Spectroscopic and Equilibrium Properties of the Indoleamine 2,3-Dioxygenase–Tryptophan–O_2 Ternary Complex and of Analogous Enzyme Derivatives. Tryptophan Binding to Ferrous Enzyme Adducts with Dioxygen, Nitric Oxide, and Carbon Monoxide†

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ABSTRACT: The dioxygen adduct of the heme protein indoleamine 2,3-dioxygenase has been generated at −30 °C in mixed solvents, and spectroscopic and equilibrium studies of its L-tryptophan (substrate) binding properties have been carried out for the first time. Comparative studies have also been performed with the NO and CO adducts of the ferrous enzyme. Under the conditions employed (−30 °C), both autoxidation and turnover (L-tryptophan + O_2 → formylkynurenine) of the ternary complex are effectively suppressed. Structural identification of the ternary complex is based on (a) the 1:1 molar stoichiometry for the substrate–oxygenated enzyme adduct formation ($K_d \sim 10^{-4}$ M), (b) the time-dependent linear product formation (turonover) at −20 °C, and (c) the quantitative conversion of the complex to the ferrous CO derivative by bubbling with CO. Binding of L-tryptophan to the oxygenated enzyme leads to decreases in the intensities of its major absorption bands (λ_{max} 415, 541, 576 nm) and to a blue shift of its Soret peak. Interestingly, among the ferrous enzyme derivatives examined, *only* the substrate-bound oxygenated enzyme exhibits solvent-dependent Soret absorption peak positions, e.g., λ_{max} 411.5 and 413.5 nm in 65% (v/v) aqueous glycerol and ethylene glycol, respectively. In addition, indole binds to the oxygenated enzyme, causing a red shift of its Soret peak in these solvents *only* in the presence of substrate (411.5 → 414 nm and 413.5 → 414.5 nm, respectively), while similar effects of indole are independent of tryptophan for the other ferrous enzyme derivatives. These results suggest that the electronic structure of the enzyme–substrate–O_2 ternary complex is especially sensitive to changes in the active site heme environment. The present equilibrium study of substrate binding to the oxygenated enzyme and the effect of pH on that process, in combination with the previously determined pH profile of the K_d value of L-tryptophan binding to the ferrous enzyme and that of the K_m value, provide strong support for the ordered catalytic sequence in which the ferrous enzyme binds L-tryptophan first, followed by dioxygen binding.

Indoleamine 2,3-dioxygenase (Hayaishi, 1971, 1976) and tryptophan 2,3-dioxygenase (Feigelson & Brady, 1974), both of which catalyze the oxidative cleavage of the indole ring of tryptophan by incorporating molecular oxygen into the organic substrate, are two of the few heme protein dioxygenases known to exist. Molecular oxygen binds reversibly to the sixth coordination position of the heme iron in the ferrous form of these enzymes (Ishimura et al., 1967, 1970; Hirata et al., 1977; Taniguchi et al., 1979). In addition, an organic substrate (tryptophan)[1] binds to the catalytic site of the dioxygenases to form the enzyme–substrate–O_2 ternary complex which is an obligatory intermediate in the catalytic cycle of the enzymes (Ishimura et al., 1970; Taniguchi et al., 1979). Subsequently, the heme-iron-bound dioxygen is incorporated into the organic substrate to form the product, formylkynurenine. It has been considered to be a common property of oxygenases that the binding of organic substrates precedes the binding of dioxygen (Hayaishi, 1974). This has clearly been demonstrated with the case of tryptophan 2,3-dioxygenase which does not readily form a dioxygen complex in the absence of tryptophan (Ishimura et al., 1967, 1970; Feigelson & Brady, 1974). In contrast, indoleamine 2,3-dioxygenase can form a relatively stable binary complex with O_2, i.e., the oxygenated enzyme, even in the absence of substrate (Hirata et al., 1977; Taniguchi et al., 1979). Thus, the order for the binding of tryptophan and O_2 to the ferrous enzyme has not yet been established for the dioxygenase. Nonetheless, previous equilibrium studies of the tryptophan binding properties of indoleamine 2,3-dioxygenase have led to the proposal that, in the catalytic cycle of the enzyme, tryptophan binds to the ferrous enzyme first, followed by dioxygen (Sono et al., 1980). In these earlier studies, the ferrous CO enzyme was used as a model for the oxygenated enzyme since, in contrast to the stable nature of the tryptophan 2,3-dioxygenase ternary complex, the indoleamine 2,3-dioxygenase–tryptophan–O_2 ternary complex is extremely autoxidizable (Hirata et al., 1977; Taniguchi et al., 1979). In addition, it has been impossible to perform equilibrium substrate titration experiments of the oxygenated enzyme since the added substrate is rapidly converted to product upon turnover of the ternary complex. This problem has been successfully overcome in this work by lowering the temperature to −30 °C using mixed solvents. Employing this subzero temperature technique, it has been possible for the first time to detect a spectral change upon tryptophan binding to oxygenated indoleamine 2,3-dioxygenase and, thus, to distinguish substrate-free and bound forms of the oxygenated enzyme with optical absorption and magnetic circular dichroism (MCD)[2] and CD spectroscopic techniques. This has

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[1] Unless otherwise specified, tryptophan refers to L-tryptophan throughout the text.

[2] Abbreviation: MCD, magnetic circular dichroism.

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also allowed the dissociation constants of tryptophan binding to oxygenated indoleamine 2,3-dioxygenase to be determined. Similar studies have not been done with oxygenated tryptophan 2,3-dioxygenase because of the reasons described above (Ishimura et al., 1967, 1970). In addition, parallel spectroscopic and equilibrium substrate-binding studies for the NO and CO adducts of ferrous indoleamine 2,3-dioxygenase are reported which provide useful data for comparison with the results for the oxygenated enzyme. This study has revealed that the spectral (i.e., electronic) properties of the enzyme–substrate–O_2 ternary complex are more sensitive to environmental factors than its substrate-free form. In addition, the equilibrium data obtained in this study have provided important information concerning the catalytic reaction sequence of the dioxygenase. A major portion of this work was presented at the 1984 International Chemical Congress of Pacific Basin Societies, Honolulu, HI, Dec 15–19, 1984, and at the joint meeting of the American Society of Biological Chemists (the 76th Annual) and the Division of Biological Chemistry of the American Chemical Society, Washington, DC, June 8–12, 1986 (Sono, 1986).

Experimental Procedures

Indoleamine 2,3-dioxygenase was purified from rabbit small intestine by the mthod of Shimizu et al. (1978) except that the final isoelectrofocusing step was omitted. Instead, step 6 (Sephadex G-100 chromatography) was repeated 2–4 times. The native ferric enzyme obtained by this procedure exhibited a A_{406}/A_{280} value of 1.7–1.8 at pH 6.0 and 24 °C and was more than 70% pure (Sono & Dawson, 1984). Dioxygen gas and dioxygen-free nitrogen gas were obtained from Matheson Air Products. Chemicals were of reagent grade (Aldrich or Fisher) and were used without further purification.

Oxygenated indoleamine 2,3-dioxygenase was prepared at −30 °C by bubbling dioxygen into a solution of the ferrous (high-spin) enzyme either in 65% (v/v) aqueous glycerol or in 65% aqueous ethylene glycol. Both mixed solvents contained 0.035 M potassium phosphate (pH 7.5).[3] The ferrous enzyme was initially prepared at ~0 °C by reducing the native ferric enzyme with a slight excess of sodium dithionite under a nitrogen atmosphere and subsequent cooling to −30 °C. Conversion of the ferrous enzyme to the oxygenated form was monitored by optical absorption spectroscopy. The oxygenated enzyme sample was allowed to stand for about 1 h at −30 °C until the bubbles disappeared and the solution became clear. The absence of dithionite (λ_{max} = 315 nm) in the medium was confirmed by the lack of a further absorbance decrease at 315 nm upon additional dioxygen bubbling. Preparation of the CO and NO (from 1 mM $NaNO_2$) adducts of the ferrous enzyme were carried out as described previously (Sono et al., 1980; Sono & Dawson, 1984).

Titrations of oxygenated indoleamine 2,3-dioxygenase with tryptophan were performed at −30 °C in open cuvettes by stepwise additions of tryptophan from a concentrated stock solution (100 mM in aqueous mixed solvents of 65% glycerol or 65% ethylene glycol containing 0.035 M potassium phosphate buffer). Stock solutions of other indole derivatives (50–100 mM) were prepared also in 65% glycerol or in 65% ethylene glycol. Under these conditions, the tryptophan-binding reaction was extremely slow in reaching equilibrium (>1 h) at low tryptophan concentrations (<0.5 mM). This was not only because of the low temperature employed but also due to the high viscosity of the mixed solvent at −30 °C which leads to very slow mixing of the added tryptophan to the enzyme solution, especially in 65% glycerol. Therefore, titrations were started with tryptophan concentrations greater than 50 μM. Essentially the same methods were used for tryptophan titrations of the ferrous CO and ferrous NO derivatives of indoleamine 2,3-dioxygenase at various temperature values and in different solvents, except that the titrations were performed in the presence of excess sodium dithionite in rubber-septa-stoppered cuvettes under CO and N_2 atmospheres, respectively. The titration data were analyzed by the use of double-reciprocal plots or Hill plots as described previously (Sono et al., 1980). For additional details, see the figure legends.

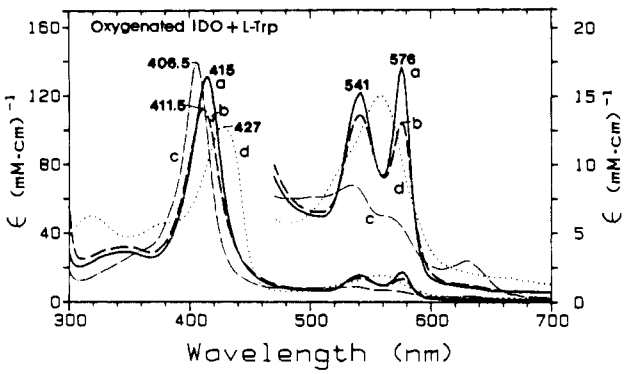

FIGURE 1: Optical absorption spectra of oxygenated indoleamine 2,3-dioxygenase (IDO) (—, spectrum a) and its L-tryptophan (L-Trp) adduct (---, spectrum b) at −30 °C. To 19.6 μM oxygenated IDO prepared as described under Experimental Procedures in 65% (v/v) glycerol/0.035 M potassium phosphate (pH 7.5), L-tryptophan was added from a 100 mM stock solution to give a final concentration of 2 mM. This resulted in the spectral change from a to b which was complete in 20 min. No detectable spectral changes were observed upon further additions of the substrate. For comparison, spectra of the native ferric (–·–, spectrum c) and ferrous (···, spectrum d) enzyme, both in the absence of tryptophan, are overplotted under the same experimental conditions. The peak at ~315 nm for the ferrous enzyme is due to the presence of dithionite in the solution. Path length was 0.2 cm.

Optical absorption spectra and MCD/CD spectra were recorded on a Varian Cary 219 spectrophotometer and a JASCO J-40 spectropolarimeter, respectively, both of which were equipped with a circulator for temperature control (±1 °C). The detailed methods and instrumental conditions have been described elsewhere (Sono et al., 1982). The temperature of the samples was directly monitored with a copper thermocouple attached to a digital indicator (Omega Engineering). All pH measurements were performed at room temperature with a Brinkman pH 101 meter (Metrohm combination electrode).

Results

Optical Absorption Spectral Characteristics of Oxygenated Indoleamine 2,3-Dioxygenase in the Presence and Absence of Substrate. As shown in Figure 1, the dioxygen adduct of the enzyme in the absence of substrate (spectrum a) exhibits Soret, β, and α absorption bands at 415, 541, and 576 nm, respectively, at −30 °C in 65% glycerol/0.035 M potassium phosphate (pH 7.5). A virtually identical spectrum is obtained at near 0 °C except for slightly lower intensities of all peaks due to the effect of temperature (Vickery et al., 1976). The spectrum at pH 7.5 and −30 °C does not differ significantly from that obtained at pH 6.0. An indistinguishable spectrum

[3] The pH value (7.5 or 6.0) of the 0.1 M potassium phosphate buffer was measured at room temperature before it was mixed with glycerol or ethylene glycol. Physicochemical properties of mixed solvents and their applications to studies of biochemical reactions have been extensively studied by Douzou and co-workers (Douzou, 1974; Eisenstein et al., 1977). For the actual proton activity values, see Douzou (1974).

Table I: Optical Absorption Bands of Ferrous Indoleamine 2,3-Dioxygenase (IDO) Adducts with O_2, NO, and CO in Their L-Tryptophan (L-Trp) Free and Bound Forms

enzyme derivative	solvent[a]	temp (°C)	λ_{nm} (ϵ_{mM})[b] δ	Soret	β	α
IDO–O_2	A	−30	344 (30)	415 (132)	541 (15.3)	576 (17.2)
	B	−30	344 (30)	415 (132)	541 (15.2)	576 (17.3)
+L-Trp (1.32 mM)	A	−30	344 (31)	411.5 (113)	541 (13.8)	576 (13.2)
+L-Trp (4 mM)	B	−30	344 (~31)	413.5 (114)	541 (14.1)	576 (13.6)
IDO–NO	A	−30	*c*	419 (134)	544 (12.6)	575 (13.3)
	A	4	*c*	419 (127)	*c*	*c*
	B	4	*c*	419 (127)	*c*	*c*
+L-Trp (1.32 mM)	A	−30	*c*	416 (112)	543 (12.4)	572 (11.2)
	A	4	*c*	416.5 (108)	*c*	*c*
	B	4	*c*	417 (114)	*c*	*c*
IDO–CO	A	4	*c*	420.5 (200)	539 (15.3)	570 (16.7)
+L-Trp (100 mM)	A	4	*c*	418 (167)	539 (14.0)	568 (15.0)

[a] Solvent A, a 65:35 (v/v) mixture of glycerol and 0.1 M potassium phosphate (pH 7.5). Solvent B, a 65:35 (v/v) mixture of ethylene glycol and 0.1 M potassium phosphate (pH 7.5). [b] Expressed in mM^{-1} cm^{-1}. [c] Not determined.

of the oxygenated enzyme is obtained in 65% ethylene glycol/0.035 M potassium phosphte (pH 7.5). Optical absorption spectral data of the oxygenated enzyme with and without tryptophan in the mixed solvents are summarized in Table I together with those of the NO and CO complexes of the ferrous enzyme. These spectral features (absorption band positions) of the oxygenated enzyme are essentially identical with those previously reported by Hirata et al. (1977) and Uchida et al. (1983) for the same enzyme derivative in aqueous potassium phosphate buffer at 24 and 4 °C, respectively. This indicates that the solvent and pH have negligible effects on the spectrum. It should be noted that the ratio (R) of the α peak intensity to that of the β peak of the oxygenated enzyme spectrum obtained in this work (R = 1.12 at −30 °C) is much greater than that observed previously, probably because the present complex is more homogeneous and free from possible contamination of the oxidized form resulting from autoxidation. At 4 °C and in 65% glycerol/0.035 M potassium phosphate, the oxygenated enzyme autoxidizes to the ferric form with half-lives of about 24 and 4 h at pH values of 7.5 and 6.0, respectively.

Upon addition of tryptophan to the oxygenated enzyme in 65% glycerol at −30 °C, the optical absorption spectrum (spectrum a) undergoes noticeable changes and yields spectrum b. The intensities of the α, β, and Soret bands decrease with a concomitant blue shift of the Soret peak position. The relative ratio of α to β peak intensities (R) also changes from $R > 1$ for the substrate-free oxygenated enzyme to $R < 1$ for its tryptophan adduct. Curiously, the Soret peak positions of the resulting enzyme species clearly differ in the two mixed solvents used: a greater blue shift is seen in 65% glycerol (λ_{max} = 411.5 nm) than in 65% ethylene glycol (λ_{max} = 413.5 nm) (Table I). This is in contrast to the solvent-independent spectral nature of the substrate-free oxygenated enzyme as mentioned above. Furthermore, in a 1:1 (v/v) mixture of these two solvents, a Soret peak of the oxygenated enzyme in the presence of 2 mM (≥20 K_d) tryptophan is found at 412.5 nm, an intermediate position between those observed in respective mixed solvents. Unfortunately, other cryogenic mixed solvents such as 50–70% aqueous methanol, ethanol, or dimethylformamide (Douzou, 1974) have caused denaturation of the enzyme. It should be emphasized that, although the tryptophan-binding reaction is quite slow in reaching equilibrium (vide supra) under the conditions employed, especially in 65% glycerol solution and at low substrate concentrations below 50 μM, the observed spectral changes are complete within 20 min with millimolar substrate concentrations. Similar spectral changes have also been seen upon substrate binding to the CO

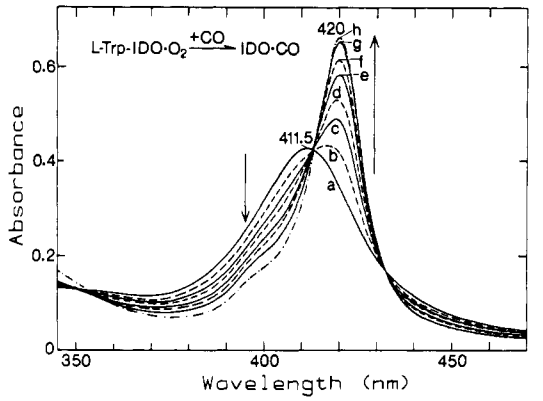

FIGURE 2: Spectral conversion of the L-tryptophan complex of oxygenated indoleamine 2,3-dioxygenase (IDO–O_2) (spectrum a) to the CO adduct (spectra g and h) of the enzyme. The starting spectrum (a) was obtained with 18.9 μM oxygenated enzyme in the presence of 2 mM L-tryptophan in the mixed solvent (see Figure 1) at −30 °C. After CO was bubbled into the oxygenated enzyme solution for about 30 s at −30 °C, spectrum a changed to b in 1 h, during which time the sample was kept under a CO atmosphere (~1 atm) in a rubber-septa-capped cuvette. Then the temperature was raised to −20 °C, and the spectral changes b → g were recorded at 0.5, 1, 2, 3, and 10 h for spectra c, d, e, f, and g following spectrum b. The final spectrum (h) was obtained by adding a small amount of sodium dithionite (~50 μM, final concentration) to the sample exhibiting spectrum g. As judged from the K_d value for tryptophan–ferrous CO enzyme complex (K_d = 3.1 mM), about 40% of the resulting ferrous CO enzyme is complexed with tryptophan. The arrows indicate the direction of the absorbance changes.

(Sono et al., 1980) and NO (Sono & Dawson, 1984) adducts of the ferrous enzyme.

Identification of the New Enzyme Derivative Generated by Addition of Tryptophan to Oxygenated Indoleamine 2,3-Dioxygenase as the Enzyme–Substrate–O_2 Ternary Complex. In order to confirm that the observed spectral change is in fact due to the specific binding of tryptophan to the oxygenated enzyme to form the enzymatically active ternary complex and is not caused by nonspecific effects of the substrate or autoxidation of the oxygenated enzyme, the following experiments have been carried out.

(*a*) *Conversion of the New Enzyme Derivative to the Ferrous CO Form.* The tryptophan complex of the oxygenated enzyme can be converted to the CO-bound form upon CO bubbling as shown in Figure 2. The ligand exchange reaction is extremely slow at −30 °C (spectrum a → b). In order to accelerate the conversion, the temperature was raised to −20 °C. A single set of isosbestic points was seen at 350.5, 413.5, and 432.5 nm during the spectral change. After conversion

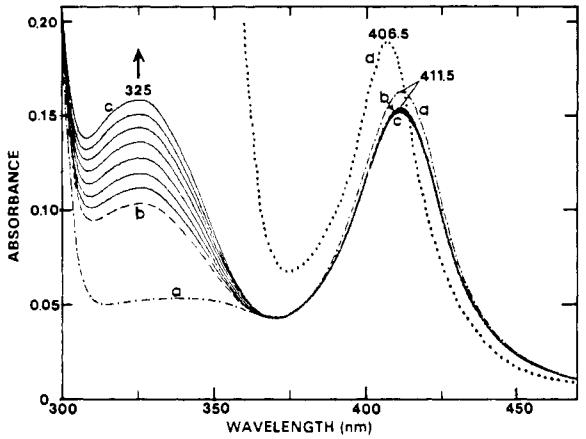

FIGURE 3: Time-dependent absorbance increase in the near UV region (300–360 nm) at −20 °C due to the product (formylkynurenine) formation from the indoleamine 2,3-dioxygenase–tryptophan–O_2 ternary complex. The tryptophan complex of the oxygenated enzyme (7.2 μM) was first prepared at −30 °C in the presence of 1.32 mM L-tryptophan (spectrum a), and then the temperature was raised to −20 °C, during which time spectrum a changed to b. After the temperature had reached −20 °C, spectral changes of the enzyme solution were recorded at 10-min intervals for 70 min (b → c). Spectrum d was obtained after further raising the temperature to about +20 °C and allowing the sample to stand for about 30 min with occasional gentle bubbling of O_2 into the solution.

of the initial spectrum a to spectrum g (≥10 h), addition of sodium dithionite caused only a very small further absorbance change (g → h), indicating that prior to the addition of dithionite the formation of the ferrous enzyme CO complex was virtually complete. Since it has been reported that CO can act as a reducing agent for several ferric heme proteins (Bickar et al., 1984), control experiments were performed in which ferric indoleamine 2,3-dioxygenase was incubated in CO-bubbled solutions and under a CO atmosphere (~1 atm) under the same experimental conditions as used for the oxygenated dioxygenase. No absorption spectral changes occurred over 12 h. These results rule out the possibility that the complex formed upon binding of tryptophan to the oxygenated enzyme could contain significant amounts of autoxidized enzyme.

(*b*) *Product Formation from the New Enzyme Species.* The absorption spectrum of the oxygenated enzyme in the presence of tryptophan in 65% glycerol as shown in spectrum a of Figure 3 (see also spectrum b of Figure 1) does not change significantly with time at −30 °C. However, increasing the temperature to −20 °C results in a time-dependent absorbance increase in the near UV region (300–360 nm) (Figure 3). A new absorption peak at 325 nm emerges which can be attributed to formation of the product, formylkynurenine (λ_{max} = 325 nm).[4] The observed absorbance increase is linear with time for at least 70 min at −20 °C (spectrum b → c). During the spectral change below 360 nm, the absorption spectrum of the enzyme in the wavelength region between 370 and 700 nm remains virtually unchanged, with the Soret peak of the enzyme being seen at 411.5 nm (Figure 3). Addition of the nonmetabolizable substrate analogues indoleacetic acid (5 mM) or α-methyl-DL-tryptophan (5 mM) to the oxygenated enzyme does not cause such time-dependent spectral changes at −20 °C. Time-dependent linear product formation is also observed in 65% ethylene glycol at −20 °C upon addition of tryptophan to the oxygenated enzyme. Interestingly, the rate of the product formation is significantly higher in 65% ethylene glycol than that in 65% glycerol by a factor of ≥4. These results are most consistent with and support the interpretation that the new enzyme derivative generated upon tryptophan addition to the oxygenated enzyme is the enzyme–substrate–O_2 ternary complex and that it turns over at −20 °C to yield the product, formylkynurenine. The linear product formation with time as presented in Figure 3 indicates that autoxidation or inactivation of the ternary complex at −20 °C is not occurring. Above −10 °C, the ternary complex autoxidizes at considerable rates as judged by the loss of the linearity of the product formation concomitant with a gradual blue shift of the Soret peak position of the enzyme (data not shown). After complete autoxidation of the enzyme (see legend to Figure 3), the absorption spectrum of the ferric enzyme with a Soret peak at 406.5 nm (spectrum d in Figure 3) is nearly quantitatively recovered. This indicates that the enzyme remains intact during the above process.

Titration of Oxygenated Indoleamine 2,3-Dioxygenase with Tryptophan. In order to determine the equilibrium dissociation constant (K_d) and the stoichiometry for the binding of tryptophan to the oxygenated enzyme, substrate titration experiments have been carried out. For comparison, tryptophan titration experiments for the ferrous NO and ferrous CO enzyme derivatives have also been performed under various conditions. Figure 4A shows the spectral changes in the Soret region (365–480 nm) upon stepwise addition of tryptophan to the oxygenated enzyme in 65% glycerol at −30 °C. The spectral changes are tryptophan concentration dependent and exhibit saturation behavior with increasing substrate concentration as indicated in a double-reciprocal plot (Figure 4A, inset). Although the spectra were collected over several hours in order to allow the binding reactions to reach equilibria (see Experimental Procedures), control experiments with the oxygenated enzyme have shown that the absence of substrate or the addition of the nonmetabolizable substrate analogues indoleacetic acid (5 mM) or α-methyl-DL-tryptophan (5 mM) does not induce similar spectral changes over the same period of time. In addition, the final spectrum obtained in the presence of 1.32 mM tryptophan after the titration (several hours) is identical with the spectrum obtained in 20 min by addition of 2 mM tryptophan to the oxygenated enzyme under the same conditions (cf. Figure 1). During the titration, a single set of isosbestic points is observed at 406 and 442 nm in the Soret region (Figures 1 and 4A) and at 522, 555, and 586 nm in the visible region (Figure 1). This set of isosbestic points is clearly distinguishable from that of the oxygenated enzyme and the native ferric state (411, 480, 524, and 591 nm, Figure 1) or from that of the oxygenated enzyme and the ferrous state (390, 423, 454, 500, 528, 547, 569, and 586 nm, Figure 1) at −30 °C. Taken together, these results eliminate any other possible reactions such as autoxidation of the oxygenated enzyme and dissociation of the bound dioxygen from the oxygenated derivative and indicate that the direct and specific binding of tryptophan to the oxygenated enzyme causes the observed spectral changes.

The double-reciprocal plot of the titration results gives a straight line with the x intercept of −14.5 mM^{-1} (Figure 4A,

[4] In control experiments in which catalytic reactions were carried out in aqueous phosphate buffer solutions at ambient temperature (25 °C) with L-tryptophan as substrate and ascorbic acid–methylene blue as cofactors (Shimizu et al., 1978), the product (formylkynurenine) that was formed by the dioxygenase also exhibited an absorption peak at 325 nm (cf. 321 nm; Feigelson & Brady, 1974). Although the reason for a slight difference in the absorption peak of the product observed in this work and reported in the literature is not clear at present, the product in this work was further confirmed spectrophotometrically by its conversion to kynurenine (λ_{max} 360 nm; isosbestic point 335 nm) upon treatment with formamidase (Shimizu et al., 1978).

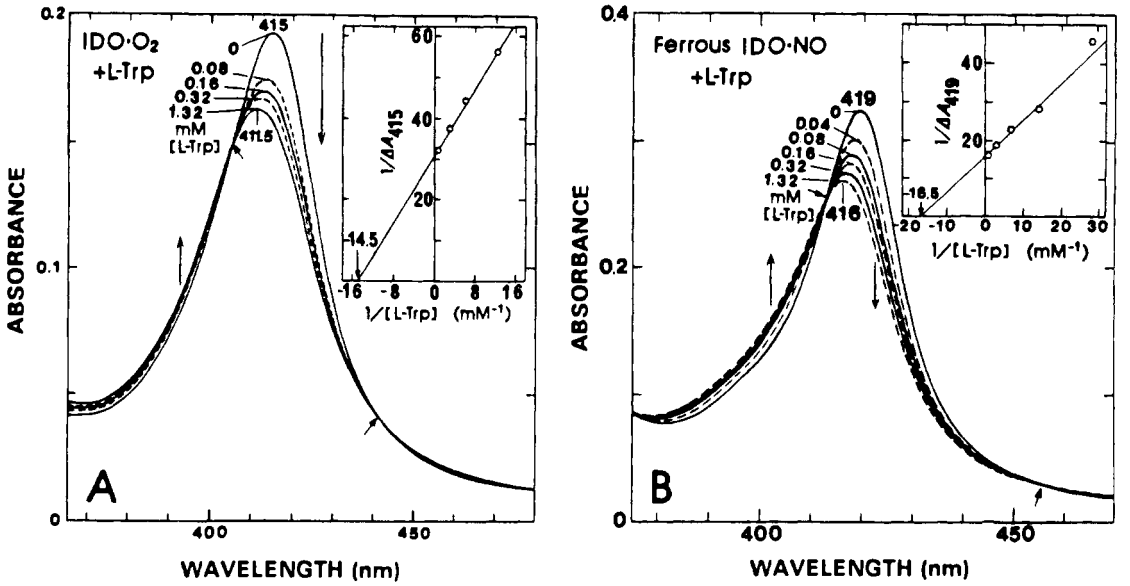

FIGURE 4: Spectrophotometric titrations of oxygenated (A) and ferrous NO (B) indoleamine 2,3-dioxygenase (IDO) with L-tryptophan (L-Trp) at −30 °C. In (A), to 7.2 μM oxygenated enzyme (spectrum with a peak at 415 nm), L-tryptophan was added in a stepwise fashion to the final concentrations indicated in the figure in 65% (v/v) glycerol/0.035 M potassium phosphate buffer (pH 7.5) at −30 °C. The absorbance changes were followed for about 2 h after the first three additions of tryptophan and for about 1 h after the last addition in order to allow the binding reactions to reach equilibrium. The directions of the absorbance changes are shown by the vertical arrows. The small arrows indicate the isosbestic points (408 and 442 nm). The double-reciprocal plot of the results is shown in the inset. In (B), the NO complex of the ferrous enzyme (11.8 μM) was titrated with L-tryptophan (0–1.32 mM). In this case, L-tryptophan was added to the enzyme first at 14 °C, at which temperature a binding equilibrium was reached within 30 s, and then the sample cuvette was brought into a cuvette holder that had been kept at −30 °C. After a new equilibrium was reached (~5 min), a spectrum was recorded. For the next addition of the substrate, the sample was brought back to 14 °C. This procedure was repeated until the final spectrum was obtained in the presence of 1.32 mM L-tryptophan. The inset shows a double-reciprocal plot of the results.

inset). A Hill plot of the same data also gives a straight line with a slope of unity (n = 1) (Figure 5). Similarly, analysis of the tryptophan titration results for the ferrous NO enzyme (Figure 4B) yields a straight line in the double-reciprocal plot (Figure 4B, inset) with the x intercept of −16.5 mM^{-1} and a straight line in a Hill plot (Figure 5) with a slope of unity. These results indicate that tryptophan binds to the O_2 and NO complexes of the ferrous enzyme in a molar ratio of 1:1 with dissociation constants of 69 and 60 μM, respectively. Similarly, in 65% ethylene glycol at −30 °C and at pH 7.5, tryptophan binds to the oxygenated enzyme with a dissociation constant of 120 μM.

Temperature, Solvent, and pH Dependence of the Dissociation Constants (K_d) for the Tryptophan Adducts of Oxygenated, Ferrous NO and Ferrous CO Indoleamine 2,3-Dioxygenase. Since the NO and CO complexes of the ferrous enzyme are stable in the presence and absence of substrate even at room temperature, the effects of temperature on tryptophan affinities of these enzyme derivatives have been studied both in the mixed solvent [65% (v/v) glycerol/0.035 M potassium phosphate (pH 7.5)] (−30 °C to +24 °C) and in aqueous solution (0.1 M potassium phosphate, pH 7.5) (4–24 °C). Hill plots of the titration results in the mixed solvent are shown in Figure 5, where a slope of unity (n = 1) is obtained for all cases studied. Unfortunately, the tryptophan-binding equilibrium experiments with the oxygenated enzyme above −30 °C are made difficult by the turnover (above −20 °C) and the autoxidation (above −10 °C) reactions of the ternary complex (vide supra). At pH 6.0 and −30 °C, nearly identical K_d values (K_d = ~55 μM) to those determined at pH 7.5 are obtained for the oxygenated enzyme, indicating the absence of significant pH effects. The pH effects are also insignificant between pH 6 and 7.5 for the ferrous NO (this work) and ferrous CO enzyme (Sono et al., 1980). The K_d values thus obtained in both solvents at various temperature values and at pH 7.5 are summarized in Figure 6. First, note

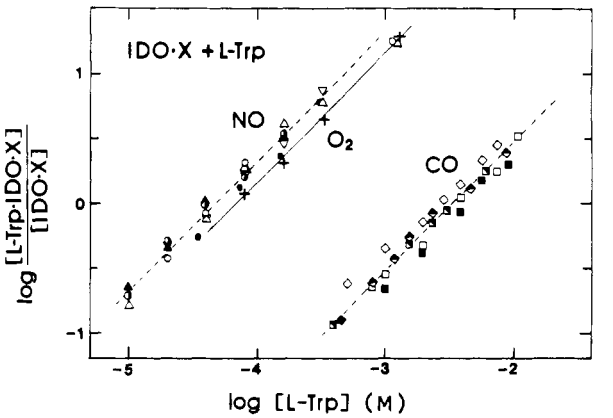

FIGURE 5: Hill plots for L-tryptophan binding to the ferrous indoleamine 2,3-dioxygenase (IDO) complexes with NO, O_2, and CO at various temperature values. All data were obtained in 65% glycerol/0.035 M potassium phosphate (pH 7.5) with enzyme concentrations of about 10 μM. The plots for the O_2 (+) and NO (●) adducts of the ferrous enzyme at −30 °C are made by using the titration results presented in Figure 4. Temperature values for the other symbols for NO complex are (○) −20 °C, (◑) −10 °C, (△) 4 °C, (▲) 14 °C, and (▽) 24 °C and those for the CO complex are (■) −20 °C, (□) −10 °C, (◪) 4 °C, (◇) 14 °C, and (◆) 24 °C. The solid straight line for the O_2 complex and the dashed straight lines for the NO and CO complexes have a slope of 1. The latter two lines are drawn at the center of the somewhat scattered points for different temperature values.

that the K_d values for the tryptophan adducts of both the ferrous CO and ferrous NO enzyme derivatives are almost temperature independent over the temperature range examined. This is especially true in the mixed solvent. The K_d values for the ferrous CO enzyme are somewhat scattered for different temperature values (Figures 5 and 6). However, no systematic trend in the K_d value with temperature is seen (Figure 6). Although such temperature independence of the equilibrium constant is unusual, the results were observed

Table II: Comparison of Optical Absorption and MCD Spectral Parameters of Tryptophan-Free and -Bound Oxygenated Indoleamine 2,3-Dioxygenase (IDO) with Those of Oxygenated Forms of Myoglobin (Mb) and Horseradish Peroxidase (HRP)[a]

	IDO-O_2[b]		IDO-O_2 + Trp[b]		Mb-O_2[c]		HRP-O_2[d]	
MCD	optical peak λ_{nm}	MCD[e] λ ($\Delta\epsilon/H$)[g]	optical peak λ_{nm}	MCD λ ($\Delta\epsilon/H$)[g]	optical peak λ_{nm}	MCD λ ($\Delta\epsilon/H$)[g]	optical peak λ_{nm}	MCD[f] λ ($\Delta\epsilon/H$)[g]
				Soret Region				
peak		406 (27)		404 (22)		411 (25)		409 (23)
crossover	415 (Soret)	415.5 (0)	411.5 (Soret)	414 (0)	418 (Soret)	419.5 (0)	417 (Soret)	418 (0)
trough		423.5 (-25)		422.5 (-21)		427 (-22)		427 (-24)
$\Delta\lambda$[h]		17.5		18.5		16		18
$\Delta(\Delta\epsilon/H)$[i]		52		43		47		47
				Visible Region				
peak		571 (49)		571 (38)		572 (46)		572 (27)
crossover	576 (α)	575.5 (0)	576 (α)	575.5 (0)	581 (α)	577.5 (0)	577 (α)	578.5 (0)
trough		581 (-64)		584 (-49)		584 (-63)		587 (-32)
$\Delta\lambda$[h]		10		10		12		15
$\Delta(\Delta\epsilon/H)$[i]		113		87		109		59
ref	*j*	*j*	*j*	*j*	*k*	*l*	*m*	*j*

[a] All MCD values were obtained at -30 °C in mixed solvents. [b] Determined in 65:35 (v/v) mixture of glycerol and 0.1 M potassium phosphate buffer (pH 7.5). [c] Determined in 50:50 (v/v) mixture of ethylene glycol and 0.1 M potassium phosphate buffer (pH 7). [d] Determined in 70:30 (v/v) mixture of dimethylformamide and 0.1 M potassium phosphate buffer (pH 7.5) with protein concentration of 11.5 μM in the presence of 2 mM H_2O_2. [e] The values are comparable to those determined at 5 °C and reported by Uchida et al. (1983). [f] The values are comparable to those determined at -40 °C and reported by Nozawa et al. (1980). [g] Expressed in nm (M^{-1} cm^{-1} T^{-1}). [h] Peak-to-trough wavelength separation in nm. [i] Peak-to-trough intensity in M^{-1} cm^{-1} T^{-1}. [j] This work. [k] Antonini and Brunori (1971). [l] Dawson and Cramer (1978). [m] Wittenberg et al. (1967).

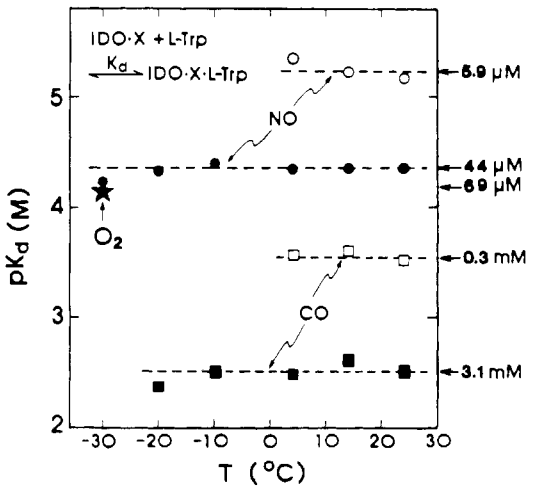

FIGURE 6: Effects of temperature and solvent on the L-tryptophan affinities (K_d values) of various derivatives of indoleamine 2,3-dioxygenase (IDO). The data points for the oxygenated enzyme and ferrous NO and ferrous CO enzyme are indicated by a star, circle, and square, respectively. For the NO and CO adducts of the ferrous enzyme, the data points shown by open symbols (○, □) were determined in 0.1 M potassium phosphate buffer (pH 7.5), and those shown by closed symbols (●, ■) in 65% (v/v) glycerol/0.035 M potassium phosphate buffer (pH 7.5). The K_d value determination for the tryptophan adduct of the oxygenated enzyme was only possible at -30 °C in the mixed solvent (see text). Since the observed temperature effect on the K_d values for NO and CO complexes of the ferrous enzyme is quite small, especially in the mixed solvents, average K_d values at the different temperature values are indicated with dashed lines in the figure and in numbers on the right-hand side of the figure. See Experimental Procedures for details.

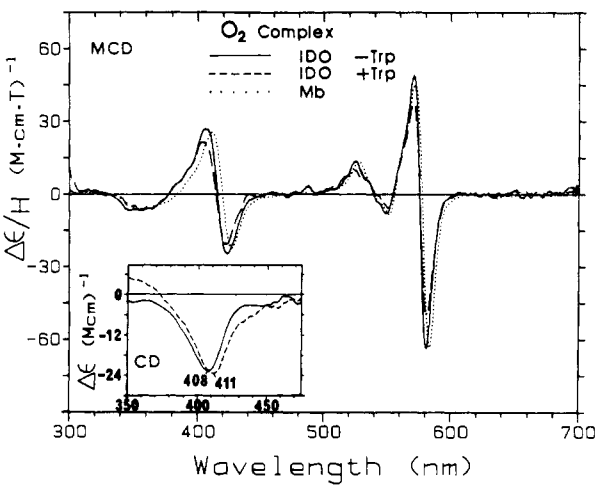

FIGURE 7: MCD and CD (inset) spectra of L-tryptophan (Trp) free (solid line) and bound (dashed line) oxygenated indoleamine 2,3-dioxygenase (IDO) and MCD spectrum of oxygenated myoglobin (Mb) (dotted line). See Figure 1 for the conditions for the dioxygenase adducts. The spectrum of oxygenated sperm whale myoglobin is taken from Dawson and Cramer (1978).

reproducibly. Second, the tryptophan affinity of the ferrous NO enzyme is considerably higher by a factor of 50–70 than that of the ferrous CO enzyme in both mixed and aqueous solvents. For the same ligand derivatives, tryptophan affinities are higher in aqueous solutions than in mixed solvents by about 1 order of magnitude. Third, the oxygenated enzyme exhibits a tryptophan affinity that is nearly the same as that of the ferrous NO enzyme. Thus, it appears that the ferrous NO enzyme is a better model for studies of substrate binding to the oxygenated derivative than is the ferrous CO enzyme.

MCD Spectral Properties of Oxygenated Indoleamine 2,3-Dioxygenase with and without Bound Tryptophan in Comparison with Those of Other Oxygenated Heme Proteins. The effects of the substrate binding to the oxygenated enzyme on its MCD spectrum have also been examined. MCD spectra of the oxygenated enzyme with and without bound tryptophan are displayed in Figure 7. For direct comparison, an MCD spectrum of oxygenated myoglobin (Vickery et al., 1976; Dawson & Cramer, 1978) generated under similar experimental conditions is overplotted on the same figure. MCD spectral parameters of the oxygenated forms of these two heme proteins and of oxygenated horseradish peroxidase (i.e., compound III) under similar conditions are summarized in Table II. The MCD spectral change of the oxygenated dioxygenase upon tryptophan binding is unexpectedly small. Only slight decreases in the peak and trough intensities with little change in the peak positions are observed. The MCD spectrum of oxygenated myoglobin is nearly identical in line shape and intensity to that of the oxygenated dioxygenase, especially to that of its substrate-free form. Among the oxygenated derivatives of these three heme proteins, only oxygenated horseradish peroxidase exhibits a considerably smaller MCD

peak-to-trough intensity (about half) in the visible region for the derivative-shaped signal centered at 578.5 nm than those observed for the other proteins (Table II). The MCD intensities in the other regions (the Soret and β peaks) and the peak-to-trough wavelength separation ($\Delta\lambda$, Table II) are not significantly different among the three oxygenated heme proteins.

Effects of Substrate Binding to the Oxygenated Enzyme on Its CD Spectrum. CD spectra of the oxygenated enzyme with and without tryptophan in 65% glycerol at −30 °C are displayed in the inset of Figure 7. Although the CD spectral line shapes in the Soret region (350–475 nm) of both the substrate-free and bound oxygenated enzyme do not differ significantly, the trough position shifts to the red (408 → 411 nm) upon tryptophan binding. This spectral shift is opposite to that observed in the optical absorption spectrum, where a blue shift (415 → 411.5 nm) was seen for the Soret peak. This indicates that conformational changes have occurred at the active site of the oxygenated enzyme as a result of complex formation with tryptophan. Substantial CD spectral changes have also been observed with other ferrous and ferric enzyme derivatives upon substrate binding (Sono & Dawson, 1984; Uchida et al., 1983).

Effects of Indole on Optical Absorption Spectra of the Enzyme–Tryptophan–O_2 Ternary Complex and Analogous Enzyme Derivatives. Interestingly, the addition of a millimolar concentration of indole to the enzyme–tryptophan–O_2 ternary complex has been found to cause the Soret peak to shift to the red by 2.5 nm in 65% glycerol (411.5 → 414 nm) as shown in Figure 8A. A similar red shift is also observed in 65% ethylene glycol (413.5 → 414.5 nm) upon addition of 2–4 mM indole to the ternary complex (not shown). The effect of indole cannot be reversed by further addition of tryptophan up to 20 mM to the complex in both solvents, suggesting that indole does not replace bound tryptophan, i.e., tryptophan and indole can bind to the oxygenated enzyme concomitantly to form a quaternary complex. This is further supported by the observation that the complex ($\lambda_{max} \sim 414$ nm) formed is enzymatically active as judged from time-dependent linear product formation (i.e., A_{325} increase) at −20 °C. In contrast, for the substrate-free oxygenated enzyme there is no such Soret peak shift observed even with 25 mM indole either in 65% glycerol (Figure 8B) or in 65% ethylene glycol. In the presence of 25 mM indole, an addition of 10 mM tryptophan to the oxygenated enzyme in 65% glycerol and in 65% ethylene glycol only slightly shifts its Soret peak to shorter wavelength (415 → 414 and 415 → 414.5 nm, respectively) (see Figure 8B for the former) in contrast to the larger blue shift (415 → 411.5 and 415 → 413.5 nm, respectively) observed with 1.32–2 mM tryptophan in the absence of indole (Figure 1). Increasing the tryptophan concentration from 10 to 20 mM has no effect. This is consistent with the above interpretation that indole binds to the substrate-bound oxygenated enzyme to form a quaternary complex. In addition, this suggests that indole also binds to the substrate-free oxygenated enzyme, most probably at a site other than the tryptophan binding site, without causing any detectable spectral change. Indole also binds to the ferrous CO and ferrous NO enzyme (K_d = 1.5–2 mM in 0.1 M potassium phosphate, pH 7.5, and at 4 °C, this work) in both the presence and absence of tryptophan. In these cases, however, slight red shifts (by 1–1.5 nm) of the Soret absorption peak positions of these enzyme derivatives are observed upon indole binding *regardless* of the presence or absence of tryptophan (this work). Tryptophan binding affinity of either enzyme derivative does not differ significantly with or without

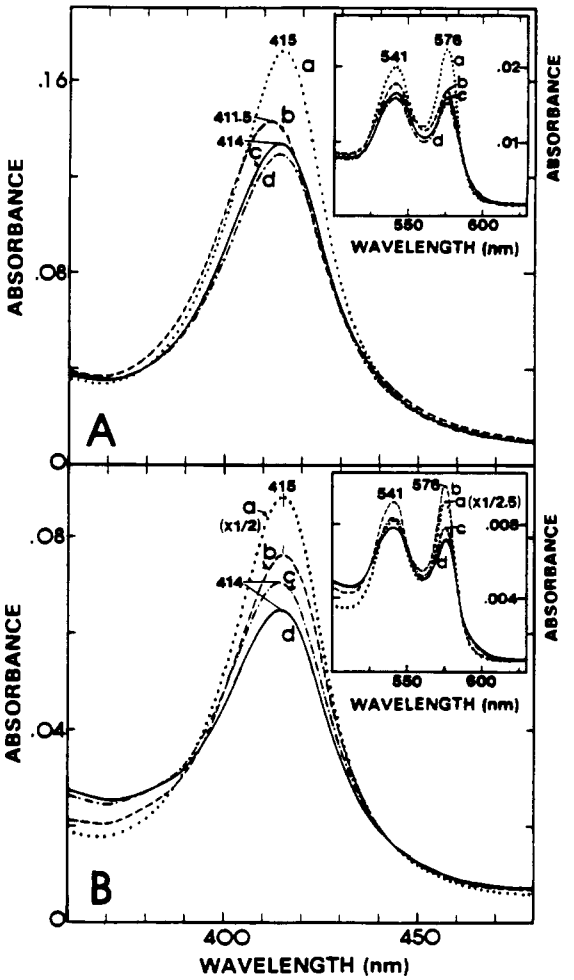

FIGURE 8: Effects of indole on the optical absorption spectrum of oxygenated indoleamine 2,3-dioxygenase (IDO–O_2) in the presence and absence of tryptophan. All measurements were performed at −30 °C in 65% (v/v) glycerol/0.035 M potassium phosphate (pH 7.5) with a path length of 0.2 cm. In (A), to the 6.51 μM oxygenated enzyme (spectrum a), 4 mM L-tryptophan was first added to obtain spectrum b. Then 2.5 mM indole was added (spectrum c), followed by the second addition of tryptophan in the final concentration of 20 mM (spectrum d). Spectral changes in the visible region (500–630 nm) are shown in the inset. Concentrations of stock solutions for L-tryptophan and indole were 100 and 50 mM, respectively, and therefore the original enzyme solution was partially diluted after each addition of these compounds. In (B), to 6.67 μM of the oxygenated enzyme (spectrum a), 25 mM indole was first added to obtain spectrum b, followed by additions of 10 mM (spectrum c) and 20 mM (spectrum d) L-tryptophan in the final concentrations. Corresponding spectral changes in the visible region are shown in the inset. Due to the large dilution during the above treatments, the final enzyme concentration was less than half of the starting value.

indole (4 mM), indicating that both the substrate and indole can concomitantly bind to these enzyme derivatives to form quaternary complexes. Indoleacetic acid (25 mM) has no detectable effects on the optical properties of any of the ferrous enzyme derivatives examined either with or without tryptophan. Thus, it seems that, unlike indole, indoleacetic acid does not readily bind to or has quite low affinity for either the substrate-free or bound oxygenated enzyme.

Discussion

In the present study, the ternary complex of ferrous indoleamine 2,3-dioxygenase with substrate and dioxygen has been successfully generated and stabilized for the first time by using a subzero temperature technique. Substrate-free oxygenated indoleamine 2,3-dioxygenase has previously been prepared at ambient temperature (5–24 °C) by reaction of the native ferric

enzyme with superoxide anion (Hirata et al., 1977) or by the binding of dioxygen to the ferrous enzyme (Hayaishi, 1974, 1976; Hirata et al., 1977; Uchida et al., 1983). In contrast, only the substrate adduct of the oxygenated form of tryptophan 2,3-dioxygenase has been previously generated and characterized with optical absorption spectroscopy (Ishimura et al., 1967, 1970). The present study fills this gap and provides new information about the effects of tryptophan binding on the heme electronic structure and the active site conformation of the oxygenated enzyme. The following aspects arising from the new findings in this work will be discussed further in relation to the reaction mechanism and catalytic cycle for dioxygen insertion into organic substrates catalyzed by these heme-containing dioxygenases.

Roles of the Enzyme and Substrate in Possible Activation of Dioxygen. Close similarities in the optical absorption spectra and, therefore, in the electronic structures have been noticed among the oxygenated derivatives of tryptophan 2,3-dioxygenase (with substrate) (Ishimura et al., 1967, 1970), indoleamine 2,3-dioxygenase (without substrate) (Hayaishi, 1976; Hirata et al., 1977), myoglobin (Antonini & Brunori, 1971), and horseradish peroxidase (Keilin & Hartree, 1951; Wittenberg et al., 1967). In comparative MCD studies of dioxygen complexes of heme proteins including myoglobin, horseradish peroxidase, and cytochrome P-450, Nozawa et al. (1980) pointed out that the ratio of the MCD peak-to-trough intensity for the visible region (α peak) to that for the Soret region decreases for these proteins in the mentioned order. They have suggested that the observed trend might be correlated with their functions in terms of dioxygen activation. However, the differences in the above MCD parameter for the oxygenated forms of myoglobin and indoleamine 2,3-dioxygenase (with and without substrate) are very small (Figure 7). Thus, the functional differences between these two dioxygen-binding heme proteins are not reflected in their electronic structures. Furthermore, the slight change of the MCD parameter upon substrate binding to the oxygenated indoleamine 2,3-dioxygenase (Table II) is not significant enough to indicate that activation of the bound dioxygen occurs by substrate binding. It therefore seems that electronic spectral properties alone cannot distinguish functional differences between indoleamine 2,3-dioxygenase and myoglobin. Unfortunately, this leaves unanswered the question as to whether the heme-iron-bound dioxygen in the dioxygenase is activated or not.

Effects of Solvents and Indole on the Spectral Properties of the Enzyme–Substrate–O_2 Ternary Complex. The solvent dependence of the Soret absorption peak position of the enzyme–tryptophan–O_2 ternary complex observed in this work is an intriguing finding. Curiously, the solvent effects have been observed for the oxygenated enzyme *only* in the presence of substrate, while the analogous ferrous enzyme ternary complexes with NO and CO do not exhibit significant differences in their optical absorption Soret peak positions either in 65% glycerol or in 65% ethylene glycol (Table I). For the oxygenated enzyme, the effects of indole are also specific to its substrate adduct (Figure 8). The formation of the quaternary complexes of indole and substrate with the ferrous enzyme adducts with O_2, NO, and CO has been revealed in this work for the first time. Although the physiological significance of the observed indole and solvent effects is not clear at the present, the cause of the effects that have been observed specifically for the dioxygen complex of the enzyme *only* in the presence of tryptophan might be correlated to relative geometric positions of the heme-iron-bound dioxygen and the enzyme-bound tryptophan in the ternary complex. The observed results indicate that the electronic structure of the enzyme–substrate–O_2 ternary complex is especially sensitive to changes in the active-site heme environment. This, in turn, may be indicative of the importance of a particular geometric structure or physical arrangement of the heme–dioxygen–substrate complex for the dioxygenation reaction to occur at the enzyme active site.

Sequence of Tryptophan and O_2 Binding to Ferrous Indoleamine 2,3-Dioxygenase during Its Catalytic Cycle. As judged from the common substrate-binding properties of the CO and NO adducts of ferrous indoleamine 2,3-dioxygenase, it is reasonable to assume that the oxygenated enzyme would behave in a manner similar to those of the other ligand adducts of the ferrous enzyme in terms of the temperature, pH, and solvent dependence. In fact, the tryptophan affinities of the ferrous enzyme complexes with CO (Sono et al., 1980), NO (this work), and ethyl isocyanide (M. Sono, unpublished results) are pH-insensitive between pH 6 and 7.5. From these data one can estimate K_d values for the tryptophan adduct of the oxygenated enzyme at room temperature in aqueous buffer media. An estimated K_d value for the oxygenated enzyme in 0.1 M potassium phosphate buffer at pH 7.5 and at 24 °C would be close to that for the ferrous NO enzyme ($K_d \sim 6$ μM) determined under the same conditions (Figure 6). This K_d value coincides closely with the Michaelis constant (K_m) for tryptophan ($K_m = 9.0$ μM) (Sono et al., 1980) under the same conditions (pH 7.5, 24 °C). On the other hand, the estimated K_d values for the oxygenated enzyme ($K_d \sim 6$ μM, this work) and for the ligand-free ferrous enzyme [$K_d = 7.5$ μM, Sono et al. (1980)] are also very close to each other. This would seem to suggest that no preference exists in the order of tryptophan binding to the ferrous or oxygenated enzyme, which is inconsistent with the previously proposed catalytic sequence of indoleamine 2,3-dioxygenase (Sono et al., 1980). However, the K_d value for the oxygenated enzyme–tryptophan adduct is pH-independent as shown in this work, while the K_d value for the ferrous enzyme and the K_m values for tryptophan exhibit parallel changes with pH (Sono et al., 1980) where a protein ionizable group with a pK_a value of 7.3 at 24 °C is involved (M. Sono, unpublished data). Thus, the K_m value and its pH dependence for tryptophan in the steady state of the catalytic reaction closely correlate with the K_d value and its pH dependence for substrate binding to the ferrous enzyme, but not for substrate binding to the oxygenated enzyme. Therefore, the previously proposed catalytic reaction sequence (Sono et al., 1980) in which tryptophan first binds to the ferrous enzyme followed by dioxygen is further supported in this work. Further studies directed toward definitely establishing the reaction sequence and distinguishing physicochemical properties of dioxygen bound to the heme iron of dioxygenases with and without organic substrate and those of dioxygen bound to other heme proteins are in progress.

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Registry No. L-Trp, 73-22-3; indoleamine 2,3-dioxygenase, 9014-51-1.

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Nonionic Detergents Increase the Stoichiometry of Ligand Binding to the Rat Hepatic Galactosyl Receptor†

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ABSTRACT: When digitonin is used to expose intracellular galactosyl (Gal) receptors in isolated rat hepatocytes, only about half of the binding activity for ^{125}I-asialoorosomucoid (ASOR) is found as compared to cells solubilized with Triton X-100. The increased ligand binding in the presence of detergent is not due to a decrease in K_d but could be due either to an increase in the number of ASORs bound per receptor or to exposure of additional receptors. Several experiments support the former explanation. (i) No additional activity is exposed even when 80% of the total cell protein is solubilized with 0.4% digitonin. It is, therefore, unlikely that receptors are in intracellular compartments not permeabilized by digitonin and inaccessible to ^{125}I-ASOR. (ii) Digitonin-treated cells are not solubilized by Triton X-100 if they are first treated with glutaraldehyde under conditions that retain specific binding activity. ^{125}I-ASOR binding to these permeabilized/fixed cells increases about 2-fold in the presence of Triton X-100 and a variety of other detergents (e.g., Triton X-114, Nonidet P-40, Brij-58, and octyl glucoside) but not with the Tween series, saponin, or other detergents. When these fixed cells are washed to remove detergent, ^{125}I-ASOR binding decreases almost to the initial level. (iii) Affinity-purified Gal receptor linked to Sepharose 4B binds approximately twice as much ^{125}I-ASOR in the presence of Triton X-100 as in its absence. The results suggest that the increase in Gal receptor activity in the presence of nonionic detergents is due to an increase in the valency of the receptor rather than to exposure of additional receptors.

The use of nonionic detergents has enabled the solubilization, purification, and characterization of a wide variety of membrane-bound proteins (Helenius & Simons, 1975; Lichtenberg et al., 1983). The nonionic detergent substitutes for phospholipids and membrane proteins and dilutes them during the process of membrane solubilization. In the final isolated protein preparations, detergent substitutes for the phospholipid

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