Studies on the Catalytic and Allosteric Sites in Modulating the Reactivity of Tryptophan Oxygenase with Heme Ligands. II. Carbon Monoxide Derivatives*

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ABSTRACT: The ferroheme moiety of *Pseudomonas acidovorans* tryptophan oxygenase binds carbon monoxide. Two distinct effects could be seen which resulted in an enhanced affinity for this ligand. In one case the effect was derived solely from substrate binding at the catalytic site while in the other it was due to effector combination at the allosteric site. Evidences in support of an enhanced reactivity of the prosthetic ferroheme iron consequent to the binding of substrate at the catalytic site were obtained from combined spectral and kinetic studies on the interaction of ferrotryptophan oxygenase with carbon monoxide, in the presence and absence of the substrate, tryptophan, and or the competitive inhibitor, 5-fluorotryptophan. The affinity of ferroheme for carbon monoxide was profoundly increased by either tryptophan, which binds at both the catalytic and allosteric sites, or 5-fluorotryptophan, which binds exclusively at the catalytic site. The value of $n = 1.0$ obtained for the interaction coefficient from the Hill plot for 5-fluorotryptophan indicates a noninteracting binding site for 5-fluorotryptophan on the enzyme molecule. In the absence of tryptophan or 5-fluorotryptophan which combine with the catalytic site, the reactivity of the ferroheme iron for carbon monoxide was not affected by α-methyltryptophan, which binds exclusively to the enzyme's allosteric site. Hence, under these conditions, the affinity for carbon monoxide is modulated solely by interactions at the catalytic site. On the other hand, the sigmoidal saturation curve observed for the equilibrium formation of the ferrotryptophan oxygenase–carbon monoxy complex as a function of tryptophan concentration converted into a hyperbolic one in the presence of α-methyltryptophan. Thus, the ferroheme reactivity with carbon monoxide is evidently influenced both by the specific interaction of tryptophan at the catalytic site, and by the allosteric effector α-methyltryptophan. The equilibrium binding of carbon monoxide to ferrotryptophan oxygenase alone was found to be independent of pH in the range 6.0–8.0. In the presence of subsaturation levels of tryptophan the enhanced affinity of the enzyme for carbon monoxide was markedly pH dependent and reflected pH dependency of the K_M for tryptophan. This evidence suggests that tryptophan interaction at the catalytic site possesses considerable ionic character. A model is proposed in which kinetic behavior and the equilibrium binding of specific ligands by the heme moiety may be accommodated by two parameters: one operative solely through the catalytic site; the other linked to cooperative interaction between the catalytic and allosteric sites.

The allosteric properties of *Pseudomonas acidovorans* tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12) derived from the kinetics of the catalytic reaction have been documented previously (Feigelson and Maeno, 1967; Koike *et al.*, 1969). In the preceding paper (Koike and Feigelson, 1971) evidence was presented for a modulation of the reactivity of the enzyme ferriheme prosthetic group with the ligand cyanide solely by saturation of the catalytic site. It was found that either the substrate, tryptophan, or a competitive inhibitor, 5-fluorotryptophan, enhanced binding of cyanide to ferriheme–enzyme, while the allosteric effector, α-methyltryptophan, was without influence.

Spectral studies (Ishimura *et al.*, 1967; Maeno and Feigelson, 1968) have been made of the effects of tryptophan upon the reaction of the ferroheme form of the enzyme with the ligands oxygen and carbon monoxide. However, no distinction could be made from those studies between effects related exclusively to binding at the catalytic site from those related to binding at the allosteric site, since tryptophan combines at both sites. The present study documents the effects of tryptophan analogues with specificity for the catalytic and allosteric sites of tryptophan oxygenase upon its affinity for carbon monoxide. The results obtained in the preceding paper, as well as those presented here confirm the existence of two valence states for the heme prosthetic group, one of which (heme iron trivalent) is insensitive to the binding by the allosteric effector, α-methyltryptophan, while the other (heme iron divalent) is sensitized to this allosteric transformation. Parallel structural alterations have recently been detected by sedimentation velocity studies of the ferri- and ferro-enzyme in the presence or absence of various heme ligands and effectors (Poillon and Feigelson, 1971). The intramolecular processes by which tryptophan oxygenase function is regulated thus depends both on the valence state of the heme iron and the conformation of the protein.

Experimental Section

Spectral Examinations. Visible absorption spectra and measurements of equilibrium binding of carbon monoxide were obtained with a Cary Model 14 recording spectrophotometer. The absorption maxima at 432 or 421 mμ, and the isosbestic point at 427 mμ in the Soret region were used to

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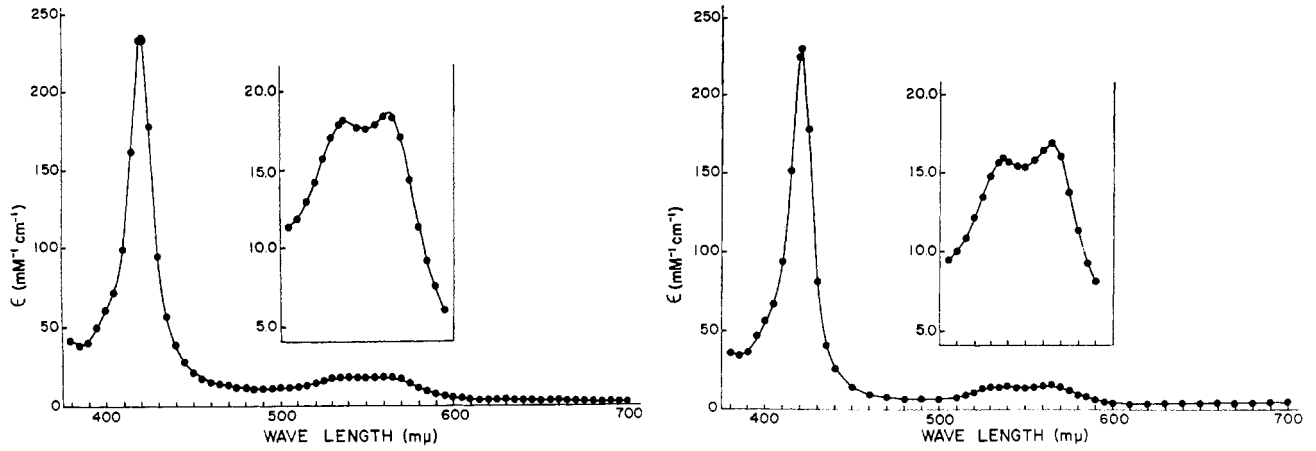

FIGURE 1: Optical spectra of ferrotryptophan oxygenase CO derivative in the presence of tryptophan or 5-fluorotryptophan. (a, left) Ferrotryptophan oxygenase was equilibrated with a gas mixture containing 40% CO and 60% N_2 in the presence of 3.0×10^{-4} M tryptophan in 0.1 M sodium phosphate buffer (pH 7.0) at 25° for 8 min. (b, right) Ferrotryptophan oxygenase was equilibrated with 50% CO and 50% N_2 in the presence of 5.0×10^{-3} M 5-fluorotryptophan in 0.1 M sodium phosphate buffer (pH 7.0) at 25° for 8 min.

measure the extent of equilibrium binding according to the equation, x% carbon monoxide derivative = [((A432) 0% − (A432) x%)/((A432) 0% − (A432) 100%)] × 100 or [((A421) 0% − (A421) x%)/((A421) 0% − (A421) 100%)] × 100; (A) 0%, (A) 100%, and (A) x% are measured optical densities of 0, 100, and x% carbon monoxide derivatives, respectively at 432 or 421 mμ. Sodium phosphate buffer (0.1 M, pH 7.0) was used throughout. Protein concentration was estimated from the millimolar extinction coefficients of the Soret and ultraviolet absorption peaks at 405 and 280 mμ (229 and 146 mM^{-1} cm^{-1}, respectively).

Purification and Activity Assay. Ferritryptophan oxygenase was purified to homogeneity from tryptophan-induced *Pseudomonas acidovorans* (ATCC 11299b) (Poillon *et al.*, 1969) and was exhaustively dialyzed at 4° against 0.1 M sodium phosphate buffer (pH 7.0) to remove exogenous tryptophan used for enzyme stabilization during purification. Ferrotryptophan oxygenase was prepared from the ferri form by the addition of a few crystals of solid sodium dithionite.

Kinetic experiments were performed at 25° in a 2.5-ml standard assay system containing 2.0 μg of tryptophan oxygenase (specific activity 16 EU/mg), 250 μmoles of sodium phosphate (pH 7.0), 1.3 μmoles of EDTA, and 1.0 μmole of sodium ascorbate; the appropriate concentrations of tryptophan and effectors are indicated in the legends to the individual figures. Enzymatic activity was measured by continuous recording of formylkynurenine formation at 321 mμ (Koike *et al.*, 1969). Other experimental details are described in the appropriate legends.

Chemicals. L-Tryptophan and 5-fluorotryptophan were obtained from Nutritional Biochemicals Co. α-Methyl-DL-tryptophan was a product of Regis Chemical Co.; Carbon monoxide was purchased from the Matheson Co.

Results

Optical Spectra of Ferrotryptophan Oxygenase Carbon Monoxide Derivative in the Presence of Tryptophan or 5-Fluorotryptophan. Ferrotryptophan oxygenase was prepared by sodium dithionite reduction and its spectrum exhibited the characteristic Soret absorption maximum at 432 mμ and peaks of weaker intensity at 554 and 588 mμ in the visible region (Ishimura *et al.*, 1967; Poillon *et al.*, 1969). The optical spectrum of the carbon monoxide derivative of the ferro-enzyme in the Soret and visible regions was, as has been previously reported, only about 70% complete, even at 100% carbon monoxide saturation (Maeno and Feigelson, 1968). The calculated dissociation constant, K_D, of about 4.0×10^{-4} M, indicates that ferrotryptophan oxygenase has much lower affinity for carbon monoxide than does horseradish peroxidase (Keilin and Hartree, 1951). In Figure 1 are shown the absorption spectra for the carbon monoxy-enzyme complex in the presence of 3.0×10^{-4} M tryptophan (part a) and in the presence of 5.0×10^{-3} M 5-fluorotryptophan (part b). Complex formation was complete at 40 and 50% carbon monoxide saturation, respectively, and the characteristic absorption bands in the Soret and visible regions, were well resolved. In the presence of either tryptophan or 5-fluorotryptophan, the Soret absorption peak occurred at 420–421 mμ, and the weaker α and β bands at 563–565 and 537 mμ, respectively. It has been shown by others (Peisach *et al.*, 1968; Blumberg *et al.*, 1968) that such optical spectra correlate with electron paramagnetic resonance spectra (actually a lack of signal as compared to $d^5{}_{/2}$ or $d^5{}_{/2}$) and can be ascribed to ferroheme iron predominantly in the low-spin electronic configuration *i. e.*, $d^6{}_0$). In such cases the α band is always of slightly greater intensity than the β band (see Figure 1).

The Effect of Tryptophan on the Ferrotryptophan Oxygenase–Carbon Monoxide Equilibria in the Presence and Absence of Allosteric Effector. In Figure 2a is shown the equilibrium formation of the ferro–enzyme–carbon monoxy complex alone and in the presence of two different concentrations of tryptophan. In the absence of tryptophan, carbon monoxide complex formation was a hyperbolic function of the carbon monoxide concentration and was only 70% complete when in equilibrium with an atmosphere of 100% carbon monoxide (Figure 2a, lower curve). In the presence of 1.0×10^{-4} M tryptophan (Figure 2a, middle curve), and 3.0×10^{-4} M tryptophan (Figure 2a upper curve) derivative formation was complete at progressively lower carbon monoxide levels as has been previously reported (Ishimura *et al.*, 1967; Maeno and Feigelson, 1968). In this respect, these results are reminiscent of those for the binding of cyanide to the ferri-enzyme, *i.e.*, tryptophan enhances affinity for the heme ligand. In each instance, the carbon monoxide saturation curve was hyper-

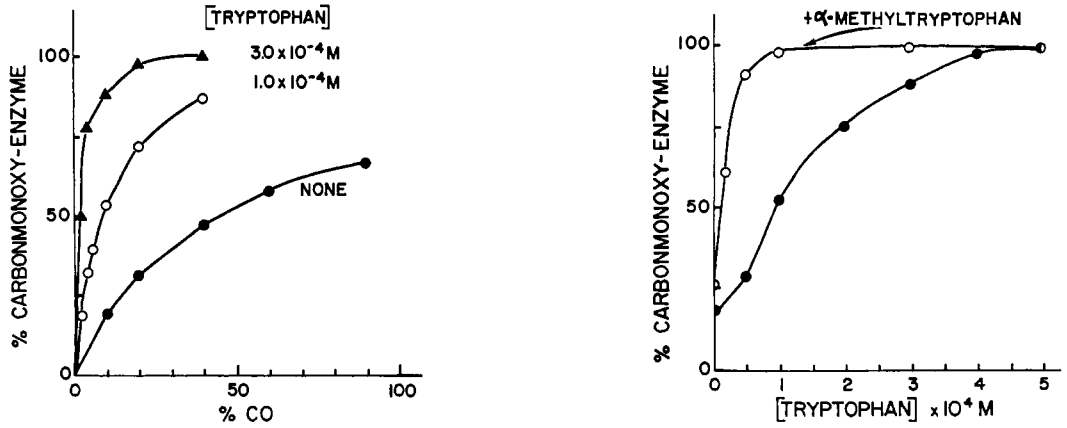

FIGURE 2: The effects of tryptophan and α-methyltryptophan upon the equilibrium formation of the carbon monoxy complex of tryptophan oxygenase. (a, left) Ferrotryptophan oxygenase (0.29 μM) was equilibrated with N_2–CO gas mixtures containing the indicated % (v/v) of CO in the absence (●) or presence of two different concentrations of tryptophan: 1.0×10^{-4} M (○) and 3.0×10^{-4} M (▲). All measurements were made in 0.1 M sodium phosphate buffer (pH 7.0) at 25° and all spectra were taken after 8-min equilibration. (b, right) Ferrotryptophan oxygenase (0.25 μM) in 0.1 M sodium phosphate buffer (pH 7.0) containing the indicated concentrations of tryptophan in the absence (●) or presence (○) of 1×10^{-3} M α-methyltryptophan were equibrated with 10% CO–90% N_2 (v/v) and absorption spectra taken.

bolic and when the data of Figure 2a were plotted according to the Hill equation (Hill, 1910, 1936), the interaction coefficient, n, for carbon monoxide binding to the ferro–enzyme is always 1.0.

In Figure 2b is shown the extent of complex formation as a function of tryptophan concentration in the presence and absence of 1.0×10^{-3} M α-methyltryptophan, at 10% carbon monoxide saturation. It is evident that in the latter case, the carbon monoxy–ferro–enzyme complex formation was a sigmoidal function of tryptophan concentration, while in the former case it was a hyperbolic one with a profound shift of the saturation curve to the left. Thus α-methyltryptophan clearly eliminates the sigmoidicity with respect to tryptophan saturation, and at low levels of tryptophan, enhances the affinity of the ferro–enzyme for carbon monoxide. In the absence of tryptophan, α-methyltryptophan is essentially without effect. These findings are reminiscent of similar effects of α-methyltryptophan upon the rate of the catalytic formation of formylkynurenine as a function of tryptophan concentration (Feigelson and Maeno, 1967). These results, however, are in direct contrast to those for the binding of cyanide to the ferri–enzyme, where α-methyltryptophan exerted no cooperative effect under analogous conditions. It should be recalled that tryptophan binds at the catalytic and allosteric sites, while α-methyltryptophan preferentially occupies the allosteric site. The parameter determining these phenomena seems to be the valence state of the heme iron, *i.e.*, when trivalent, the influence of the catalytic site on the heme reactivity with ligands is insensitive to occupancy of the allosteric site while when divalent, heme reactivity is linked to the allosteric transition evoked by α-methyltryptophan or tryptophan.

Competitive Inhibition by 5-Fluorotryptophan and the Allosteric Effect of α-Methyltryptophan on the Catalytic Activity. As previously reported (Feigelson and Maeno, 1967), when enzymatic activity is measured as a function of tryptophan concentration, an S-shaped saturation curve is observed, indicating the existence of two or more interacting tryptophan binding sites on the enzyme. The addition of α-methyltryptophan results in the conversion of the nonhyperbolic tryptophan saturation curve into a hyperbolic one which can be treated according to classical Michaelis–Menten kinetics.

In Figure 3 are shown kinetic data for tryptophan oxygenase activity in the presence of the inhibitor 5-fluorotryptophan and the allosteric effector, α-methyltryptophan. The nonhyperbolic nature of the plot of initial reaction rate as a function of tryptophan concentration remains even in the presence of 5×10^{-3} M 5-fluorotryptophan (Figure 3a) but is converted into a hyperbolic one in the presence of 1.6×10^{-3} M α-methyltryptophan (Figure 3b). For clarity of presentation these data are shown as double-reciprocal plots. It can be seen that the double-reciprocal plots were nonlinear in the presence and absence of 5-fluorotryptophan and that the inhibition could be overcome by increasing tryptophan concentration. By saturating the allosteric site with α-methyltryptophan, the linear double-reciprocal plots shown in Figure 3b were obtained. The intersection at the same point on the y axis, in either the absence or the presence of 1.6×10^{-3} M α-methyltryptophan, indicates that 5-fluorotryptophan competes with tryptophan for the catalytic site. As 5-fluorotryptophan was a potent competitive inhibitor and gave a hyperbolic inhibition curve, the apparent order of the reaction was determined with respect to 5-fluorotryptophan by use of the Hill equation. The equation applied was $\log [\alpha/(1 - \alpha)] = \log K_D - n \log$ (5-fluorotryptophan), where, α = the degree of inhibition, n = interaction coefficient, and K_D = dissociation constant. As shown in Figure 3c, the plot of $\log [(1 - \alpha)/\alpha]$ *vs.* $-\log$ (5-fluorotryptophan), was a straight line for which the slope, n, was 1.0 even in the absence of α-methyltryptophan. From Figure 3c, the dissociation constant, K_D, for combination of 5-fluoro-DL-tryptophan with the enzyme in the presence of 2.4×10^{-4} M L-tryptophan was calculated to be 2.0×10^{-3} M. The addition of 1.6×10^{-3} M α-methyltryptophan did not change the slope of the Hill plot (n = 1.0), thus indicating that 5-fluorotryptophan binds only at a noninteracting catalytic site and indicates the absence of competition between 5-fluorotryptophan and α-methyltryptophan for the allosteric site.

Effect of pH on Ferrotryptophan Oxygenase–Carbon Monoxy Complex Formation in the Presence or Absence of Tryptophan. The equilibrium formation of the carbon monoxide derivative of ferrotryptophan oxygenase was studied as a function of pH on the presence and absence of tryptophan. As shown in Figure 4, in the absence of tryptophan, the

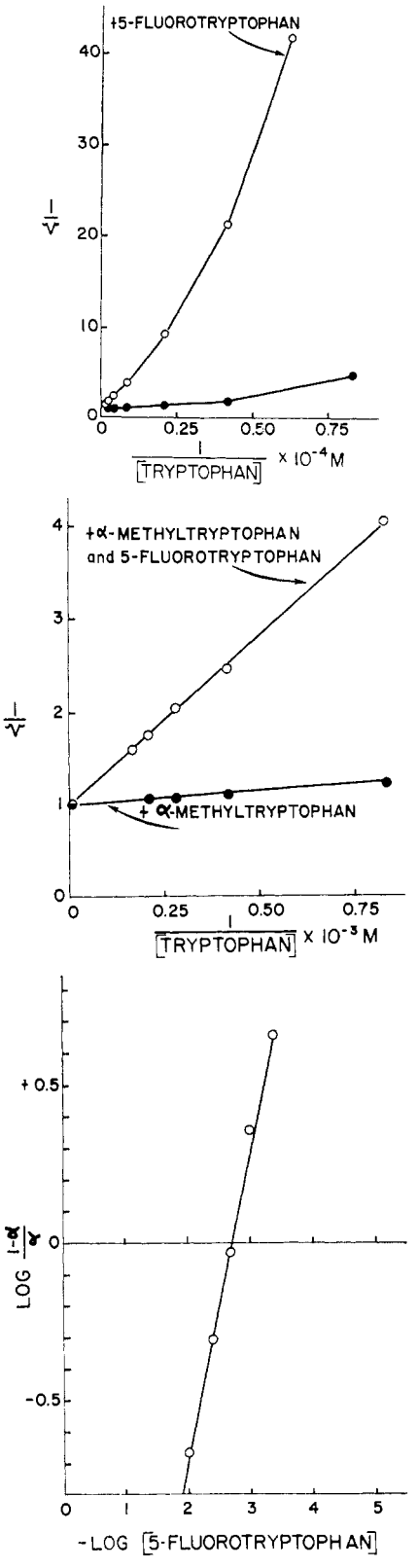

FIGURE 3: Competitive inhibition of tryptophan oxygenase by 5-fluorotryptophan and the allosteric effect or α-methyltryptophan on the enzymatic activity. (a, top) Activity measurements were carried out in the 2.5-ml standard assay system as a function of tryptophan concentration in the presence (○) or absence (●) of 5.0×10^{-3} M 5-fluorotryptophan. (b, middle) Activity measurements at the indicated tryptophan levels were carried out in the 2.5-ml standard assay system supplemented with 1.6×10^{-3} M α-methyltryptophan, in the presence (○) or absence (●) of 5.0×10^{-3} M 5-fluorotryptophan. (c, bottom) A plot of log $[(1 - \alpha)/\alpha]$ *vs.* − log [5-fluorotryptophan] obtained from activity measurements carried out in the 2.5-ml standard assay system modified to contain 2.4×10^{-4} M tryptophan and the indicated molar concentrations of 5-fluorotryptophan (α = the fractional inhibition).

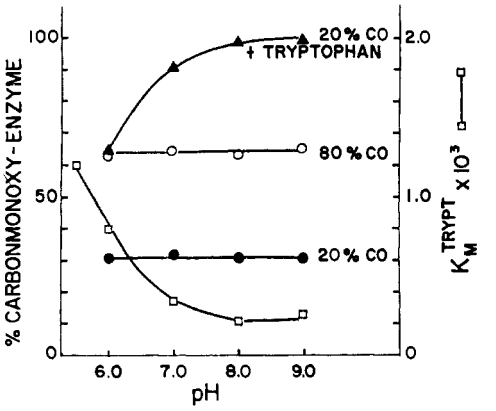

FIGURE 4: Effects of pH on the formation of carbon monoxy derivative of ferrotryptophan oxygenase in the presence or absence of tryptophan and upon the K_M^{Trp}. The Michaelis–Menten constant for tryptophan, K_M^{Trp} (□), was determined by measuring enzyme activities in the standard assay system, as a function of tryptophan concentration, at pH 5.5 in 0.1 M sodium acetate buffer, at pH 6.0–8.0 in 0.1 M sodium phosphate buffer, and at pH 9.0 in 0.1 M sodium glycoolate buffer. For determination of the extent of carbon monoxy–ferrotryptophan oxygenase formation 0.33 μM of reduced enzyme were, in the appropriate buffer, equilibrated with 80% CO–20% N_2 (○) or with 20% CO–80% N_2 in the absence (●) or presence of 1.5×10^{-4} M tryptophan (▲).

equilibrium formation of the carbon monoxy–ferrotryptophan oxygenase complex, in equilibrium with either 20 or 80% CO, was independent of pH variation between 6.0 and 9.0. However, in the presence of 1.5×10^{-4} M tryptophan, at 20% carbon monoxide the extent of ferro–enzyme–carbon monoxy complex formation was markedly dependent on pH (Figure 4, uppermost curve). At each pH value the K_M for tryptophan was also evaluated by measurements of catalytic activity; as depicted in Figure 4, the K_M decreased as the pH was raised. It is evident, therefore, that at the concentration of tryptophan used in this study (1.5×10^{-4} M), which is insufficient to saturate the enzyme, the proportion of the ferro–enzyme saturated with tryptophan increased as the pH was raised and that there was a corresponding increased formation of the carbonmonoxyferro–enzyme complex. These findings indicate that the increased saturation of the ferro–enzyme by tryptophan, evoked by an elevated pH, results in an augmented binding of CO by the heme. Parenthetically, it should be noted that between pH 6.0 and 9.0 the Hill coefficient, n, was shown to remain 1.0 for CO.

Effect of 5-Fluorotryptophan on the Ferrotryptophan Oxygenase–Carbon Monoxy Complex Formation. As tryptophan combines with both the allosteric and catalytic sites of tryptophan oxygenase, studies were undertaken to directly determine whether a compound which binds solely at the catalytic site would influence the affinity of ferrotryptophan oxygenase for carbon monoxide. 5-Fluorotryptophan is ideally suited for this purpose since it has been shown to be a competitive inhibitor which binds exclusively at the catalytic site of the tryptophan oxygenase molecule (Figure 3). The effect on the equilibrium binding of carbon monoxide was therefore studied as a function of 5-fluorotryptophan concentration at 10% carbon monoxide. The resulting series of optical spectra indicated a progressive increase in the proportion of the enzyme as ferro–enzyme–carbon monoxy complex as the 5-fluorotryptophan concentration was elevated. The fraction of carbon monoxy–enzyme complex formed at each concentration of 5-fluorotryptophan was calculated and the data were plotted

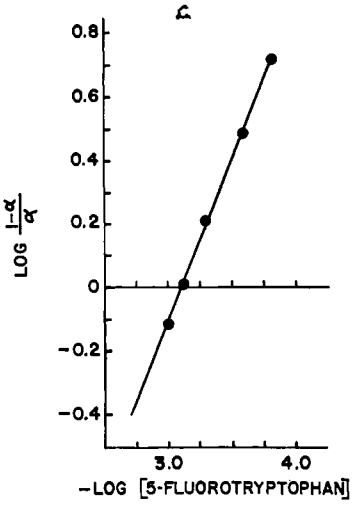

FIGURE 5: Effect of 5-fluorotryptophan on carbon monoxy ferrotryptophan oxygenase complex formation. A plot of log [(1 − α)/α] *vs.* − log [5-fluorotryptophan] was obtained from spectral experiments in which 0.29 μM ferrotryptophan oxygenase in equilibrium with 10% CO and 90% N_2 was incubated in 0.1 M sodium phosphate buffer (pH 7.0) at 25° for 8 min with the indicated molar concentrations of 5-fluorotryptophan. α = The fraction of ferrotryptophan oxygenase liganded by carbon monoxide.

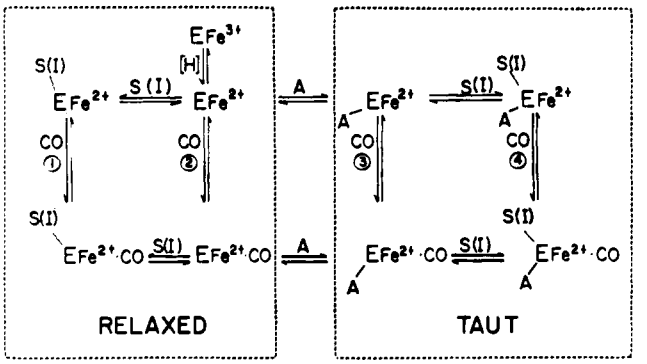

FIGURE 6: Interactions between carbon monoxide and substrate and/or effector-saturated ferrotryptophan oxygenase.

and analyzed using the Hill equation. The plot of log [(1 − α)/α] *vs.* −log (5-fluorotryptophan) obtained (Figure 5) was linear with slope n = 1.0 confirming the presence of either a single site or more than one noninteracting binding sites for 5-fluorotryptophan on the ferro–enzyme molecule which when occupied enhanced the affinity of the ferrotryptophan oxygenase for carbon monoxide.

Discussion

Tryptophan oxygenase is a special allosteric protein in that one molecule, tryptophan, may bind at two topographically and functionally distinct sites on the enzyme. One, the catalytic site(s), the other, a catalytically inactive allosteric site(s) (Feigelson, 1969). Furthermore, a cooperative interaction exists between these two distinct binding sites which results in an enhanced affinity for the substrate oxygen when the allosteric site is occupied by either tryptophan or its analog, α-methyltryptophan (Maeno and Feigelson, 1968). These two different functional sites may be considered "linked" in the sense defined by Wyman (1948, 1964). A well-known example of such a "linked function" is the Bohr effect in which the dissociation of certain side-chain protons of hemoglobin are inextricably linked to the sequential oxygenation of its four heme groups.

A principle aim of the studies described in this paper was to ascertain the degree to which the binding of carbon monoxide to ferrotryptophan oxygenase was regulated by interactions at the catalytic site(s) alone as was the case for the binding of cyanide to the ferri-enzyme (Koike and Feigelson, 1971), or by interactions at the allosteric site(s) alone or whether each of these sites played separate roles in modulating the affinity of the ferroheme moiety of the enzyme for carbon monoxide. Early experiments indicated that binding of tryptophan to the enzyme increased its affinity for carbon monoxide (Ishimura *et al.*, 1967; Maeno and Feigelson, 1968). As tryptophan serves both as substrate, *i.e.*, saturates a catalytic site, and as effector, *i.e.*, saturates a catalytically inactive allosteric site, the use of tryptophan alone could not distinguish between effects upon these distinct sites. The availability however, of two tryptophan analogs each capable of specifically saturating either the substrate or allosteric site(s) provided an experimental probe to investigate the role of these portions of the enzyme in modulating the affinity of ferrotryptophan oxygenase for the ligand carbon monoxide. 5-Fluorotryptophan is an inhibitor of tryptophan oxygenase activity which is competative with the substrate, tryptophan, and not competitive with the effector, α-methyltryptophan. The latter compound, on the other hand, at the concentrations herein employed, does not inhibit tryptophan oxygenase, but on the contrary increases its catalytic activity by converting an S-shaped tryptophan concentration *vs.* velocity curve to a hyperbolic one and lowers the $K_M^{O_2}$ (Feigelson and Maeno, 1967). Thus, 5-fluorotryptophan and α-methyltryptophan, respectively, bind to the catalytic and allosteric sites of tryptophan oxygenase.

In Figure 6 is depicted a schematic representation of the various species encountered during the equilibrium binding of carbon monoxide to reductively generated ferrotryptophan oxygenase in the presence or absence of agents which saturate the catalytic and allosteric enzymic sites. This simplified formulation which makes no assumptions concerning the stoichiometry of substrate and allosteric sites includes the so-called "relaxed," effector-free, and "taut," effector-saturated, conformational isomers of an allosteric protein (Monod *et al.*, 1965; Koike *et al.*, 1969). The studies herein presented indicate that progressive saturation of the catalytic site by either tryptophan (S) or the competitive inhibitor 5-fluorotryptophan (I) results in augmented affinity of the ferroheme moiety of tryptophan oxygenase for carbon monoxide, 1 > 2. Furthermore, in the presence of subsaturation levels of tryptophan, saturation of the allosteric site(s) by α-methyltryptophan (A) leads to marked enhancement in the affinity of carbon monoxide for the ferro–enzyme 4 $\gg$ 2. Thus, in addition to enhanced reactivity of the prosthetic ferroheme with carbon monoxide consequent to saturation of the catalytic site, a synergistic effect linked to the allosteric isomerization is also operative. In view of the absence of an allosteric effect upon the affinity of the ferriheme form of tryptophan oxygenase for cyanide (Koike and Feigelson, 1971) whereas the ferroheme tryptophan oxygenase does manifest a synergistically enhanced affinity for carbon monoxide upon saturation of its allosteric site(s) one may conclude that certain allosteric transitions are dependent on the valence state of the heme prosthetic group.

These conclusions are supported by earlier observations

that only with the ferroheme enzyme, and not with the ferriheme enzyme, can saturation of the allosteric site(s) evoke a conformational change reflected as an elevated $s_{20,w}$ (Poillon and Feigelson, 1971). Thus, presumably it is this allosterically induced taut configuration of the ferroheme tryptophan oxygenase which manifests enhanced affinity for the artificial ligand, carbon monoxide, and for its normal substrate–ligand, oxygen.

Acknowledgments

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References

Blumberg, W. E., Peisach, J., Wittenberg, B. A., and Wittenberg, J. B. (1968), *J. Biol. Chem. 243*, 1854.

Feigelson, P. (1969), *Advan. Enzyme Reg. 7*, 119.

Feigelson, P., and Maeno, H. (1967), *Biochem. Biophys. Res. Commun. 28*, 289.

Hill, A. V. (1910), *J. Physiol. (London) 40*, 4P.

Hill, R. (1936), *Proc. Roy. Soc., Ser. B 120*, 427.

Ishimura, Y., Nozaki, M., Hayashi, O., Tamura, M., and Yamazaki, I. (1967), *J. Biol. Chem. 242*, 2574.

Keilin, D., and Hartree, E. F. (1951), *Biochem. J. 61*, 153.

Koike, K., and Feigelson, P. (1971), *Biochemistry 10*, 3378.

Koike, K., Poillon, W. N., and Feigelson, P. (1969), *J. Biol. Chem. 244*, 3457.

Maeno, H., and Feigelson, P. (1968), *J. Biol. Chem. 243*, 301.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Peisach, J., Blumberg, W. E., Wittenberg, B. A., and Wittenberg, J. B. (1968), *J. Biol. Chem. 243*, 1871.

Poillon, W. N., and Feigelson, P. (1971), *Biochemistry 10*, 753.

Poillon, W. N., Maeno, H., Koike, K., and Feigelson, P. (1969), *J. Biol. Chem. 244*, 3447.

Wyman, J. (1948), *Advan. Protein Chem. 4*, 410.

Wyman, J. (1964), *Advan. Protein Chem. 19*, 223.

Aspartate Transcarbamylases of *Citrobacter freundii**

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ABSTRACT: Two aspartate transcarbamylases (EC 2.1.3.2) which have different molecular weights can be separated from each other when an extract of *Citrobacter freundii* ATCC 8090 is subjected to gel filtration chromatography. Both enzymes have very similar, but not identical, kinetic characteristics and are inhibited by most nucleotides and by inorganic ortho- and pyrophosphate. They differ, however, in their response to ATP. ATP activates the large aspartate transcarbamylase but has little effect on, or is an inhibitor of, the small aspartate transcarbamylase when aspartate concentrations are below saturation. The larger enzyme can be converted to the smaller enzyme *in vitro*, suggesting that they possess a common subunit. The proportion of the two enzymes *in vivo* is not constant but varies such that the larger enzyme increases during logarithmic growth until it is the sole enzyme present in stationary phase of culture.

Aspartate transcarbamylases (ATCase)[1] derived from various bacterial species can be grouped into three distinct classes as a result of both the kinetic and gel filtration characteristics of the particular enzyme (Neumann and Jones, 1964; Bethell and Jones, 1969). Bethell and Jones (1969) found that most bacteria studied contained a single ATCase; however crude extracts of *Citrobacter freundii* ATCC 8090 and *Proteus vulgaris* ATCC 8427 contained both a class B (mol wt 300,000; ATCase activity that is inhibited by CTP) and a class C enzyme (mol wt 100,000; ATCase activity that is not affected by CTP) in nearly equal amounts. The latter author's preliminary study of the kinetic characteristics of the two *Citrobacter* enzymes showed that CTP inhibited only the class B ATCase at pH 7.0 which suggested that of these two ATCases only the larger one was under "feedback control."

At least two different possibilities would explain why *Citrobacter* should simultaneously possess significant amounts of two ATCases. It was possible that the two *Citrobacter* enzymes were chemically and physiologically distinct. In this case the enzymes would not share a common structural gene or a common polypeptide chain. A second possibility would be that the smaller *Citrobacter* ATCase contained a polypeptide(s)

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[1] Abbreviations used are: carbamyl phosphate:L-aspartate carbamyltransferase (EC 2.1.3.2), ATCcase; carbamyl-L-aspartate, CAA; carbamyl phosphate, CAP; *p*-chloromercuribenzoate, *p*CMB.