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Studies on the Roles of the Catalytic and Allosteric Sites in Modulating the Reactivity of Tryptophan Oxygenase with Heme Ligands. I. Cyanide Derivatives*

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ABSTRACT: Spectral studies on bacterial tryptophan oxygenase and its cyano complex were performed. Ferritryptophan oxygenase at pH 7.0 exhibits spectral and electron paramagnetic resonance properties indicative of a single ferriheme moiety in a high-spin state ($d^5_{5/2}$). The monocyano complex, however, exhibits spectral and electron paramagnetic resonance properties typical of ferriheme in a low-spin state ($d^5_{1/2}$). From equilibrium binding data, dissociation constants (K_D), Hill coefficients (n), and standard free-energy changes were determined for the binding of cyanide to ferritryptophan oxygenase in the presence and absence of tryptophan. Either the substrate, tryptophan, or the competitive inhibitor, 5-fluorotryptophan, enhanced the affinity of ferriheme for cyanide (*i.e.*, lowered K_D), while the allosteric effector, α-methyltryptophan, was incapable of affecting this binding equilibrium (*i.e.*, K_D unchanged). It has been shown that α-methyltryptophan stabilizes the quaternary structure of the native enzyme and converts the "S"-shaped substrate saturation curve into a hyperbolic one. Hence, the affinity of the heme for cyanide is insensitive to certain cooperative interactions among subunits which modulate the catalytic activity of the native tetrameric enzyme molecule but rather seems to depend solely on saturation of the catalytic site by the substrate (tryptophan) or the competitive inhibitor (5-fluorotryptophan).

Tryptophan oxygenase is a dioxygenase, catalyzing the reaction between tryptophan and oxygen, yielding formylkynurenine (Tanaka and Knox, 1959; Feigelson *et al.*, 1965; Ishimura *et al.*, 1967). The tryptophan oxygenase of *Pseudomonas acidovorans* is a tetrameric heme protein of molecular weight 122,000 (Poillon *et al.*, 1969). Recent studies indicate the heme prosthetic group may oscillate in valence during catalysis (Forman and Feigelson, 1971). Its prosthetic heme also reacts with suitable ligands to form both ferri and ferro derivatives with characteristic absorption and epr spectra. In a recent report (Ishimura *et al.*, 1967), a close similarity was noted of the ternary complex (tryptophan–oxygen–ferrotryptophan oxygenase) detected spectrally to those of oxymyoglobin and horseradish peroxidase (III).

Evidence has been accumulating indicating that tryptophan oxygenase from *P. acidovorans* possesses regulatory as well as catalytic site(s) (Feigelson and Maeno, 1967; Koike *et al.*, 1969). The substrate, tryptophan, is capable of binding to both the regulatory and catalytic site(s); whereas, under

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appropriate conditions, the tryptophan analog, α-methyltryptophan, binds only to the regulatory site(s) (Koike *et al.*, 1969). Previous studies have shown that tryptophan markedly stabilizes the enzyme and enhances the affinity of its prosthetic heme iron for various ligands (Feigelson *et al.*, 1965; Ishimura *et al.*, 1967; Maeno and Feigelson, 1968; Koike *et al.*, 1969). It remained uncertain whether this was a consequence of tryptophan binding to the catalytic or allosteric site(s) of the enzyme. The present studies were designed to explore the respective roles and underlying chemical properties of the catalytic and allosteric sites of tryptophan oxygenase in the regulation of the interaction between its ferriheme and cyanide.

Materials and Methods

Absorption spectra and measurements of binding equilibria were obtained using a Cary Model 14 recording spectrophotometer equipped with a 0–0.1-absorbance slide-wire. The extinction coefficients of ferritryptophan oxygenase and its monocyanide derivative, as well as the isosbestic points for the two Soret bands, were independently used to measure the extent of the equilibrium formation of the ferricyano complex. (Details are given in the legend of Figure 2.) Sodium phosphate buffer (0.1 M, pH 7.0) was used in all spectrophotometric experiments and at least 8 min were allowed to elapse before spectra were recorded to ensure that ferricyano–enzyme complex formation had reached equilibrium at 25°. Protein concentrations were estimated from the ultraviolet absorption at 280 mμ or the Soret maximum at 405 mμ, using previously determined millimolar extinction coefficients of 146 and 229, respectively (Poillon *et al.*, 1969), or by a microturbidometric method (Layne, 1957; Poillon *et al.*, 1969).

Tryptophan oxygenase was purified to homogeneity from substrate-induced *P. acidovorans* (ATCC 11299b) as previously described (Poillon *et al.*, 1969). Homogeneity was verified by ultracentrifugal and analytical acrylamide gel (pH 8.2) examination. Prior to use ferritryptophan oxygenase was exhaustively dialyzed at 4° against 0.1 M sodium phosphate (pH 7.0) to remove the exogenous tryptophan used to stabilize the enzyme during purification.

L-Tryptophan, indole, 5-fluoro-DL-tryptophan, and other analogs were obtained from Nutritional Biochemical Co.; α-methyl-DL-tryptophan was a product of Regis Chemical Co.

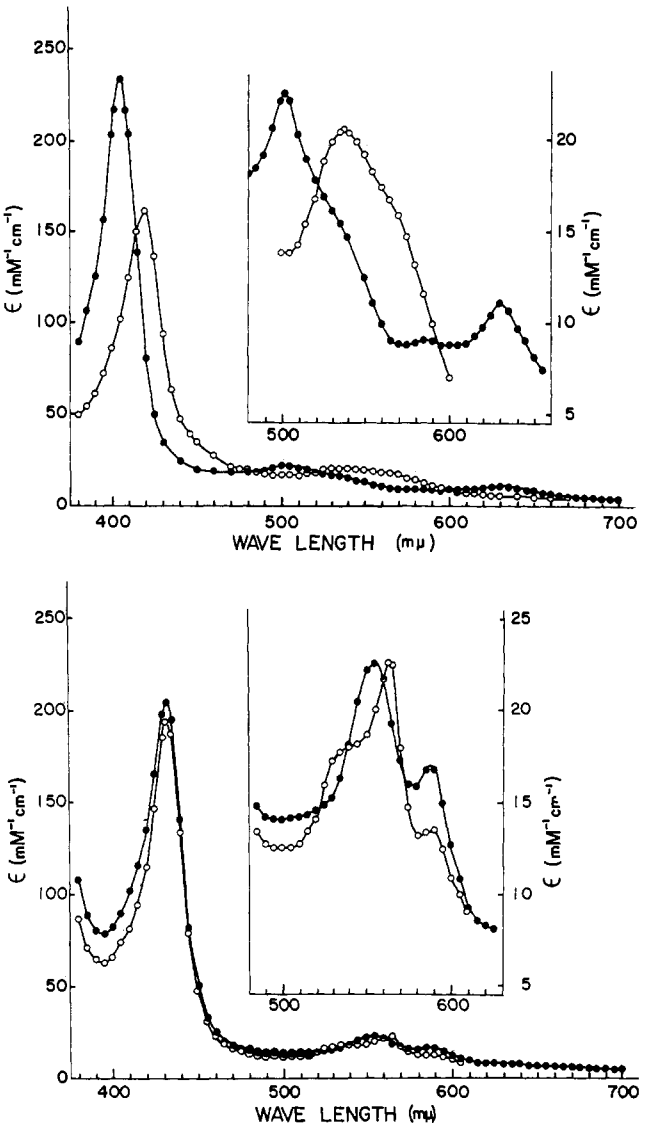

FIGURE 1: Optical spectra of ferri- and ferrotryptophan oxygenase and their cyanide derivatives. (a, top) Ferritryptophan oxygenase in 0.1 M sodium phosphate (pH 7.0, ●). Ferritryptophan oxygenase in 0.1 M sodium phosphate (pH 7.0) containing 0.01 M KCN (○). (b, bottom) Ferrotryptophan oxygenase in 0.1 M sodium phosphate (pH 7.0) prepared by reduction of the ferri–enzyme with a few crystals of sodium dithionite (●). Ferrotryptophan oxygenase in 0.1 M sodium phosphate containing 0.0476 M KCN (○).

Results

The optical spectra of ferri- and ferrotryptophan oxygenase were determined in 0.1 M sodium phosphate (pH 7.0) at 25°. The optical spectrum of ferritryptophan oxygenase exhibits maxima at 405 mμ in the Soret region and at 502 and 632 mμ, with a shoulder near 534 mμ in the visible region (Figure 1a). The ferro–enzyme was prepared by the addition of a few crystals of sodium dithionite to the ferri–enzyme. In the spectrum of ferrotryptophan oxygenase, the Soret–absorption maxima is shifted to 432 mμ and α and β peaks of weaker intensity are seen in the visible region at 554 and 588 mμ (Figure 1b). These spectra closely resemble those reported by Keilin and Hartree (1951, 1955) for ferri- and ferrohorseradish peroxidase (HRP)[1] and ferrimyoglobin, and by Yonetani and Ray (1965) for ferricytochrome *c* peroxidase.

The absorption spectrum of the cyanoferritryptophan oxygenase exhibits a maximum at 419 mμ in the Soret region and a distinct peak at about 537 mμ, as well as an unresolved shoulder at 570 mμ in the visible region (Figure 1a). This spectrum is also reminiscent of the ferri-HRP–cyano complex reported by Keilin and Hartree (1951, 1955). The cyanoferrotryptophan oxygenase complex exhibits a maximum at 432 mμ in the Soret region, and peaks of weaker intensity at 564 and 590 mμ, with a shoulder near 535 mμ in the visible region (Figure 1b).

The optical spectra of the cyanide derivatives of ferritryptophan oxygenase in the presence of either 1.5 mM tryptophan or 5 mM 5-fluorotryptophan in the Soret and α,β regions were also recorded. The ferritryptophan oxygenase cyanide derivative in the presence of tryptophan showed absorption maxima at 420 mμ in the Soret region and at 541 mμ, with a shoulder evident near 572 mμ (Table I). The ferritryptophan

[1] Abbreviations used are: HRP, horseradish peroxidase; Gdn·HCl, guanidine hydrochloride.

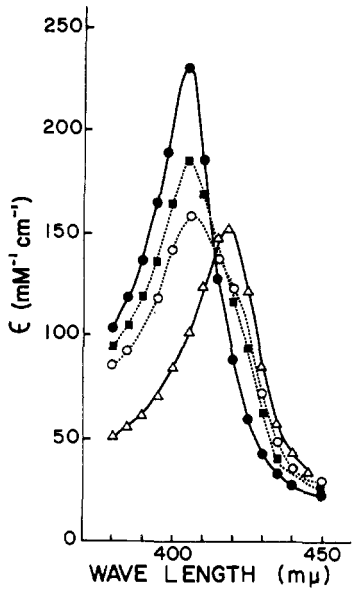

FIGURE 2: Optical spectra of ferritryptophan oxygenase in the Soret region as a function of increasing cyanide concentration. Ferritryptophan oxygenase (0.24 μM) in 0.1 M sodium phosphate (pH 7.0) containing 2.0×10^{-4} M L-tryptophan (20°) with no KCN (●); 2×10^{-5} M KCN, 39% cyano-enzyme derivative (■) 6×10^{-5} M KCN, 57% cyano-enzyme derivative (○); 1×10^{-2} M KCN, 100% cyano-enzyme derivative (Δ). Optical spectra were taken after equilibrium *vs.* optical blanks lacking the enzyme. Based on these spectra one can calculate the portion of the ferritryptophan oxygenase as its cyano complex as follows: x% cyanide derivative = (A405) 0% − (A405)%/(A405) 0% − (A405) 100% × 100 or = (A419) 0% − (A419)%/(A419) 0% − (A419) 100% × 100. (A) 0%, (A) 100%, and (A) x% are measured optical densities of 0%, 100%, and x% cyanide derivatives, respectively, at 405 or 419 mμ.

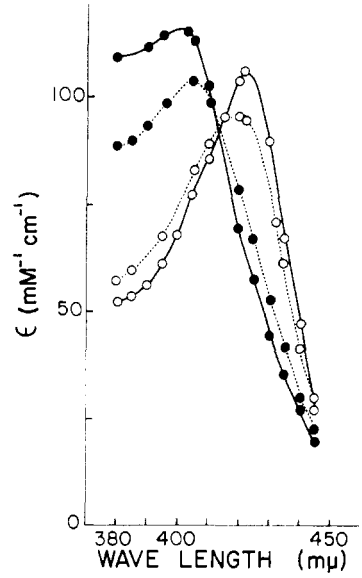

FIGURE 3: Soret spectra of guanidine-treated ferritryptophan oxygenase as a function of cyanide concentration. Denatured ferritryptophan oxygenase (0.66 μM) in 4.2 M Gdn·HCl in 0.1 M sodium phosphate (pH 7.0) and no KCN, 0% cyanide derivative (●—●); in the presence of 4×10^{-3} M KCN, 24% cyanide derivative (●····●); with 1×10^{-2} M KCN, 78% cyanide derivative (○····○); with 2×10^{-2} M KCN, 100% cyanide derivative (○—○). Ferritryptophan oxygenase (0.66 μM) was treated with 4.6 M Gdn·HCl and after 60 min the indicated amounts of KCN were added into the denatured ferritryptophan oxygenase. Spectra were taken after equilibrium *vs.* blanks lacking the enzyme. Based on these spectra the portion of the denatured ferritryptophan oxygenase as cyanide derivative was calculated as follows: x% cyanide derivative = (A422) 0% − (A422) x%/(A422) 0% − (A422) 100% × 100. (A422) 0%, (A422) 100%, and (A422) x% are measured optical densities of 0%, 100%, and x% cyanide derivatives at 422 mμ, respectively.

oxygenase cyanide derivative in the presence of 5-fluorotryptophan showed optical maxima at 419 mμ in the Soret region and at 537 mμ, with a shoulder at 570 mμ (Table I). The addition of tryptophan to the cyanoferritryptophan oxygenase complex caused a very slight hypochromic red shift of a few millimicra in the Soret and α,β regions, which was reminiscent of that observed when tryptophan was added to native ferritryptophan oxygenase (Maeno and Feigelson, 1967; Ishimura *et al.*, 1967). Upon removal of cyanide from a solution of the ferritryptophan oxygenase cyanide complex by exhaustive dialysis against 0.1 M sodium phosphate buffer (pH 7.0), the optical spectrum was converted into one essentially indistinguishable from that of the ferri-enzyme alone.

TABLE I: Spectral Properties of Tryptophan Oxygenase Complexes.

Enzyme State	Predominant Epr Signal	Optical Absorption Maxima
Ferri-TO	High spin[a]	405, 502, 532(s), 632
Ferro-TO		432, 554, 588
Cyanoferri-TO	Low spin[a]	419, 537, 570(s)
Cyanoferro-TO		432, 535(s), 564, 590
Cyanoferri-TO + 1.5 mM tryptophan		420, 541, 572(s)
Cyanoferri-TO + 5.0 mM 5-F-tryptophan		419, 537, 570(s)
Ferri-HRP	High spin[b]	403, 500, 643
Ferro-HRP	None[b]	432, 557, 588(s)
Cyanoferri-HRP	Low spin[b]	421, 539
Cyanoferro-HRP	None[b]	432, 536, 566

[a] Feigelson *et al.* (1968). [b] Blumberg *et al.* (1968), optical absorption maxima at 20°.

The epr spectrum of ferritryptophan oxygenase at pH 7.0 was characteristic of heme iron in the high-spin configuration (X band, 14°K, $g = 6.00$) which, upon addition of cyanide, changed to the low-spin type, with a broadened absorption in the high magnetic field (Feigelson *et al.*, 1968). Similar broad and unresolved low-spin signals have been reported in the epr spectra of the cyanide derivatives of ferri-HRP and ferricytochrome *c* peroxidase (Blumberg *et al.*, 1968; Ehrenberg, 1966). In Table I are summarized the visible and epr spectral data for the native and cyanide derivatives of ferri- and ferrotryptophan oxygenase, as well as similar data for HRP as reported by other authors (Keilin and Hartree, 1951, 1955; Blumberg *et al.*, 1968). These data indicate that high-spin ferritryptophan oxygenase combines reversibly with cyanide to form a low-spin derivative with a visible spectrum quite similar to that of the cyanoferri-HRP complex.

The addition of increments of cyanide to the ferri-enzyme generated a series of spectra which exhibited an isosbestic point at 414 mμ in the Soret region (Figure 2). This result indicates the formation of a single species of cyanoferritryptophan oxygenase and precludes the existence of spectrophotometrically identifiable intermediates or side products at

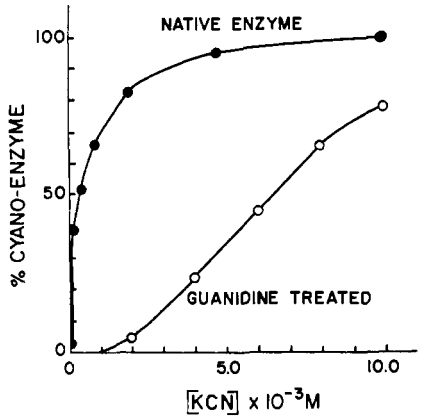

FIGURE 4: The formation of the cyano–enzyme complex of native and Gdn·HCl-treated ferritryptophan oxygenase as a function of cyanide concentration. In replicate cuvets 0.34 μM ferritryptophan oxygenase was treated with the indicated concentrations of KCN (●). Ferritryptophan oxygenase (0.66 μM) in 4.2 M Gdn·HCl, was similarly treated with the indicated amounts of KCN (○). Per cent cyanide derivative was calculated from the optical density change after equilibrium according to the principal of the equations shown in Figures 2 and 3.

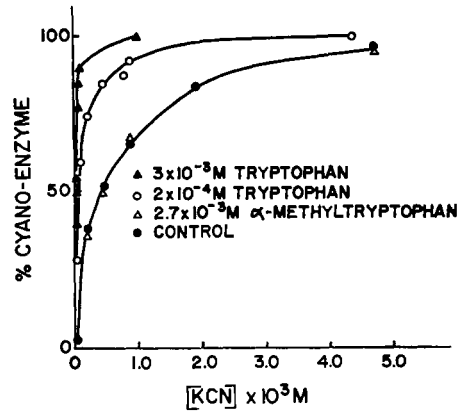

FIGURE 5: The effects of tryptophan and α-methyltryptophan upon cyanoferritryptophan oxygenase formation. Ferritryptophan oxygenase (0.34 μM) was treated with the indicated concentrations of KCN in 0.1 M sodium phosphate (pH 7.0) in the absence of tryptophan and α-methyltryptophan (●); in the presence of 2.7×10^{-3} M α-methyltryptophan (Δ); 2.0×10^{-4} M tryptophan (○), or 3×10^{-3} M tryptophan (▲). All spectra were taken after equilibrium and per cent cyanide derivatives were calculated as previously described.

any stage during the chemical reaction. These spectral changes provided the basis for quantitative evaluation of the proportional amount of cyanide derivative present under various experimental conditions (see legend to Figure 2).

Gdn·HCl has been shown to cause ferritryptophan oxygenase to dissociate from a tetrameric structure (mol wt 122,000, $s_{20,w}$ = 6.26 S) to a monomeric species (mol wt 33,200, $s_{20,w}$ = 1.73 S) (Poillon *et al.*, 1969). Low levels of Gdn·HCl cause a hypochromic red shift of the Soret absorption peak of the ferri–enzyme from 405 to 411 mμ. At the higher concentrations of Gdn·HCl utilized in the study depicted in Figure 3, a blue shift of the Soret absorption maximum from 411 to 401 mμ occurs which is accompanied by a further decrease in intensity (compare Figure 1a to Figure 3). These spectral changes in the Soret region evoked by Gdn·HCl are similar to those reported for metmyoglobin (Schecter and Epstein, 1968). The rate of alteration by Gdn·HCl was evaluated optically as described in the legend of Figure 3 and was found to be dependent on the concentration of Gdn·HCl. At the levels of Gdn·HCl herein employed, equilibrium values were reached within 1 hr. Treatment of these preparations of enzyme with increasing amounts of cyanide caused hypochromic shifts of the Soret absorption peak from 401 mμ to longer wavelengths (Figure 3). The extent of the change in the Soret region was dependent on the cyanide concentration with the maximum ultimately shifted to 421 mμ when the cyano–enzyme complex formation was complete. All spectra had an isosbestic point at 413 mμ. Furthermore, the absorption spectrum of the denatured cyanoferri–enzyme was similar to those of other dinitrogenous base–iron porphyrin complexes (Hogness *et al.*, 1937; Harbury and Loach, 1959), suggesting that the reaction system may consist of only two components, free heme and its dicyanide derivative.

In a manner analogous to that described in the legends to Figure 2, the hypochromicity and Soret absorption peak shift which accompanies conversion of the Gdn·HCl-treated ferri–enzyme into its cyanide derivative may be used to quantitate the extent of cyano complex formation as a function of cyanide concentration (see legend to Figure 3). In Figure 4 are plotted the data for the degree of cyano complex formation as a function of cyanide concentration for both the native and Gdn·HCl-treated ferri–enzyme. A hyperbolic relationship is evident for the equilibrium binding between native ferritryptophan oxygenase and cyanide. Spectral changes equivalent to the conversion of 50% of the enzyme into its monocyanide derivative occurred at a concentration near 0.3 mM cyanide. In contrast, a sigmoidal relationship is evident for the equilibrium binding of cyanide to the Gdn·HCl-treated enzyme. This required a cyanide concentration of about 6.5 mM to achieve conversion of 50% of the heme into its cyanide derivative. This "S"-shaped saturation curve supports the spectral data indicating that the Gdn·HCl-treated ferri–enzyme has two interacting cyanide binding sites (n = 1.2). Thus, the structural alteration of the enzyme evoked by Gdn·HCl treatment frees the fifth ligand of the heme iron from its normal ligand in the protein thereby enabling it as well as the sixth coordination position of the heme iron to react with cyanide.

Evidence that tryptophan enhances the affinity of tryptophan oxygenase for both carbon monoxide and for cyanide has been reported (Ishimura *et al.*, 1967; Maeno and Feigelson, 1968; Koike and Feigelson, 1971). Furthermore, these previous studies indicate that tryptophan is capable of binding to both the catalytic and allosteric sites of the enzyme. It is therefore of import to determine whether the affinity of the enzyme's heme prosthetic group for its ligands is subject to the control at the catalytic or the allosteric site or both. The results of an experiment designed to answer this question for the ferri–enzyme are depicted in Figure 5. It may be seen that the inclusion of 2.7 mM α-methyltryptophan in the reaction system causes no enhancement in cyanide binding, relative to the control. Conversely, a profound augmentation of cyano–enzyme complex formation is seen at both 0.2 and 3 mM tryptophan concentrations. Hence, the allosteric effector, α-methyltryptophan, present at a concentration known to saturate the regulatory site (Koike *et al.*, 1969) does not detectably influence the binding of cyanide to the ferri–enzyme whereas saturation of the catalytic site by tryptophan does.

Under these conditions, a reaction scheme for the formation of the cyano complexes of ferritryptophan oxygenase, in the

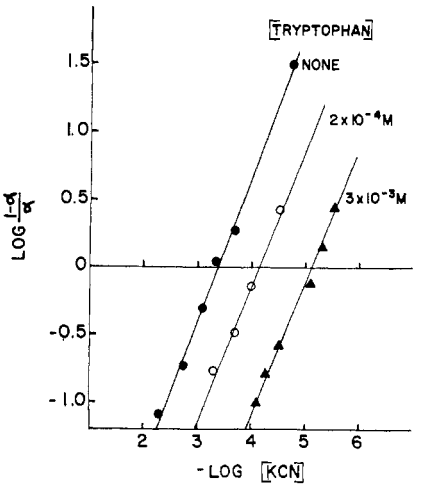

FIGURE 6: Hill plots of cyanoferritryptophan oxygenase formation in the absence and presence of tryptophan. At each of the indicated levels of KCN, 0.34 μM ferritryptophan oxygenase in 0.1 M sodium phosphate (pH 7.0) was incubated in the absence of tryptophan (●); in the presence of 2.0×10^{-4} M tryptophan (○); or 3.0×10^{-3} M tryptophan (▲).

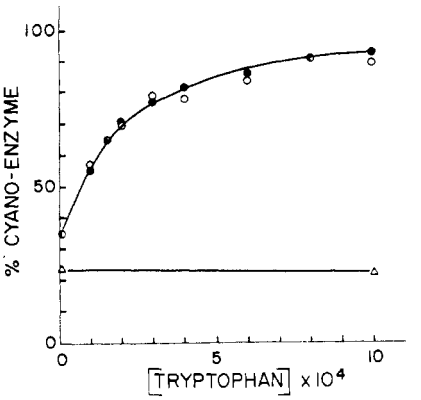

FIGURE 7: The effect of tryptophan and α-methyltryptophan upon the formation of cyanoferritryptophan oxygenase. Ferritryptophan oxygenase (0.24 μM) in 0.1 M sodium phosphate (pH 7.0), containing the indicated amount of tryptophan in the absence of (●) or presence of 5.0×10^{-3} M α-methyltryptophan (○) was treated with 2.0×10^{-4} M KCN. Ferritryptophan oxygenase (0.27 μM) denatured by exposure for 60 min to 4.8 M Gdn·HCl in 0.1 M sodium phosphate (pH 7.0) was then treated with 4.0×10^{-3} M KCN in the presence or absence of 1.0×10^{-3} M tryptophan (Δ). All spectra were taken after equilibrium was reached and per cent cyanide derivatives were calculated.

absence and presence of tryptophan may be formulated thus

$$\begin{array}{ccc} EFe^{3+}\cdot(KCN)_n & \overset{+n(KCN)}{\rightleftharpoons} & EFe^{3+} \\ +S\uparrow\downarrow & & \downarrow\uparrow+S \\ SEFe^{3+}\cdot(KCN)_n & \underset{+(nKCN)}{\rightleftharpoons} & SEFe^{3+} \end{array}$$

The formation of the cyano-enzyme complex in the absence or presence of tryptophan can be expressed in the manner described by Hill (Hill, 1910; Wyman, 1948, 1964) as $K_D = [(1 - \alpha)/\alpha](KCN)^n$, where K_D is an apparent equilibrium constant for the dissociation of cyanide from the cyanoferri-enzyme or tryptophan cyanoferri-enzyme complex; n is the Hill coefficient of this reaction and α is the fraction of the total ferri-enzyme present as cyanide derivative. This derivation is an expression of the equilibrium state of an overall thermodynamic process in the presence or absence of tryptophan and intermediate states are not relevant.

Thus, when $\log [(1 - \alpha)/\alpha]$ is plotted against $-\log$ (KCN), the slope of the resulting linear line indicates n and the ordinate intercept the $\log K_D$. Data from such optical studies on the ferri-enzyme alone and in the presence of K_M and saturation levels of tryptophan are shown in Figure 6. It may be seen that the three lines obtained are essentially parallel with slope (n) near one. The calculated Hill coefficients, n, and the apparent dissociation constants, K_D, for the formation of the ferri-enzyme cyanide derivatives in the absence and presence of tryptophan are shown in Table II, together with the calculated change in standard free energy, ΔF, for the formation of these ferri-enzyme cyanide derivatives.

TABLE II: Effect of Tryptophan upon the Binding of Cyanide to Ferritryptophan Oxygenase.

[Trp], M	Hill Coef (n)	Constant ($K_D \times 10^5$)	ΔF (cal/mole)[a]
None	1.06	25.00	−4912
2.0×10^{-4}	1.06	6.00	−5757
3.0×10^{-3}	1.02	0.79	−6958
1.0×10^{-2}	1.00	0.32	−7493
HRP (Keilin and Hartree, 1955)	1.00	0.20	−7771

[a] $\Delta F = -RT \ln (K_D)^{-1}$.

The calculated Hill coefficient, $n = 1$, for these reactions is compatable with one cyanide molecule being bound to one high-spin ferriheme iron. That the binding affinity of the high-spin ferriheme for cyanide is enhanced by tryptophan is reflected by the progressive diminution in the apparent dissociation constant, K_D and consequently the progressive increase in the standard free energy of binding of ~2600 cal/mole as the concentration of tryptophan is increased from 0 to 10 mM (Table II).

The spectra of the ferri, ferro, and cyano complexes of tryptophan oxygenase and HRP were remarkably similar (Table I), suggesting that the electronic configurations of the ferriprotoporphyrin IX of these two enzymes were in a similar electronic state. However, a dissociation constant of 0.2×10^{-5} M has been reported for the cyanide derivative of HRP (Keilin and Hartree, 1951, 1955). This value differs by two orders of magnitude from that of cyanoferritryptophan oxygenase. Thus, either stereochemical or chemical differences exist in the environment of the hemes of these enzymes.

Although α-methyltryptophan alone was incapable of enhancing the binding of cyanide to ferritryptophan oxygenase (Figure 5), experiments were undertaken to determine whether the conformational alteration accompanying α-methyltryptophan binding to the allosteric site of the ferri-enzyme might in some manner synergize with the augmentation in cyanide affinity for the enzyme which accompanies saturation of its catalytic site. Accordingly, the equilibrium formation of the ferritryptophan oxygenase cyanide derivative was measured as a function of tryptophan concentration, in the presence

and absence of α-methyltryptophan (Figure 7). Incubation of the ferri–enzyme with 0.2 mM cyanide leads to the conversion of 37% of the enzyme into its cyanide derivative. The cyano–enzyme complex increases hyperbolically as ferritryptophan oxygenase becomes saturated with tryptophan. This hyperbolic relationship is in marked contrast to the results cited in the accompanying study (Koike and Feigelson, 1971) which show that the binding of carbon monoxide to the ferroenzyme is a sigmoidal function of tryptophan concentration. The concentration of tryptophan required to effect a 50% enhancement of the cyanoferritryptophan oxygenase complex formation, about 2.0×10^{-4} M, is slightly lower than the 3.3×10^{-4} M tryptophan required to obtain 50% of the maximal catalytic activity when it is measured as a function of tryptophan concentration.

When α-methyltryptophan is added to the system, at a level previously shown to both abolish the sigmoidal tryptophan saturation curve in the catalytic reaction (Feigelson and Maeno, 1967) and to evoke structural stabilization (Koike *et al.*, 1969) no alteration in the extent of cyanoferritryptophan oxygenase formation is detectable (Figure 7). These results indicate that at all tryptophan levels the equilibrium between ferriheme and its cyanide derivative is independent of the presence or absence of α-methyltryptophan and that no synergistic effect is detectable. Similar experiments were performed to evaluate the binding between Gdn·HCl-denatured ferri–enzyme and cyanide in the absence and presence of tryptophan. Once the native structure of tryptophan oxygenase is altered by 4.8 M Gdn·HCl, the extent of cyano–enzyme complex formation is no longer responsive to the presence of tryptophan (Figure 7), as would be expected.

Inasmuch as 5-fluorotryptophan behaves kinetically as a strict competitive inhibitor with respect to tryptophan, it presumably binds exclusively to the catalytic site (Koike and Feigelson, 1971). It was therefore of interest to determine whether its presence would influence the affinity of the ferri–enzyme for cyanide. The results of such as experiment are presented in Figure 8. It may be seen that on treating the ferri–enzyme with 5×10^{-4} M cyanide in the absence of 5-fluorotryptophan, 43% of the enzyme exists as the cyano–enzyme complex. As the concentration of 5-fluorotryptophan was raised to 5 mM, the affinity of the ferri–enzyme for cyanide increased, which was a hyperbolic function of the 5-fluorotryptophan concentration. When the data were calculated according to the Hill equation, a Hill coefficient on $n = 1.1$ for 5-fluorotryptophan was obtained. These results are analogous to those obtained with tryptophan (Figure 7) and indicated that the competitive inhibitor, 5-fluorotryptophan, is equally as effective as the substrate, tryptophan, in enhancing the affinity of the ferri–enzyme for cyanide.

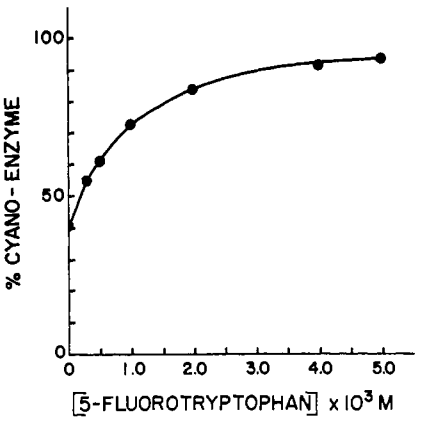

FIGURE 8: The augmentation of cyanoferritryptophan oxygenase formation as a function of 5-fluorotryptophan concentration. Ferritryptophan oxygenase (0.31 μM) was treated with 2.5×10^{-4} M KCN in 0.1 M sodium phosphate (pH 7.0) in the presence of the indicated concentrations of 5-fluorotryptophan. Spectra were taken after equilibrium was reached and the per cent cyanide derivatives were calculated.

Discussion

The optical absorption spectrum of ferritryptophan oxygenase in the Soret and α,β regions is strikingly similar to that of ferri-HRP (Keilin and Hartree, 1951, 1955), ferricytochrome *c* peroxidase (Yonetani and Ray, 1965), and ferrimyoglobin (Keilin and Hartree, 1951, 1955). This suggests that the electronic configuration of the ferritryptophan oxygenase heme iron is approximately the same as that of high-spin heme iron (*i.e.*, heme $d^{5}{}_{/2}$ in the nomenclature of Peisach *et al.*, 1968). The visible spectrum of the ferri–enzyme cyanide derivative is also reminiscent of that for the cyano complexes of these same three proteins in which the heme iron is in the low-spin state (*i.e.*, heme $d^{5}{}_{1/2}$ Peisach *et al.*, 1968). The visible spectrum of the ferrotryptophan oxygenase cyanide derivative is similarly reminiscent of cyanoferro-HRP in which the heme iron is diamagnetic and may be written as heme $d^{6}{}_{0}$ (Shulman and Sugano, 1965; Zerner *et al.*, 1966). The cyanide derivatives of ferritryptophan oxygenase in the presence of tryptophan or 5-fluorotryptophan are not distinguishable optically from the ferri–enzyme cyanide derivative alone. However, their paramagnetic properties remain to be elucidated.

In view of these similarities of ferri-, cyanoferri-, and cyanoferrotryptophan oxygenase to these three proteins it seems reasonable to conclude that the fifth ligand of the heme of tryptophan oxygenase arises from the protein and is nitrogenous (imidazole?) in nature (Smith and Williams, 1970). The sixth ligand position is probably occupied by water in the resting enzyme and by molecular oxygen in the ternary complex. The tryptophan enhancement of equilibrium binding of cyanide may be reasonably ascribed to a difference in the electronic character of the fifth coordination position of the heme iron. This has been shown in the CO binding studies of Alben and Caughey (1962, 1966) and oxygen binding studies of Rossi-Fanelli and Antonini (1957). What remains unknown and speculative is the degree to which the alteration in the electronic environment of the heme is due to a direct interaction between the heme and the tryptophan (or 5-fluorotryptophan) which is bound to the catalytic site or to alterations in protein conformation which in turn influence and determine the affinity of the heme for cyanide. What is evident is that this phenomenon is regulated by events occurring at the catalytic site and is uninfluenced by saturation of the allosteric site. Chemical combination of the enzyme with cyanide is in contrast to that which is observed with respect to carbon monoxide binding, which, as will be shown in the accompanying paper, is affected by saturation of both the allosteric and catalytic sites.

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