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Kinetic Evidence Indicating the Absence during Catalysis of an Unbound Ferroprotoporphyrin Form of Tryptophan Oxygenase*

Henry Jay Forman and Philip Feigelson†

ABSTRACT: A kinetic study of the mechanism of catalysis of tryptophan oxygenase using carbon monoxide as an inhibitor is presented. The study was carried out with the aid of α-methyltryptophan to saturate the allosteric site thereby allowing formulation of simplified rate equations and the generation of linear reciprocal kinetic plots. Various proposed mechanisms are evaluated; the data are incompatible with the existence during the steady-state reaction of any tryptophan-free form of ferrotryptophan oxygenase which is capable of binding either oxygen or carbon monoxide. The mechanism which is compatible with all the data involves an ordered addition of tryptophan to the enzyme followed by oxygen. As oxygen will not bind to trivalent heme, one possible explanation for this requirement is the reduction, during catalysis, of the enzyme-bound ferriprotoporphyrin IX moiety by tryptophan.

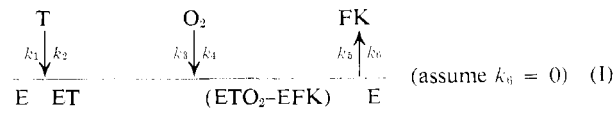
Tryptophan 2,3-dioxygenase (EC 1.13.1.12), a heme protein, catalyzes the reaction of the pyrrole moiety of tryptophan with oxygen resulting in the formation of formylkynurenine.

Maeno and Feigelson (1967) and Hayaishi (1969) have carried out studies which show that, in the absence of tryptophan, the trivalent and divalent forms of heme in tryptophan oxygenase are, respectively, unaffected and slowly autooxidized by oxygen. When dithionite reduction of the ferriheme moiety of the enzyme is performed the ferrotryptophan oxygenase obtained is capable of binding carbon monoxide in the absence of tryptophan. All trivalent forms of ferritryptophan oxygenase will not bind carbon monoxide. No form of the enzyme in its divalent state has been found as yet which will not bind CO. The valence state of the heme iron of the enzyme during various stages of catalysis and particularly the valence state to which it returns after releasing formylkynurenine before tryptophan is bound again has remained the most interesting and difficult problem concerning the catalytic mechanism of this dioxygenase. In this paper a new approach to the problem was applied which confirmed the order of binding of the substrates as tryptophan before oxygen, and supports the concept of fluctuation of the valence state of the heme iron during catalysis (as depicted in Figure 1A). The properties of enzyme which has been chemically reduced by dithionite were compared to those found for the enzyme during the steady state resulting in rejection of the former as a participating form in the catalytic cycle.

Kinetic Theory

In previous papers (Feigelson *et al.*, 1969; Koike *et al.*, 1969) the allosteric properties of tryptophan oxygenase were discussed. As shown in these papers, α-methyltryptophan reduces the complex behavior of the enzyme such that the simple mechanisms below need only be considered. The concentration of α-methyltryptophan herein employed (9.6×10^{-4} M) is such that it saturates the allosteric site and does not combine with the catalytic site. Thus, in these experiments considerations of allosterism are eliminated from further consideration.

Various mechanisms for the catalytic reaction of tryptophan oxygenase could be postulated without consideration of its valence state (eq I–III).



(assume $k_6 = 0$) (I)

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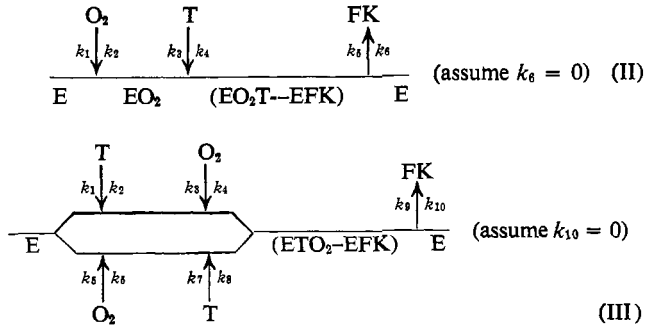

where E = tryptophan oxygenase, T = L-tryptophan, O_2 = oxygen, and FK = formylkynurenine.

The use of kinetics can distinguish among these mechanisms. In this experiment CO was used as a dead end inhibitor which by virtue of earlier studies including photochemical action spectra is known to be competitive with O_2 (Maeno and Feigelson, 1968). The variable substrate was L-tryptophan.

Rate equations can be derived for these alternative mechanisms which also encompass CO binding competitively with O_2 to the enzyme (King and Altman, 1956).

In mechanism I, v = observed velocity and V is maximal velocity.

$$v = \frac{k_5}{\frac{k_2(k_4+k_5)}{k_1k_3(T)(O_2)} + \left[\frac{k_4+k_5}{k_3(O_2)}\right]\left[1+\frac{(CO)}{K_{TCO}}\right] + \frac{k_5}{k_1(T)} + 1} \quad (1)$$

and if $K_T = k_5/k_1$, $K_{O_2} = (k_4 + k_5)/k_3$, $K_{iT} = k_2/k_1$, and $V = k_5$

$$v = \frac{V}{\frac{K_{iT}K_{O_2}}{(T)(O_2)} + \left[\frac{K_{O_2}}{(O_2)}\right]\left[1+\frac{(CO)}{K_{TCO}}\right] + \frac{K_T}{(T)} + 1} \quad (2)$$

Mechanism II:

$$v = \frac{k_5}{\left[\frac{k_2(k_4+k_5)}{k_1k_3(T)(O_2)} + \frac{k_5}{k_1(O_2)}\right]\left[1+\frac{(CO)}{K_{CO}}\right] + \frac{(k_4+k_5)}{k_3(T)} + 1} \quad (3)$$

and if $K_T = (k_4 + k_5)/k_3$, $K_{iT} = k_2(k_4 + k_5)/k_3k_5$, $K_{O_2} = k_5/k_1$, $K_{iO_2} = k_2/k_1$, and $V = k_5$

$$v = \frac{V}{\left[\frac{K_{iO_2}K_T}{(T)(O_2)} + \frac{K_{O_2}}{(O_2)}\right]\left[1+\frac{(CO)}{K_{CO}}\right] + \frac{K_T}{(T)} + 1} \quad (4)$$

Mechanism III involves a combination of the first and second mechanisms. If the assumptions of a rapid equilibrium mechanism and release of formylkynurenine as the rate-limiting step are made we arrive at

$$v = \frac{k_9}{\left[\frac{k_2k_4}{k_1k_3(T)(O_2)}\right]\left[1+\frac{(CO)}{K_{CO}}\right] + \frac{k_4}{k_3(O_2)}\left[1+\frac{(CO)}{K_{TCO}}\right] + \frac{k_2k_4k_5}{k_1k_3k_6(T)} + 1} \quad (5)$$

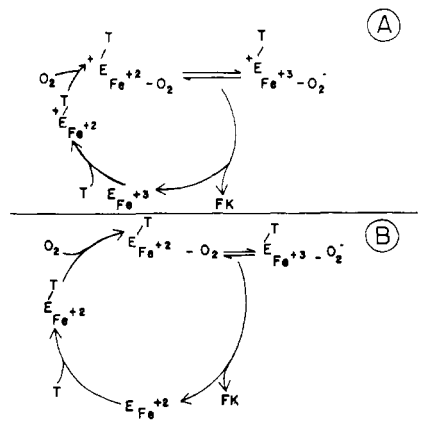

FIGURE 1: Two proposed mechanisms for tryptophan oxygenase.

Where K_{CO} and K_{TCO} are the binding constants for interaction of CO with enzyme and enzyme–tryptophan complex, respectively, and if $K_1 = k_2/k_1$, $K_2 = k_4/k_3$, $K_3 = k_6/k_5$, $V = k_9$

$$v = \frac{V}{\left[\frac{K_1K_2}{(T)(O_2)}\right]\left[1+\frac{(CO)}{K_{CO}}\right] + \frac{K_2}{(O_2)}\left[1+\frac{(CO)}{K_{TCO}}\right] + \frac{K_1K_2}{K_3(T)} + 1} \quad (6)$$

Methods and Materials

Tryptophan oxygenase activity was measured with continuous spectrophotometry in a Cary 14 recording spectrophotometer at 321 nm as described previously (Feigelson *et al.*, 1965). The specific activity was determined under standard assay conditions with formation of 1 μmole of formylkynurenine per min at 25° defined as one enzyme unit and protein concentration measured by a turbidometric method (Layne, 1957). The enzyme was purified to homogeneity by the method developed in this laboratory (Poillon *et al.*, 1969). α-Methyltryptophan was supplied by Regis Chemical Co. High-purity CO and N_2 were supplied by the Matheson Co. The other experimental details are described in the legend of Figure 2.

Results

Reciprocal plots of $1/v$ *vs.* 1/[Trp] in the absence of and at various concentrations of CO are shown to be parallel (Figure 2). This may also be described as an intercept effect with no slope effect indicating uncompetitive inhibition by CO with respect to tryptophan. These results may be compared to the previously derived rate equations.

Mechanism I: rearranging eq 2, we find for the reciprocal plot

$$\frac{1}{v} = \frac{1 + \frac{K_{O_2}}{(O_2)}\left[1+\frac{(CO)}{K_{TCO}}\right]}{V} + \frac{K_T + \frac{K_{iT}K_{O_2}}{(O_2)}}{V}\frac{1}{(T)} \quad (7)$$

For this sequential mechanism the plot of $1/v$ *vs.* 1/[T] the intercept depends on CO concentration, while the slope is independent of CO.

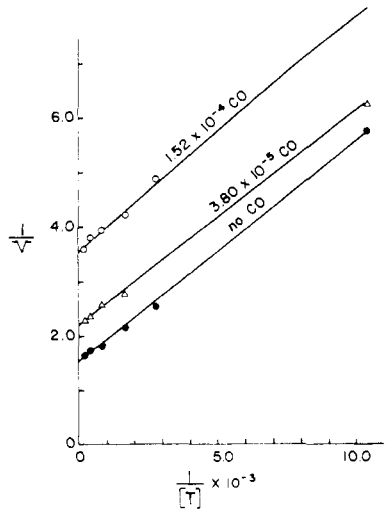

FIGURE 2: The inhibition of tryptophan oxygenase by carbon monoxide with tryptophan as variable substrate. The reaction mixture with a total volume of 2.5 ml in a sealed cuvet contained 4 μmoles of sodium ascorbate, 2.4 μmoles of DL-α-methyltryptophan, 1.3 μmoles of EDTA, 164 μmoles of sodium phosphate buffer (pH 7.0), 0.1 ml of tryptophan oxygenase (6.4 specific activity), and tryptophan in the various concentrations indicated. CO-saturated buffer was varied with N_2-saturated buffer to give a volume of 0.4 ml of the total 2.5 ml. In this way CO levels could be varied while maintaining a constant O_2 concentration. The velocity was measured as change in optical density at 321 mμ/4 min. The lines are drawn with a leasts-square fit. "t" tests were done to show that the lines were parallel within a 95% confidence interval.

$$\text{intercept} = \left[1 + \frac{K_{O_2}}{(O_2)}\left[1 + \frac{(CO)}{K_{TCO}}\right]\right]\Big/V \qquad (8a)$$

$$\text{slope} = \left[K_T + \frac{K_{iT}K_{O_2}}{(O_2)}\right]\Big/V \qquad (8b)$$

Mechanism II: rearranging eq 4, we find for the reciprocal plot

$$\frac{1}{v} = \frac{1 + \left[\frac{K_{O_2}}{(O_2)}\right]\left[1 + \frac{(CO)}{K_{CO}}\right]}{V} + \frac{\left[\frac{K_i(O_2)}{(O_2)}\left[1 + \frac{(CO)}{K_{CO}}\right] + 1\right]K_T}{V}\frac{1}{(T)} \qquad (9)$$

$$\text{intercept} = \left[1 + \frac{K_{O_2}}{(O_2)}\left[1 + \frac{(CO)}{K_{CO}}\right]\right]\Big/V \qquad (10a)$$

$$\text{slope} = \left[\left[\frac{K_i(O_2)}{(O_2)}\left[1 + \frac{(CO)}{K_{CO}}\right] + 1\right]K_T\right]\Big/V \qquad (10b)$$

Mechanism III: rearranging eq 6, we find for the reciprocal plot

$$\frac{1}{v} = \frac{1 + \frac{K_2}{O_2}\left[1 + \frac{(CO)}{K_{TCO}}\right]}{V} + \frac{\left[\frac{K_1K_2}{(O_2)}\left[1 + \frac{(CO)}{K_{CO}}\right] + \frac{K_1K_2}{K_3}\right]\frac{1}{(T)}}{V} \qquad (11)$$

$$\text{intercept} = \frac{1 + \frac{K_2}{(O_2)}\left[1 + \frac{CO}{K_{TCO}}\right]}{V} \qquad (12a)$$

$$\text{slope} = \frac{\left[\frac{K_1K_2}{(O_2)}\right]\left[1 + \frac{(CO)}{K_{CO}}\right] + \frac{K_1K_2}{K_3}}{V} \qquad (12b)$$

In mechanisms II and III both intercept and slope of the double-reciprocal plot, $1/v$ *vs.* $1/T$, are dependent on the CO concentration.

As can be seen in the double-reciprocal plot (Figure 2), the intercept but not the slope is dependent on the CO concentration. These results are compatible with mechanism I and incompatible with mechanism II or III. Furthermore, other alternative mechanisms, wherein CO binds when O_2 cannot, are all precluded as these would result in apparent noncompetitive inhibition by CO with respect to tryptophan which is contrary to the experimental observations.

The dissociation constant, K_{TCO}, found for interaction of CO with the enzyme–tryptophan complex is 1.8×10^{-5} M based on three experiments on three different preparations of enzyme. This value was calculated using eq 8a, where K_{O_2} was 3.8×10^{-5} M as previously obtained in this laboratory (Feigelson and Maeno, 1967), and O_2 and CO concentrations were calculated assuming similar solubilities in the assay mixture and water (Lange, 1956).

Discussion

Inhibition of tryptophan oxygenase by CO with O_2 as the variable substrate have shown previously that CO is competitive with O_2; furthermore, the photochemical action spectrum indicates that CO combines with the heme prosthetic group of the enzyme (Maeno and Feigelson, 1967). The present studies were done using CO as a dead-end inhibitor and tryptophan as the variable substrate. This use of kinetics has been described by Cleland (1963), and is useful in determining the order of binding of substrates where by virtue of unfavorable equilibrium constants this information cannot be obtained by product inhibition or isotope-exchange studies. The conclusion reached on the basis of the present findings is that the binding of tryptophan to the enzyme during catalysis is necessary before carbon monoxide, and hence, inferentially, oxygen can bind. A number of questions arise concerning the interpretation of earlier work wherein dithionite-reduced enzyme was employed to study the tryptophan oxygenase mechanism (Maeno and Feigelson, 1968; Ishimura *et al.*, 1967). Hayaishi's group showed that this form of the enzyme could bind CO and concentrations for half-maximal conversion into CO complex were at 5×10^{-6} and 2.8×10^{-4} M in the presence and absence of tryptophan, respectively. In our study the CO concentration for half-maximal conversion of the enzyme with tryptophan bound is approximately 1.8×10^{-5} M and is infinity for the tryptophan-free enzyme; *i.e.*, during catalysis CO cannot bind to the enzyme lacking tryptophan. We may consider two possible explanations for these divergent results (Figure 1). The first is that the valence state of the heme of the enzyme may cycle during the reaction, with tryptophan responsible for the reduction of the heme iron (Figure 1A).

The second possibility is that the heme of the enzyme is in the divalent state before tryptophan binds but that this form does not interact at detectable rates with carbon monoxide or

oxygen (Figure 1B). However, the existence during catalysis of a reduced form of the enzyme which in the absence of tryptophan reacts at significant rates with CO or O_2 is incompatible with the present evidence and may no longer be considered in proposing reaction mechanisms. Thus, the dithionite-treated enzyme, which has been shown to bind CO in the absence of tryptophan, does not seem to be a normal participant in catalysis. As no form of the divalent enzyme which cannot bind CO has yet been described, we therefore conclude, on the basis of existent evidence, that the second possibility is improbable (Figure 1B) and that oscillation in valence of the heme of tryptophan oxygenase probably occurs during the catalytic process (Figure 1A).

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Chemical and Immunological Characterization of a Unique Antigenic Region in Lysozyme*

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ABSTRACT: A peptide composed of the amino acid sequence 60–83, containing one intrachain disulfide bond (between half-cystine residues 64 and 80), and denoted "loop"-peptide, was obtained from hen egg-white lysozyme. The peptide was identified by amino acid analysis, determination of its amino- and carboxy-terminal residues, and the identification of the half-cystine residues which constitute the disulfide bond. Antibodies specific toward this region were prepared either by their isolation from the totality of anti-lysozyme antibodies with an immunoadsorbent containing the loop-peptide, or by immunization with a synthetic conjugate composed of multichain poly-DL-alanine to which the loop-peptide was attached (denoted loop-A—L). In the last case the anti-loop antibodies, namely, those recognizing the natural loop, were isolated on a lysozyme immunoadsorbent. The antibodies to the loop region, prepared according to these two approaches, showed restricted heterogeneity as compared to the totality of anti-lysozyme antibody population. This was manifested both in the acrylamide electrophoresis of their respective light chains and in the isoelectric focusing of the intact antibodies. The reactivity and specificity of the anti-loop antibodies, as compared to that of anti-lysozyme antibodies, was assessed both by their capacity to bind the labeled free loop-peptide as well as labeled intact lysozyme, and by their ability to inactivate modified bacteriophage, to which either the loop-peptide or lysozyme were chemically attached. In both these methods the anti-loop antibodies were indeed shown to react with the loop region of lysozyme exclusively and not with other parts of the molecule. Unfolding of the peptide chain of the loop-peptide, achieved by either reduction and alkylation or by performic acid oxidation, resulted in a drastic decrease in the reactivity with the specific antibodies.

These findings are indicative of the decisive role played by the spatial conformation in the antigenic specificity of this unique region in the lysozyme molecule.

Studies on the antigenic structure of hen egg-white lysozyme have been reported in recent years from several laboratories (*e.g.*, Shinka *et al.*, 1967; Imanishi *et al.*, 1969; Atassi and Habeeb, 1969; Habeeb and Atassi, 1969; Arnon, 1968; Bonavida *et al.*, 1969; Strosberg and Kanarek, 1970). In a previous communication from this laboratory (Arnon and Sela, 1969) we reported the preparation of antibodies with specificity directed toward a unique region, denoted loop-peptide,[1] in the lysozyme molecule. This peptide included the loop between half-cysteines-64 and -80. We describe now

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[1] Abbreviations used are: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; dansyl, 1-dimethylaminonaphthalene-5-sulfonyl; PFU, plaque-forming units; loop, amino acid sequence 60–83 of lysozyme; A—L, multichain poly-DL-alanyl—poly-L-lysine; loop-A—L, the loop conjugate of A—L; 1-CM-loop, loop-peptide carboxymethylated at cysteine residue 76. RCM-peptide, completely reduced and carboxymethylated peptide of the sequence 60–83 of lysozyme.