butyl isocyanide (only 64–70% saturation within its solubility; λ_{max} = 426 nm, see Table I) to form a low-spin complex. The dioxygenase complex thus formed is autoreducible as is the phosphine complex (see above), while the analogous ferric HRP complex [λ_{max} ($\epsilon_{mM^{-1}cm^{-1}}$) = 421.5 (~93), 538 (~10)] (this work) is not.

pH Dependence of the Ligand Affinity of Indolamine 2,3-Dioxygenase. Shown in Figure 2 is the pH dependence of the apparent dissociation constants (K_d^{app}) of the various ligand adducts of the ferric (solid line) and ferrous dioxygenases (dashed line). Among the ligands for the ferric enzyme, only cyanide (pK_a = 9.2) and 1-propanethiol (pK_a = 10.5), two weakly acidic anionic ligands [see Sono et al. (1986) for the pK_a values], exhibit highly pH dependent affinity. The log K_d^{app} value decreases linearly with an increase in pH between pH 6 and 8 with a slope of −1. In contrast, the K_d^{app} values for fluoride (pK_a = 3.2) and azide (pK_a = 4.7), moderately acidic anionic ligands, and for the neutral ligand dimethylphenylphosphine are constant between pH 6 and 8.

Nitrogen donor ligands including *N*- and 4-phenylimidazoles, 4-phenylpyridine, and norharman exhibit log K_d^{app} vs pH profiles (results not shown) that can be explained only by assuming that the deprotonated (neutral) form of the conjugated acids of these ligands binds to the enzyme in a pH-independent manner between pH 6 and 8 (Sono & Cady, 1989). At pH 7.0 in the absence of Trp, the affinity (K_d^{app} values shown in parentheses) of the ferric dioxygenase for the

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[3] A uniquely red-shifted Soret peak position (λ_{max} = 457 nm) of the ferrous dioxygenase–phosphine complex (Figure 1B) as compared with the Soret peak positions of the other ligand complexes of the ferrous enzyme (Table II) is very similar to those of analogous derivatives not only of ferrous myoglobin but also of ferrous cytochrome P-450$_{cam}$ and chloroperoxidase (Dawson et al., 1983; Sono et al., 1985).

Table III: Effects of Indole Derivatives on the Dissociation Constants (K_d) of the Ferric–Azide and Ferrous–Ethyl Isocyanide (EtNC) Complexes of Indolamine 2,3-Dioxygenase (IDO)[a]

indole derivative	concn of indole derivatives (mM)	ferric IDO–azide complex K_d (mM)	 rel affinity	concn of indole derivatives (mM)	ferrous IDO–EtNC complex K_d (μM)	 rel affinity
none	0	5.3	1.0	0	12.6	1.0
L-Trp	10	0.58	9.14	0.2	17.5	0.72
D-Trp	10	3.63	1.46	0.2	12.6	1.0
5-hydroxy-L-Trp	10	3.31	1.60	0.5	15.9	0.79
				8	32.4	0.39
5-hydroxy-D-Trp	10	3.98	1.33	1	16.3	0.77
tryptamine	10	2.63	2.02	0.2	12.6	1.0
serotonin	10	5.0	1.06	0.2	12.6	1.0
α-methyl-DL-Trp	10	3.98	1.33	0.2	12.0	1.05
indole-3-acetic acid	10	5.3	1.0	0.2	12.3	1.02

[a] The experiments were performed in 0.1 M potassium phosphate buffer at pH 6.0 for the ferric enzyme and at pH 7.0 for the ferrous enzyme.

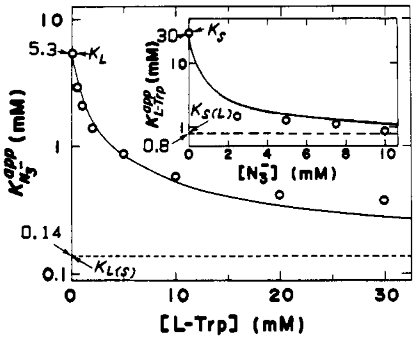

FIGURE 3: Positive cooperativity in the binding of azide and L-Trp (inset) to ferric indolamine 2,3-dioxygenase (IDO) at pH 6.0. The solid line was drawn according to eqs 8 and 12 (for the inset) by using the following constants determined by the method described in the Appendix: K_L = 5.3 mM, K_S = 30 mM, $K_{L(S)}$ = 0.14 mM, and $K_{S(L)}$ = 0.8 mM.

ligands listed below increases in the order fluoride (200 mM) < imidazole (10 mM) < azide (5.3 mM) < 1-propanethiol (130 μM) < dimethylphenylphosphine (6.3 μM) < cyanide (1.5 μM).

For the ferrous enzyme, the apparent affinity (K_d^{app}) of the two neutral ligands, *n*-butyl isocyanide (K_d^{app} = 0.29 μM) and dimethylphenylphosphine (K_d^{app} = 10 μM) is relatively high and appears to be constant between pH 6 and 8 (Figure 2, dashed line).

Effects of Indolamine Substrates on the Ligand Affinity of Indolamine 2,3-Dioxygenase. Effects of organic substrates on the ligand affinity of the ferric and ferrous dioxygenases were examined. For the ferric dioxygenase, the addition of L-Trp markedly enhances the affinity of the enzyme for small ligands including azide, cyanide, and fluoride as previously reported (Sono & Hayaishi, 1980). In Figure 3, a typical example for the azide case is shown. The K_d^{app} value of the azide complex drastically decreases with an increase in the concentration of L-Trp (0 → 30 mM) by a factor of 14 (K_d^{app} = 5.3–0.38 mM) at pH 6.0. The corresponding factor for fluoride was 5300 (K_d^{app} = 200–0.038 mM). A factor for cyanide was not determined in this work since the rate for cyanide binding to the ferric enzyme becomes extremely slow in the presence of L-Trp. Nevertheless, a considerable increase in cyanide affinity upon addition of L-Trp was easily detectable (Table I). These results are indicative of the existence of the positive cooperativity between L-Trp and each of these three ligands for their binding to the active site of the ferric dioxygenase. This suggests that azide, fluoride, and cyanide also enhance the L-Trp affinity of the enzyme in a reciprocal manner. The results shown in the inset of Figure 3, where the K_d^{app} value of the L-Trp–enzyme adduct is plotted as a function of the azide concentration, prove that this is the case. L-Trp affinity of the ferric enzyme also dramatically increases on binding of cyanide by a factor of 1850 at pH 6.0 (K_d^{app} = 30 mM–16 μM, see below).

Effects of other indolamine substrates on the azide affinity of the ferric dioxygenase were also examined. Results are summarized in Table III. When compared at a 10 mM concentration, L-Trp exerts by far the greatest effect as seen by the increase (9.1 times) in the relative azide affinity (*R*) of the dioxygenase. Under the conditions employed (pH 6.0, 25 °C), tryptamine, 5-hydroxy-L-Trp, D-Trp, and 5-hydroxy-D-Trp exhibit significant but relatively small effects (*R* = 1.3–2.0) (Table III) with decreasing magnitude in order as listed. A significant effect of α-methyl-DL-Trp (*R* = 1.3), a nonmetabolizable indolamine, is noted.

In contrast to the case of the ferric dioxygenase described above, the binding of L-Trp and heme ligands to the ferrous enzyme is negatively cooperative for all the ligands examined, i.e., L-Trp and heme ligands decrease each other's affinity for the ferrous enzyme in a reciprocal manner. A search for competitive ligands for the ferrous dioxygenase with respect to L-Trp was thus unsuccessful. Typical examples for the negatively cooperative binding have been previously reported between 4-phenylimidazole and L-Trp and between norharman and L-Trp (Sono & Cady, 1989). Ethyl isocyanide and 4-phenylpyridine also behave in a similar manner (this study).

The effects of various indolamine compounds on the ethyl isocyanide affinity of the ferrous enzyme were examined as a possible indicator for their specific interaction with the active site of the dioxygenase. Unlike the case of the azide binding to the ferric enzyme (see above), the indole derivatives except for L-Trp and 5-hydroxy-L-Trp exert very small effects on ethyl isocyanide binding to the ferrous enzyme as summarized in Table III. This is partially because the highest accessible concentration of these compounds is limited to ~0.2 mM due to the instability of the ligand-free ferrous enzyme in the presence of >0.2 mM indole derivatives (Sono et al., 1980). Fortunately, 5-hydroxy-L-Trp and 5-hydroxy-D-Trp can be used even for concentrations >0.5 mM (Sono et al., 1980). For these two substrates significant negative cooperativity was observed at 0.5–1 mM concentrations (Table III).

The effects of L-Trp on the affinity of small ligands such as O_2, CO, and NO for the ferrous enzyme cannot be easily examined since the ferrous–O_2 enzyme is not stable, especially in the presence of L-Trp (Hirata et al., 1977), and since the CO and NO affinity of the enzyme is extremely high for equilibrium titration experiments. However, the L-Trp affinity of the ferrous enzyme (K_d^{app}) in the presence and absence of CO can be readily determined. The K_d^{app} values at pH 7.0 have been reported to be 13 μM (–CO) and 350 μM (+CO) (Sono et al., 1980). This indicates that the binding of L-Trp and CO to the ferrous enzyme is negatively cooperative. This,

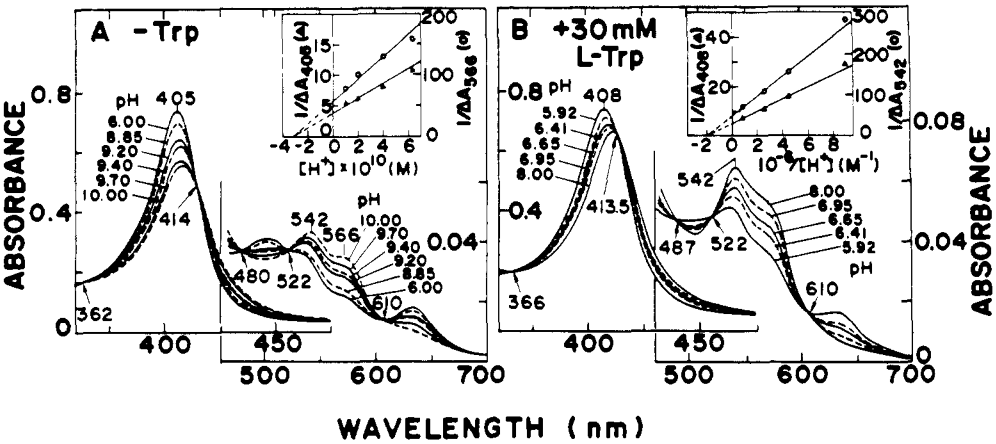

FIGURE 4: Absorption spectral transition for the acidic–alkaline forms of ferric indolamine 2,3-dioxygenase in the absence (A) and presence of 30 mM L-Trp (B). The pH values for the respective spectra are indicated in the figure. The insets show the analysis of the results for the determination of the pK_a values according to eq 18 for (A) and eq 20 for (B) as described in the Appendix. Note that the wavelength scale in the Soret region (350–475 nm) is expanded 2 times as compared with that in the visible region (475–700 nm) for both (A) and (B).

in turn, suggests that the CO affinity of the enzyme decreases upon L-Trp binding by a factor of ~27 (=350/13).

Effects of pH on L-*Trp Affinity of the Native Ferric Dioxygenase and of Its Cyanide Adduct.* It has been reported that the L-Trp affinity of the native ferric enzyme increases with an increase in pH (Sono et al., 1980). The previous K_d^{app} determination at the pH values 6.0, 7.0, and 8.0 was further expanded in more detail in this work for a wider pH range (pH 5.5–8.8). A plot of the log K_d^{app} value vs pH (not shown) yields a feature that can roughly fit in overall shape a theoretical curve (not shown) which is drawn on the basis of the assumption that a single ionizable group with a pK_a value of 9.5 (see below) for the Trp-free enzyme (and 5.5 for its L-Trp adduct) is involved for the L-Trp binding [see the Appendix in Sono et al. (1986) for data analysis].[4] Small deviations of the data points from the solid theoretical curve are seen between pH 6.5 and 7.0 (upward), between pH 7.5 and 8.0 (downward), and between pH 8.0 and 8.5 (upward). Nevertheless, the L-Trp affinity determined at pH 8.8 (K_d^{app} = 0.081 mM) is about 650 times higher than that at pH 5.5 (K_d^{app} = 52.5 mM).

In contrast to the native ferric dioxygenase case described above, L-Trp affinity (K_d^{app}) of the ferric enzyme–cyanide complex does not vary markedly between pH 5.6 and 8.4 (plot not shown). The K_d^{app} values determined at the pH values 6.0, 7.0, and 7.9 are 16.2, 11.7, and 14.5 μM, respectively. The small pH dependence of the K_d^{app} value, especially between pH 6 and 6.5 and between pH 7.5 and 8.0, appears to reflect an indirect influence of an ionizable group(s) on the L-Trp binding.

Effects of pH on the L-*Trp and 5-Hydroxy-*L-*Trp Affinity of the Ligand-Free Ferrous Dioxygenase, on the* L-*Trp Affinity of the Ferrous–CO Enzyme, and on the* K_m *Value for* L-*Trp.* A previous equilibrium study has shown that the L-Trp affinity (K_d^{app}) of the ligand-free ferrous enzyme noticeably increases with an increase in pH similar to that of the native ferric enzyme. In contrast, K_d^{app} of the L-Trp complex of the ferrous–CO enzyme has been shown not to change significantly between pH 6 and 8 (Sono et al., 1980). In addition, the K_m value for L-Trp has been found to be nearly identical with the K_d^{app} value of the L-Trp–ferrous enzyme adduct (Sono et al., 1980). The previous study was further extended over a wider pH range (pH 5.1–9.7) and in more detail in this work.

The K_d^{app} value of the L-Trp–ferrous dioxygenase adduct is nearly constant above pH 8 (K_d^{app} = 4.4 μM at pH 8.5) and increases inversely with pH below this pH value (K_d^{app} = 107 μM at pH 5.5). The experimental data above pH 6.5 fit fairly well to the theoretical curve (not shown) that is drawn by assuming the involvement of a single ionizable group with a pK_a value of 7.3 and by using a limiting low K_d^{app} value of 4.4 μM at alkaline pH [see the Appendix in Sono et al. (1986) for data analysis, where pK_{a1} = 7.3 and K_L^b = 4.4 μM for eq 13]. The K_m value for L-Trp also fits the same theoretical curve [K_m = 250 (pH 5.5), 50 (pH 6.0), 25 (pH 6.5), 13 (pH 7.0), 9 (pH 7.5), and 6.5 μM (pH 8.0)]. Due to the instability of the ferrous enzyme in the presence of >0.2 mM L-Trp, experimental values below pH 5.5, where K_d^{app} > 0.2 mM, were not obtained. Nevertheless, a rough estimate of the pK_{a2} value of 5.6 for the ionizable group in the L-Trp-bound ferrous enzyme (cf. pK_{a1} = 7.3 for the L-Trp-free enzyme) and of a limiting high K_d^{app} value of 220 μM (=K_L^a) yielded a theoretical curve for a K_d^{app} vs pH profile that fits fairly well to the experimental values [see eq 12 in Sono et al. (1986)].

The affinity of 5-hydroxy-L-Trp for the ferrous enyzme was also examined in detail between pH 5 and 8 in this work. 5-Hydroxy-L-Trp has been found to have a relatively high affinity for the ferrous enzyme and a relatively small K_m value that are similar to those for L-Trp (Shimizu et al., 1978; Sono et al., 1980). Interestingly, the pH dependence of the K_d^{app} value for 5-hydroxy-L-Trp is completely opposite to that for L-Trp. Thus, the K_d^{app} value for 5-hydroxy-L-Trp increases with an increase in pH above pH 6.0; K_d^{app} values are 46, 50, 64, 105, and 204 μM for the pH values of 4.85, 5.8, 7.0, 7.5, and 7.9, respectively. The K_d^{app} value appears to have reached a limiting low value of 45 μM at pH 5.0. Analysis of the data (K_d^{app} vs pH) in a manner similar to that for L-Trp as described above (but $K_L^a < K_L^b$, and pK_{a1} < pK_{a2}) yielded the pK_{a1} value of 7.3, suggesting that the same ionizable residue is involved for 5-hydroxy-L-Trp and L-Trp binding to the ferrous dioxygenase.

pH Dependence of the Absorption Spectrum of the Ferric Dioxygenase in the Presence and Absence of L-*Trp.* Careful reexamination of the pH effects on the optical absorption spectrum of the substrate-free native ferric enzyme in the present study showed that no significant spectral change is detectable between pH 6 and 8.[5] Furthermore, the present

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[4] The best fit was obtained by using the following constants for eq 12 in Sono et al. (1986): pK_{a1} = 9.5, pK_{a2} = 5.5, K_L^b (=K_{d2}) = 0.011 mM [and K_L^a (=K_{d1}) = 112 mM].

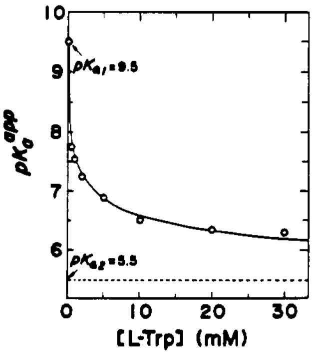

FIGURE 5: L-Trp concentration dependence of the apparent pK_a (pK_a^{app}) value of the acidic–alkaline form spectral transition of ligand-free ferric indolamine 2,3-dioxygenase. See the legend to Figure 4 and Experimental Procedures for the determination of pK_a^{app} values. The solid line was drawn according to eq 15 (Appendix) by using the following constants: $pK_{a1} = 9.5$, $pK_{a2} = 5.5$, $K_{d1} = 112$ mM (and $K_{d2} = 0.011$ mM).

study revealed that above pH 8.5 the native ferric enzyme undergoes a pH-dependent spectral change from a high-spin to a low-spin form as shown in Figure 4A. Unlike ferric myoglobin, however, the dioxygenase does not form a stable "alkaline form" above pH 8.5; following the initial fast spectral change upon pH increase, a slower secondary conversion to another low-spin-type spectrum occurs. The extent of the secondary spectral change appears to be greater at higher pH. Isosbestic points for the native ferric and the first low-spin species (see Figure 4A) seem to slightly but significantly differ from those for the first and the secondary low-spin forms of the dioxygenase (not shown).

When the pH of a sample was quickly brought back to pH ~6.0 within 2 min following the dilution of an acidic (pH 6.0) enzyme stock solution in an alkaline buffer (pH >8.5), near quantitative spectral conversion from the low-spin form to the original high-spin form was observed. However, when the enzyme solution was kept at alkaline pH until the secondary spectral change reached the maximal extent (≥30 min), very little of the original high-spin spectrum of the native ferric enzyme was recovered upon the acidification of the enzyme solution. These results suggest that only the conversion of the native ferric enzyme to the initial alkaline form of the ferric enzyme is reversible. The secondary spectral change seems to reflect an irreversible conversion of the ferric enzyme to another alkaline form. Analysis of the pH titration results for the initial reversible spectral change (shown in Figure 4A, inset) yields a pK_a value of 9.5 [x intercept: $-[H^+] = -3.2 \times 10^{-10}$ M (see the Appendix)].

In the presence of L-Trp, on the other hand, a pH-dependent spectral change between high-spin and low-spin forms readily occurs below pH 8.0. A typical example is shown in Figure 4B, where the absorption spectrum of the ferric dioxygenase was recorded in the presence of 30 mM L-Trp by changing the pH from 5.92 to 8.00. A single set of isosbestic points is seen during the spectral conversion as indicated in the figure. The resulting low-spin species at pH 8.0 is the L-Trp complex (>98% saturated) of the ferric enzyme (Sono et al., 1980). From the replot of the titration results (Figure 4B, inset), the pK_a value was determined to be 6.3 (x intercept: $-1/[H^+] = -2.0 \times 10^6$ M^{-1}) (see the Appendix). The pK_a value in the presence of 30 mM D-Trp was 7.90.

In Figure 5 the apparent pK_a value (pK_a^{app}) for the spectral transition of the ferric enzyme as a function of L-Trp concentration (0–30 mM) is plotted by using open circles. The solid line is a theoretical curve based on the estimated pK_a^{app} values of 9.5 in the absence of Trp and of 5.5 in the presence of an infinite concentration of L-Trp (see the Appendix).

DISCUSSION

Indolamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase are the only two heme proteins known to exist that catalyze the incorporation of the heme-ligand dioxygen into an organic substrate (L-Trp) (Hayaishi et al., 1975; Feigelson & Brady, 1971; Ishimura et al., 1980). As would be postulated, indolamine 2,3-dioxygenase has a considerably larger heme pocket than myoglobin and HRP. This is judged from the greater accessibility of the dioxygenase than myoglobin and HRP to relatively large heme ligands such as norharman (Sono & Cady, 1989) and 4-phenylpyridine (this work). Obviously, the requirement of a binding site for the organic substrate Trp at the active site of the dioxygenase explains this difference. Even between indolamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase, the heme pocket size of the former has been suggested to be somewhat larger than that of the latter (Eguchi et al., 1984; Sono & Cady, 1989). This likely reflects the difference in their substrate specificities (Feigelson & Brady, 1974; Shimizu et al., 1978).

Tryptophan 2,3-dioxygenase has two binding sites for L-Trp, a catalytic site and a regulatory site (Feigelson & Brady, 1974). Although indolamine 2,3-dioxygenase seems to possess an effector binding site for some indole derivatives (see below), it is still not clear whether L-Trp also occupies the effector site or not. In any case, certain ligands and Trp binding properties are shared by these two dioxygenases. In the ferric (catalytically inactive) state, L-Trp and the typical heme ligand cyanide bind to the catalytic site in a positively cooperative manner for both dioxygenases. In the ferrous state, L-Trp and CO have shown negative cooperativity for indolamine 2,3-dioxygenase (Sono et al., 1980; this work), while the CO affinity is markedly enhanced upon addition of L-Trp for tryptophan 2,3-dioxygenase (Feigelson & Brady, 1974). For both dioxygenases, these results suggest the close proximity of the Trp binding site to the heme iron. The binding of L-Trp is a prerequisite for the O_2 binding to tryptophan 2,3-dioxygenase (Ishimura et al., 1970), while O_2 can bind readily to Trp-free ferrous indolamine 2,3-dioxygenase (Hirata et al., 1977). Since the ternary complex L-Trp–ferrous enzyme–O_2 is a common obligatory intermediate for the catalytic cycles of both dioxygenases (Ishimura et al., 1970; Sono et al., 1980), the fundamental position of the heme iron bound O_2 relative to the catalytic site bound L-Trp in that complex might not be significantly different for the two dioxygenases. Hence, the observed differences in the effects of L-Trp might be caused by subtle differences in the microscopic structures of their heme iron vicinities in the L-Trp-free state, e.g., in the difference in the position of a distal side residue(s) that might be involved in the binding of O_2 or Trp.

Except for cytochrome c, ferric heme proteins known to readily bind $O_2^{\bullet -}$ are also H_2O_2-utilizing enzymes such as catalase (Shimizu et al., 1984), HRP (Shimizu et al., 1989), and myeloperoxidase (Kettle et al., 1988). On the other hand, O_2-utilizing (binding) heme proteins such as myoglobin and

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[5] The previously reported pH-dependent spectral change for the Trp-free ferric dioxygenase below pH 8.5 (with apparent $pK_a = 8.1$; Sono et al., 1980) might have been caused by the contamination of a trace amount (<0.1 μM) of a heme ligand (most likely cyanide) that has considerably higher affinity for the enzyme at an alkaline pH than at an acidic pH (see Figure 2). The same undesirable problem is often encountered at pH >7.5, especially when a low concentration (<2 μM) of the dioxygenase is used. However, partial effects of the "true" acidic–alkaline spectral transition ($pK_a = 9.5$), which will be described in the text on the spectrum of the Trp-free ferric dioxygenase at pH 8, cannot be completely neglected.

hemoglobin (Ilan et al., 1976) and cytochrome P-450_{cam} (Debey et al., 1979) have very low reactivity, if any, toward $O_2^{\bullet-}$. There seems to have been a correlation between the anionic ligand binding property (see the introduction) and the ability to bind $O_2^{\bullet-}$. However, the present study revealed that, despite the high reactivity of the ferric dioxygenase with $O_2^{\bullet-}$ (Hirata et al., 1977; Taniguchi et al., 1979; Kobayashi et al., 1989), the affinity of anionic ligands (cyanide, azide, fluoride) for the dioxygenase changes with pH in a manner similar to that of ferric myoglobin rather than that of peroxidases or catalase. Hence, it appears that another factor other than the one which is detectable in the anionic ligand binding property [e.g., an electrostatic gate of the heme pocket (Davies et al., 1980)] is influencing the $O_2^{\bullet-}$ binding to the heme iron of indolamine 2,3-dioxygenase. The heme environmental structure, hydrophobicity, or basic/acidic residue(s) in the heme pocket may play a key factor in determining the accessibility of the heme iron to $O_2^{\bullet-}$.

The existence of an effector binding site in indolamine 2,3-dioxygenase that is different from the catalytic site for L-Trp has been revealed in previous studies (Sono, 1989b; Eguchi et al., 1984). The binding of indole to the effector site is positively cooperative not only with the binding of the heme ligands cyanide and azide to the heme iron but also with the binding of L-Trp to the catalytic site (Sono, 1989b). As judged from the cooperative or noncompetitive binding of acetate or benzhydroxamate with cyanide and with L-Trp to the ferric enzyme, acetate and benzhydroxamate appear to bind to the effector binding site.

The pH-dependent absorption spectral conversion from the acidic form to the alkaline form(s) was observed only above pH 8.5 in the absence of Trp. However, due to the instability of the initially formed alkaline form, the heme iron coordination structures of these two high-pH forms of the Trp-free dioxygenase remain unclear. For the L-Trp-bound dioxygenase, hydroxide has been suggested as the sixth ligand trans to histidine on the basis of MCD and electron paramagnetic resonance studies (Sono & Dawson, 1984). At present, it is not quite certain whether the estimated pK_a values in the presence ($pK_a = \sim 5.5$) and absence of L-Trp (pK_a = 9.5) are attributable to the same residue or not. The observed pH profile of the K_d^{app} value for the L-Trp binding to the native ferric enzyme could reflect the participation of more than one ionizable group having different pK_a values. Hence, the fitting of the data with the theoretical curves (solid line) in Figure 5, which are based on the assumption that a single ionizable group is involved, should be considered as a tentative analysis. Further studies with different spectroscopic techniques might be required for a final analysis.

On the other hand, the pH dependence of the L-Trp affinity of the ligand-free ferrous dioxygenase as well as that of the K_m value for L-Trp are clearly affected by an ionizable group in the enzyme possessing a pK_a value of 7.3 (−Trp). This ionizable residue becomes more acidic upon L-Trp binding (pK_a = 7.3 → ~5.6). This is further supported by the observation of an identical pK_a value for 5-hydroxy-L-Trp binding to the ferrous enzyme. The opposite pH dependence of the affinity of these two substrates for the ferrous enzyme might reflect either a possible interaction of the substituents (H or OH) at the 5-position on the indole ring with a catalytic site residue or somewhat different interactions for L-Trp and 5-hydroxy-L-Trp with the catalytic site. Further studies on the interaction between indolamine substrates and the catalytic site of the dioxygenase using several substituted Trps are underway at present.

ACKNOWLEDGMENTS

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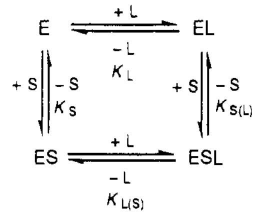
APPENDIX: DATA ANALYSIS

(*I*) *Cooperative Binding of Ligand* (*L*) *and Substrate* (*S*) *to the Enzyme* (*E*). The equilibria for the ligand and substrate binding to the enzyme can be described as



where E, L, and S are the free forms of the enzyme, ligand, and substrate, respectively, and EL, ES, and ESL are enzyme–ligand, enzyme–substrate, and enzyme–substrate–ligand complexes, respectively. From the above scheme, the following four microscopic equilibrium dissociation constants are defined.

$$K_L = [E][L]/[EL] \qquad (1)$$

$$K_S = [E][S]/[ES] \qquad (2)$$

$$K_{L(S)} = [ES][L]/[ESL] \qquad (3)$$

$$K_{S(L)} = [EL][S]/[ESL] \qquad (4)$$

Combination of eqs 1–4 yields

$$K_L K_{S(L)} = K_S K_{L(S)} \qquad (5)$$

(*a*) *Titration of the Enzyme with Ligand at a Given Substrate Concentration*. The apparent ligand equilibrium dissociation constant (K_L^{app}) is defined by the eqs 6a, 6b, and 7, which are expressed by eq 8 using the eqs 1–4. In eq 7, ΔA is the absorbance change caused by incremental additions of the ligand to the initially ligand-free enzyme (E + ES) and ΔA_∞ is the absorbance change for the complete formation of the enzyme–ligand complex (EL + ESL). Since [E]/[ES] (see eq 2) and [EL]/[ESL] (see eq 4) are constant at a given [S], (E + ES), and (EL + ESL), each should behave as if they were a single species, resulting in spectral changes that would accompany one set of isosbestic points throughout the titration. By rearranging eq 7 and by taking the logarithm of eq 7, eqs 9 and 10 are obtained, respectively. The K_L^{app} value for a given [S] can be determined either from a double-reciprocal plot (eq 9, $1/\Delta A$ vs $1/[L]$) or from a Hill plot [eq 10, $\log \{\Delta A/(\Delta A_\infty - \Delta A)\}$ vs $\log [L]$] [see Sono et al. (1980)]. Using

$$K_L^{app} = [\text{ligand-free enzyme}][\text{free ligand}]/[\text{ligand-bound enzyme}] \qquad (6a)$$

$$K_L^{app} = \{([E] + [ES])[L]\}/\{[EL] + [ESL]\} \qquad (6b)$$

$$K_L^{app} = [L](\Delta A_\infty - \Delta A)/\Delta A \qquad (7)$$

$$K_L^{app} = \{K_L(1 + [S]/K_S)\}/\{1 + [S]/K_{S(L)}\} \qquad (8)$$

$$1/\Delta A = K_L^{app}/\Delta A_\infty[L] + 1/\Delta A_\infty \qquad (9)$$

$$\log \Delta A/(\Delta A_\infty - \Delta A) = \log [L] - \log K_L^{app} \qquad (10)$$

the K_L (eq 1) and K_S (eq 2) values that have been separately determined and several pairs of K_L^{app} vs [S] that are now available, the $K_{S(L)}$ value can be determined as the average of several values obtained from eq 11, which is derived by

$$K_{S(L)} = [S]/\{K_L(1 + [S]/K_S)/K_L^{app} - 1\} \qquad (11)$$

rearranging eq 8. A theoretical curve for K_L^{app} versus [S] can then be drawn according to eq 8 (see Figure 3).

(*b*) *Titration of the Enzyme* (*E*) *with Substrate* (*S*) *at a Given Ligand* (*L*) *Concentration.* In a manner similar to the above case, the apparent substrate equilibrium dissociation constant (K_S^{app}) is given by eq 12.

$$K_S^{app} = \{K_{S(L)}(K_L + [L])\}/\{K_{S(L)}K_L/K_S + [L]\} \qquad (12)$$

By use of the experimentally determined K_L (eq 1) and K_S (eq 2) values and the $K_{S(L)}$ value determined above (eq 11), a theoretical curve for K_S^{app} vs [L] can be obtained (see Figure 3, inset).

(*II*) *pH Profiles of Substrate Binding to the Enzyme and the Acidic Form–Alkaline Form Spectral Transitions of the Enzyme.* The equilibria for the substrate binding to the enzyme and the protonation and deprotonation of an ionizable group linked to the binding of substrate can be represented in a scheme which is essentially the same as that previously shown in the Appendix of Sono et al. (1986) except that L (ligand) is replaced by S (substrate).

(*a*) *Optical Transition between the Acidic Form* (*EH*) *and Alkaline Form* (*E*$^-$) *of the Enzyme at a Given Substrate* (*S*) *Concentration.* Using eqs 5–8 in the Appendix of Sono et al. (1986) after replacing L by S and K_L^a by $K_{\alpha 1}$ (and K_L^b by $K_{\alpha 2}$), one can derive eqs 13–15 for an apparent pK_a (pK_a^{app}) of the spectral transition of the enzyme.

$$K_a^{app} = [H^+][\text{alkaline form}]/[\text{acidic form}] \qquad (13)$$

$$K_a^{app} = \{[H^+]([E^-] + [E^-\cdot S])\}/\{[EH] + [EH\cdot S]\} \qquad (14)$$

$$K_a^{app} = \{K_{a1} + K_{a2}[S]/K_{d1}\}/\{1 + [S]/K_{d1}\} \qquad (15)$$

By taking the logarithm of eq 13, eq 16 is obtained.

$$pK_a^{app} = pH - \log\,[\text{alkaline form}]/[\text{acidic form}] \qquad (16)$$

The pK_a^{app} value can be determined spectrophotometrically for the two different situations described below.

(*1*) *When a Complete Alkaline Form Cannot Be Obtained.* This is the case of the ferric enzyme in the absence of substrate (Figure 4A)

$$K_a^{app} = [H^+][\text{alkaline form}]/[\text{acid form}] \qquad (13)$$

$$K_a^{app} = [H^+][\Delta A]/(\Delta A_\infty - \Delta A) \qquad (17)$$

where ΔA and ΔA_∞ represent the absorbance changes due to the partial and complete conversion of the acidic form to the alkaline form, respectively. Rearranging eq 17 gives

$$1/\Delta A = [H^+]/K_a^{app}\Delta A_\infty + 1/\Delta A_\infty \qquad (18)$$

A plot of $1/\Delta A$ versus [H$^+$] should yield a straight line with a slope of $1/(K_a^{app}\Delta A_\infty)$, an x intercept of $-K_a^{app}$, and a y intercept of $1/\Delta A_\infty$ (see Figure 4A, inset).

(*2*) *When a Complete Acidic Form Cannot Be Obtained.* This is the case of the ferric enzyme in the presence of substrate (Figure 4B)

$$K_a^{app} = [H^+](\Delta A_\infty - \Delta A)/\Delta A \qquad (19)$$

where ΔA and ΔA_∞ represent the absorbance changes due to the partial and complete conversion of the alkaline form to the acidic form, respectively. Rearrangement of eq 19 yields

$$1/\Delta A = K_a^{app}/\Delta A_\infty[H^+] + 1/\Delta A_\infty \qquad (20)$$

A plot of $1/\Delta A$ vs $1/[H^+]$ should yield a straight line having a slope of $K_a^{app}/\Delta A_\infty$, an x intercept of $-1/K_a^{app}$, and a y intercept of $1/\Delta A_\infty$ (see Figure 4B, inset).

Registry No. L-Trp, 73-22-3; D-Trp, 153-94-6; indolamine 2,3-dioxygenase, 9014-51-1; formate, 64-18-6; fluoride, 16984-48-8; benzhydroxamate, 495-18-1; cyanide, 57-12-5; azide, 14343-69-2; *N*-phenylimidazole, 7164-98-9; 4-phenylpyridine, 939-23-1; *n*-butyl isocyanide, 2769-64-4; dimethylphenylphosphine, 672-66-2; bis(hydroxymethyl)methylphosphine, 5958-52-1; 1-propanethiol, 107-03-9; nitric oxide, 10102-43-9; ethyl isocyanide, 624-79-3; benzyl isocyanide, 10340-91-7; 5-hydroxy-L-Trp, 4350-09-8; 5-hydroxy-D-Trp, 4350-07-6; tryptamine, 61-54-1; serotonin, 50-67-9; α-methyl-DL-Trp, 153-91-3; indole-3-acetic acid, 87-51-4.

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Observation by ^{13}C NMR of the EPSP Synthase Tetrahedral Intermediate Bound to the Enzyme Active Site

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ABSTRACT: Direct observation of the tetrahedral intermediate in the EPSP synthase reaction pathway was provided by ^{13}C NMR by examining the species bound to the enzyme active site under internal equilibrium conditions and using [2-^{13}C]PEP as a spectroscopic probe. The tetrahedral center of the intermediate bound to the enzyme gave a unique signal appearing at 104 ppm. Separate signals were observed for free EPSP (152 ppm) and EPSP bound to the enzyme in a ternary complex with phosphate (161 ppm). These peak assignments account for our quantitation of the species bound to the enzyme and liberated upon quenching with either triethylamine or base. A comparison of quenching with acid, base, or triethylamine was conducted; the intermediate could be isolated by quenching with either triethylamine or 0.2 N KOH, allowing direct quantitation of the species bound to the enzyme. After long times of incubation during the NMR measurement, a signal at 107 ppm appeared. The compound giving rise to this resonance was isolated and identified as an EPSP ketal [Leo et al. (1990) *J. Am. Chem. Soc.* (in press)]. The rate of formation of the EPSP ketal was very slow, 3.3×10^{-5} s^{-1}, establishing that it is a side product of the normal enzymatic reaction, probably arising as a breakdown product of the tetrahedral intermediate. A slow formation of pyruvate was also observed and is attributable to the enzymatic hydrolysis of EPSP, with 5% of the enzyme sites occupied by EPSP and hydrolyzing EPSP at a rate of 4.7×10^{-4} s^{-1}. To look for additional signals that might arise from a covalent adduct which has been postulated to arise from reaction of enzyme with PEP, an NMR experiment was performed with an analogue of S3P lacking the 4- and 5-hydroxyl groups. Enzyme was incubated with 4,5-dideoxy-S3P and [2-^{13}C]PEP and examined by ^{13}C NMR. Only the signal for PEP was observed. All of these results reaffirm our identification of the tetrahedral species as the only observable intermediate in the EPSP synthase reaction.

EPSP synthase is an enzyme in the shikimic acid pathway which catalyzes the unusual transfer of an enolpyruvoyl moiety from PEP to S3P with the elimination of inorganic phosphate. It is the target enzyme of the commercially important herbicide glyphosate [*N*-(phosphonomethyl)glycine] (Franz, 1985; Steinrucken & Amrhein, 1980). In previous papers, we have described the isolation and structure determination of a tetrahedral intermediate formed at the active site of the enzyme from the nucleophilic attack of the 5-OH of the S3P on the C-2 position of PEP and have provided a complete kinetic description of the EPSP synthase reaction pathway (Anderson et al., 1988b,c). Recently, we have established the kinetic properties of this intermediate in solution and when added back to enzyme (Anderson & Johnson, 1990).

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