Tryptophan and α-Methyltryptophan Facilitation in the Interaction of Cyanide with Tryptophan Oxygenase*

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ABSTRACT: The ferriprotoporphyrin form of tryptophan oxygenase of *Pseudomonas* reacts with cyanide in a cooperative manner to form a complex with an absorption maximum at 420 mμ and an isosbestic point at 415 mμ. Spectral and catalytic activity studies demonstrate that the affinity of the trivalent (Fe^{3+}) form of the enzyme for cyanide is enhanced four- to fivefold by the presence of the substrate, L-tryptophan. Furthermore, α-methyl-L-tryptophan, an effector substance which is neither a substrate nor an inhibitor of the enzyme, similarly facilitates the binding of cyanide. Tryptophan oxygenase appears to be subject to a type of allosteric modulation whereby either L-tryptophan or α-methyl-L-tryptophan may bind to a site which is separate and distinct from the catalytic site. The binding of either compound at this allosteric site results in the enhancement of binding of appropriate ligands which are specific for either the divalent (oxygen, carbon monoxide) or trivalent (cyanide) state of iron in the heme prosthetic group.

The first step in the catabolism of L-tryptophan in microorganisms is catalyzed by the enzyme tryptophan oxygenase (EC 1.13.1.12), a dioxygenase which contains both hematin and copper prosthetic groups (Greengard and Feigelson, 1962; Maeno and Feigelson, 1965). Recent reports from this laboratory have demonstrated that the enzyme purified from substrate-induced *Pseudomonas acidovorans* shows a marked dependence of the K_M for oxygen on tryptophan concentration. However, in the presence of α-methyl-L-tryptophan, a substrate analog which is neither a substrate nor an inhibitor of the enzyme, the K_M for oxygen is minimal and independent of the tryptophan level (Feigelson and Maeno, 1967). Similarly both tryptophan and α-methyltryptophan enhance the affinity of the enzyme for carbon monoxide in spectral studies designed to measure the equilibrium formation of the carbonmonoxy–ferroprotoporphyrin enzyme complex (Maeno *et al.*, 1967; Maeno and Feigelson, 1968). Furthermore, the catalytic activity is a sigmoidal function of the tryptophan concentration and this sigmoidal relationship is abolished by α-methyltryptophan which converts the substrate saturation curve into a hyperbolic one (Maeno and Feigelson, 1968; Feigelson *et al.*, 1967). On the basis of these findings, it was proposed that in addition to the catalytic site, tryptophan oxygenase contains an allosteric site to which either tryptophan or α-methyltryptophan may bind, presumably causing an alteration in molecular conformation enhancing the intrinsic reactivity of the ferroprotoporphyrin at the catalytic site.

As both oxygen and carbon monoxide presumably bind exclusively to the ferroprotoporphyrin form of the enzyme and as the iron of the heme prosthetic group of tryptophan oxygenase undergoes oscillation in valence state during the catalytic process (Feigelson *et al.*, 1964, 1965; Feigelson and Maeno, 1966; Maeno and Feigelson, 1967), it was of import to determine whether the regulation of the functional capacity of the enzyme in this manner was limited to the divalent state or was a property of the ferriprotoporphyrin enzyme as well. Cyanide was eminently suitable for this purpose, since this ligand has been shown to inhibit and bind to the ferriprotoporphyrin form of the enzyme (Tanaka and Knox, 1959). The present paper reports a facilitation by the substrate L-tryptophan and the allosteric effector, α-methyltryptophan, in the equilibrium binding of cyanide to the ferriprotoporphyrin form of the enzyme.

Materials and Methods

Tryptophan oxygenase was extracted from *Pseudomonas acidovorans* by sonic oscillation and purified as described previously (Maeno and Feigelson, 1967, 1968). Enzyme activity was measured by continuous recording of formylkynurenine formation at 321 mμ at 25° with a Cary spectrophotometer Model 14 (Maeno and Feigelson, 1967). Protein concentration was determined turbidimetrically (Layne, 1959). The specific activities are expressed as micromoles of formylkynurenine formed per minute per milligram of protein at 25°. Other experimental details are described in the appropriate legends.

Results and Discussion

In an attempt to ascertain the wavelength most suit-

* From the Departments of Biochemistry and Medicine, College of Physicians and Surgeons, Columbia University, New York, New York 10032. *Received November 27, 1967.* This study is supported by research grants from the U. S. Public Health Service (CA 02332) and the National Science Foundation (G 20878).

† Career Investigator I-104, Health Research Council of the City of New York.

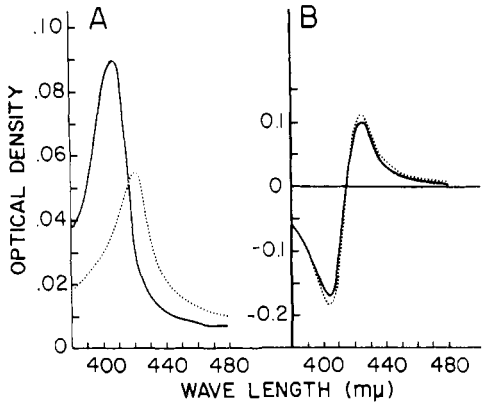

FIGURE 1: Spectral studies of the cyano-tryptophan oxygenase complex. (A) Absolute spectra. Enzyme solution (1 ml) with specific activity 17.0 in 0.05 M sodium phosphate buffer (pH 7.0) contained 3×10^{-4} M tryptophan and 34 μg of protein in the absence (——) and presence (--------) of 0.01 M KCN. (B) Difference spectral studies of the cyano-enzyme complex. The optical density difference was taken between cuvets containing 137 μg of enzyme/ml of phosphate buffer (specific activity, 17.0) in the absence and presence of 8.1×10^{-3} M KCN (——). This was repeated in the presence of 6×10^{-4} M tryptophan in both cuvets (· · · ·).

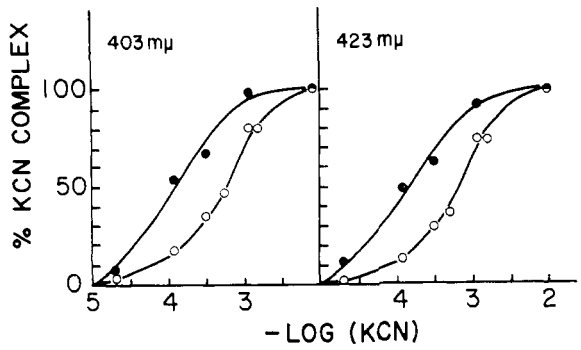

FIGURE 2: The effect of tryptophan upon the formation of the cyano-tryptophan oxygenase complex. The indicated molar concentrations of KCN (O——O) were added to 147 μg of enzyme protein (specific activity 17.0) in 1 ml of 0.05 M phosphate buffer (pH 7.0), and the optical densities were measured at 403 and 423 mμ. This was repeated in the presence of 9×10^{-4} M tryptophan (●——●).

able for measuring formation of the cyano-enzyme complex, the absolute and difference spectra of this complex were determined. As depicted in Figure 1A, the Soret peak, which is at 407 mμ for the tryptophan-ferriprotoporphyrin enzyme complex (Maeno and Feigelson, 1967), shifts to 420 mμ for the tryptophan-cyano-ferriprotoporphyrin enzyme complex, with an isosbestic point at 415 mμ. In the difference spectra shown in Figure 1B, a minimum and a maximum are found at 403 and 423 mμ, respectively, both of which are uninfluenced by the presence of tryptophan. As shown in Figure 2, formation of the cyano-enzyme complex may be determined equally well by measuring the optical density decrease at 403 mμ or the optical density increase at 423 mμ. The conversion of the enzyme into its cyano complex is a sigmoidal function of the cyanide concentration, indicating cooperative interaction between the ferriprotoporphyrin moieties of the enzyme. Although tryptophan does not modify the spectrum of the fully formed cyano-enzyme complex, it does affect the affinity of the enzyme for cyanide at less than saturation levels of cyanide. As can be seen in Figure 2, in the absence of tryptophan the cyanide concentration required for half-maximal formation of cyano-enzyme complex is 4.6×10^{-4} M and is decreased to 1.2×10^{-4} M in the presence of 9×10^{-4} M tryptophan. Tryptophan therefore evokes a fourfold enhancement in the affinity of the ferriprotoporphyrin of the enzyme for cyanide. This is in contrast to the 30-fold potentiation by tryptophan in the binding affinity of the ferroprotoporphyrin form of the enzyme for carbon monoxide (Maeno and Feigelson, 1968). A recent communication (Ishimura *et al.*, 1967), using an enzyme preparation of lower purity than that used in this study, briefly alluded to a 200-fold enhancement by tryptophan in the affinity of tryptophan oxygenase for cyanide. However, neither the data nor the experimental conditions were described.

It is well known that cyanide inhibits tryptophan oxygenase activity (Tanaka and Knox, 1959). When this inhibition was studied as a function of cyanide concentration, it was found that the cyanide level required to achieve half-maximal inhibition was an inverse function of the tryptophan concentration of the medium. As shown in Figure 3, the cyanide concentrations which resulted in 50% inhibition of the enzyme activity were 1.4×10^{-4} M KCN at 36×10^{-4} M tryptophan and 5.9×10^{-4} M KCN at 2.4×10^{-4} M tryptophan, values which are in good agreement with the spectral quantitation of cyano-enzyme complex formation, and again indicate severalfold enhancement by tryptophan in the affinity of the ferriprotoporphyrin of the enzyme for cyanide. These catalytic studies also confirm that cyanide binds preferentially to enzyme molecules which are in combination with tryptophan

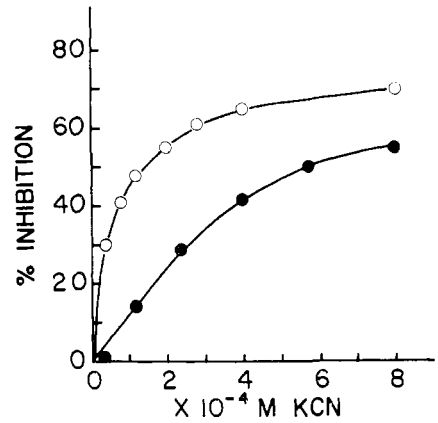

FIGURE 3: The inhibition of tryptophan oxygenase activity as a function of cyanide concentration at low and high tryptophan levels. The reaction mixture contained 360 μmoles of sodium phosphate buffer (pH 7.0), 0.92 mμmoles of hematin, 4.0 μmoles of ascorbate, the indicated amounts of cyanide, and 2.4×10^{-4} M tryptophan (●——●) or 36×10^{-4} M tryptophan (O——O). The reaction was initiated by the addition of 3.7 μg of enzyme protein (specific activity 13.0).

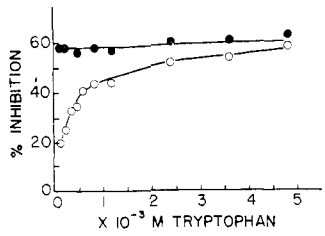

FIGURE 4: The inhibition of tryptophan oxygenase activity by cyanide at various tryptophan concentrations in the presence and absence of α-methyltryptophan. The reaction mixture in a total volume 2.5 ml contained 360 μmoles of sodium phosphate (pH 7.0), 0.92 mμmole of hematin, 4.0 μmoles of ascorbate, 0.13 μmole of EDTA, 2.1 μg of enzyme with specific activity 14.5, 0.25 μmole of KCN, and the indicated tryptophan concentrations in the absence (○——○) and presence of 3.6×10^{-3} M α-methyltryptophan (●——●). The reaction was initiated by the addition of enzyme.

rather than to the free enzyme. Cyanide thus manifests certain of the properties of an "uncompetitive" inhibitor (Reiner, 1959). When the inhibition of catalytic activity was determined as a function of tryptophan concentration at a fixed intermediate concentration of cyanide, it was evident that the inhibitory efficacy of cyanide increased as the substrate level rose (Figure 4). Extrapolation of this curve to the vertical axis indicates an inhibition of approximately 15% at zero tryptophan concentration which rises to a plateau level of approximately 60% inhibition as the enzyme becomes saturated with its substrate. This again suggests that, relative to the free enzyme, an approximately fourfold higher affinity for cyanide occurs when the ferriprotoporphyrin of the enzyme exists as part of the enzyme–substrate complex.

Evidence has been previously presented suggesting the presence of at least two discrete binding sites for tryptophan on the enzyme, one catalytic and one allosteric. The latter, and not the former, also binds α-methyltryptophan which is neither a substrate nor an inhibitor of this enzyme (Feigelson and Maeno, 1967; Feigelson *et al.*, 1967; Maeno and Feigelson, 1968). It was of considerable interest therefore to determine whether the tryptophan augmentation of cyanide binding was a consequence of combination of tryptophan with the catalytic or the allosteric site of the enzyme. As shown in Figure 4, the inhibition by cyanide in the presence of α-methyltryptophan was maximal and entirely independent of the tryptophan concentration, in contrast to the behavior in the absence of the analog. It is evident that α-methyltryptophan enhances the reactivity of the ferriprotoporphyrin of the enzyme and inferentially that this is a consequence of its binding to the enzyme's allosteric site. Presumably then the binding of either tryptophan or α-methyltryptophan at this site modifies the environment of the ferriprotoporphyrin in such a manner as to increase its affinity for cyanide. Furthermore, this same mechanism seems to be operative for the binding of either cyanide to the ferriprotoporphyrin form or carbon monoxide and oxygen to the ferroprotoporphyrin form of the enzyme (Feigelson and Maeno, 1967; Maeno and Feigelson, 1968).

In an attempt to determine whether tryptophan resulted in gross structural alterations of the enzyme, sucrose gradient centrifugation and gel filtration was undertaken in the presence and absence of 5×10^{-3} M tryptophan. As previously reported (Feigelson *et al.*, 1967; Maeno *et al.*, 1967) the sedimentation and chromatography of the enzyme were uninfluenced by the presence of tryptophan, indicating that tryptophan induces no gross changes in the quatenary structure of *Pseudomonas* tryptophan oxygenase, as has already been reported for mammalian liver tryptophan oxygenase (Schimke *et al.*, 1965; Cho-Chung and Pitot, 1967). Accordingly, the enhancement of ligand reactivity by tryptophan and similar effectors is likely due to an as yet undetermined conformational alteration of the internal molecular structure of the enzyme.

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