Allosteric Modulation of the Transient Kinetics of Carboxytryptophan Oxygenase Complex Formation after Laser Flash Photolysis

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SUMMARY

The effect of catalytic and allosteric site ligands upon the transient kinetics of formation of the carboxyenzyme complex of l-tryptophan-2,3-dioxygenase (EC 1.13.1.12) from Pseudomonas acidovorans has been studied by laser pulse, flash photolysis. Previous studies demonstrated that saturation of the catalytic and allosteric sites of the enzyme by l-tryptophan resulted in a 28-fold increase in the equilibrium constant for carboxyenzyme complex formation (MAENO, H., AND FEIGELSON, P. (1968) J. Biol. Chem. 243, 301-305). The present studies have shown that the predominant change brought about by l-tryptophan is an 18- to 20-fold increase in the rate constant for the "on" reaction, while the rate constant for the "off" reaction decreases only slightly.

With the use of the nonsubstrate analogues of l-tryptophan, 5-fluorotryptophan (specific ligand for the catalytic site) and a-methyltryptophan (ligand for the allosteric site), it was possible to determine separately the effects on \( k_{on} \) and \( k_{off} \) of saturation of the two sites. Saturation of the allosteric site alone with a-methyltryptophan does not affect \( K_{on} \) or \( k_{on} \), i.e., does not cause conversion of tryptophan oxygenase from the slow reacting species to the rapid reacting species. Saturation of the catalytic site alone with 5-fluorotryptophan does increase \( K_{on} \) and \( k_{on} \). At a nonsaturating level of 5-fluorotryptophan saturation of the allosteric site with a-methyltryptophan serves to increase the proportion of the enzyme present as the rapid reacting species. Thus, the consequences of the binding of l-tryptophan or a-methyltryptophan to the allosteric site of tryptophan oxygenase is to alter the enzyme increasing the affinity of its catalytic site for l-tryptophan or 5-fluorotryptophan, which in turn increases the proportion of the enzyme which reacts rapidly with CO.

Tryptophan oxygenases (l-tryptophan-2,3-dioxygenase, EC 1.13.1.12) of rat liver and Pseudomonas acidovorans are allosteric, tetrameric enzymes containing 2 moles of heme and 2 g atoms of copper per mole of enzyme (1). They catalyze the dioxygenase reaction

\[
l \text{-Tryptophan} + \text{O}_2 \rightarrow \text{N-formylkynurenine}
\]

The reaction is an Ordered Bi Uni kinetic process with l-tryptophan first binding to the enzyme to form the enzyme-tryptophan complex which then binds the second substrate, \( \text{O}_2 \), generating the enzyme-tryptophan-\( \text{O}_2 \) active complex which then forms formylkynurenine. Furthermore, although the velocity is a hyperbolic function of the \( \text{O}_2 \) concentration, a sigmoidal saturation behavior is manifested with respect to the l-tryptophan concentration.

The catalytic activity of tryptophan oxygenase is inhibited by carbon monoxide with the formation of the spectrophotometrically detectable, carboxyferroheme enzyme complex (2-6). This inhibition is kinetically competitive with respect to \( \text{O}_2 \) (3) and uncompetitive with respect to l-tryptophan (7). The equilibrium constant for this carboxyenzyme complex formation has been determined for the pseudomonad enzyme (3), and the consequences of saturation of the catalytic site is a 28-fold augmentation in the affinity of the ferroheme enzyme for CO (3, 4). As the enzyme has been purified to homogeneity from both sources (8, 9) in sufficient quantities for biophysical measurements, and as the carboxyferroheme enzyme complex has been shown to be photodissociable (3, 6) and has a characteristic absorption spectrum, it seemed possible to employ a laser (10) to flash photolyze the complex and directly measure the time course of the "on" reaction. Since the equilibrium constant is known, the rate constant for the "off" reaction could then be calculated directly. In this way, the effects of saturation of the catalytic or allosteric, or both, sites of tryptophan oxygenase on the rate constant for the formation of the carboxyferroheme enzyme complex could be directly observed and the effects on the dissociation of the carboxyenzyme complex calculated. This would enable the determination of the degree to which the
allosteric conformational change evoked by L-tryptophan was an attribute of augmented avidity of the enzyme-tryptophan complex for CO or due to increased stability of the carboxy-enzyme-tryptophan complex. Furthermore, as L-tryptophan binds to both the allosteric and catalytic sites of the enzyme, comparable studies employing tryptophan analogues which respectively bind exclusively to the catalytic site, 5-fluorotryptophan (4), and to the allosteric site, α-methyltryptophan (11), should elucidate the conformational and kinetic consequences of the respective saturation of these sites.

MATERIALS AND METHODS

Homogeneous tryptophan oxygenase from P. acidovorans (ATCC 11299b) was prepared as previously described (9) with some modifications (12). Enzymic assays (13) and protein determinations (14) were done as described.

All chemicals were reagent grade quality and were usually obtained from Fisher Scientific Co. L-Tryptophan was obtained from Ajinomoto Co., New York, New York. 5-Fluorotryptophan and sodium ascorbate were obtained from Sigma Co. DL-α-Methyltryptophan was obtained from Regis Chemical Co., Chicago, Ill.

Tryptophan oxygenase was purified in the presence of L-tryptophan to stabilize the enzyme. For these experiments, purified tryptophan oxygenase was freed of L-tryptophan by passage of 1 to 3 ml of the enzyme through a Sephadex G-25 fine column (0.9 × 40 cm), equilibrated with 0.1 M potassium phosphate, pH 7.0. The enzyme solution thus obtained was concentrated to approximately 10 μM in enzyme heme. The enzyme was reduced to the ferroheme(Cu+)2 form with dithionite prior to utilization (12, 15) with a concomitant removal of residual oxygen. To an aliquot of the reduced enzyme in a 5-mm cuvette was added L-tryptophan or the appropriate tryptophan analogues; the mixture was saturated with gaseous 100% CO by bubbling and sealed; and after temperature equilibration it was subjected to flash photolysis. Further details are in the legend of each figure.

The Soret peak of the ferroheme(Cu+)2 form of the enzyme is 432 nm and that of its carboxy complex is 421 nm. Carbon monoxide recombination kinetic data were obtained by monitoring transmission changes of the sample following photolysis of the carboxyferroheme enzyme complex by an intense monochromatic laser pulse of light. The spectrophotometric monitoring system consisted of light from a tungsten iodide lamp which traversed the 5-mm sample cuvette perpendicular to the laser light path and passed through a 436 ± 3 nm interference filter and a type 47 Wratten guard filter to a photomultiplier which was coupled to an amplifier and oscilloscope. The oscilloscope was triggered at the same instant as the laser flash (10). The oscilloscope traces recording the optical changes corresponding to the reformation of the carboxy-enzyme complex were recorded photographically. A cylindrical flashlamp pumped rhodamine 6-G-ethanol liquid dye laser operating in the Gaussian mode at 580 ± 5 nm with a 1-μs, 600-mJ output was used as the photolysis source. The center portion of the Gaussian laser pulse was allowed to impinge upon one side of the cuvette, and the enzyme concentration was adjusted so that a relatively uniform and significant fraction of the enzyme-CO complex underwent photolytic dissociation. The degree of photolytic dissociation obtained in these experiments ranged from 26 to 57%.

The photographed oscilloscope traces were digitized with the use of a television scanner-record digitizer (16), and the experimental data were then fitted by computer to a sum of two exponentials in order to obtain the rate constants and initial absorbance changes. All experiments were conducted under pseudo-first order conditions with carbon monoxide concentration always in considerable excess of the enzyme concentration. The sum of exponentials was taken as

$$\Delta A = \Delta A_1 e^{-\gamma_1 t} + \Delta A_2 e^{-\gamma_2 t}$$

where ΔA is the total change in absorbance, ΔA1 and ΔA2 are the initial change in absorbance and the pseudo-first order rate constant for the enzymic species which rapidly combines with CO (rapid phase); ΔA1 and λ1 are the initial change in absorbance and the pseudo-first order rate constant for the enzymic species which slowly combines with CO (slow phase); and t is time. λ1 was determined from the data in Fig. 1 for the slow phase (-Trp), where in the absence of L-tryptophan all the enzyme exists in the form which has the slow combination rate with CO. The λ1 thus calculated was imposed on the above equation. ΔA, ΔA2, and λ1 were varied continuously until the best computer fit of the experimental data was obtained.

RESULTS

L-Tryptophan is both a substrate and a positive allosteric effector of tryptophan oxygenase. The consequence of saturation of tryptophan oxygenase by L-tryptophan is a decrease in Km for the other substrate, O2 (11). Similarly, a large increase (28-fold) is seen in the equilibrium constant (Kes) for the formation of the carboxytryptophan oxygenase complex when the enzyme is saturated with L-tryptophan (3). Fig. 1 shows the results of an experiment in which the rate of formation, in the absence and presence of tryptophan, of the carboxyferroheme enzyme complex was monitored after flash photolysis. As can be seen, the presence of a near saturating (3.0 mM) amount of L-tryptophan (+Trp) dramatically increases the rate of formation of the carboxy-enzyme complex as compared with L-tryptophan-free ferroheme enzyme. Fig. 2 is a computer-derived presentation of the data of the slow (-Trp) tracing of Fig. 1. The essential linearity of the plot in Fig. 2 of log of the absorbance versus time indicates that this is a pseudo-first order kinetic process.

![Fig. 1 (left). Transmission change which occurs after 580 nm laser photolysis of carboxytryptophan oxygenase (1.0 μM in heme, specific activity, 11) in 0.1 M potassium phosphate, pH 7.0, 944 μM CO at 24°, and measured at 436 ± 3 nm. The upper trace (rapid phase) is enzyme in the presence of 3 mM L-tryptophan (Trp), while the lower trace (slow phase) is the enzyme in the absence of L-tryptophan. Pseudomonad tryptophan oxygenase was reduced with a few crystals of dithionite and then saturated with CO by continuous bubbling with 100% CO for 2 min. An absorption spectrum obtained with a replicate sample indicated that a complete conversion of ferroheme tryptophan oxygenase (Soret, 432 nm) to carboxyferroheme enzyme complex (Soret, 421 nm) had occurred under these experimental conditions.](http://www.jbc.org/content/250/8/5268)
As can be seen, $k_{on}$ determined for the fast species of carboxyferroheme-enzyme complex formation is relatively independent of the concentration of enzyme or of the concentration of CO. This assumption that a pseudo-first order process is being observed on the data of Fig. 3 (see "Materials and Methods"). The rapid phase pseudo-first order rate constant thus obtained from Fig. 4 is 6.38 s$^{-1}$. From this the second order rate constant (X = 6.38 s$^{-1}$) from Fig. 2 is calculated from Fig. 2 is 6.38 s$^{-1}$. From this the second order rate constant for the slow phase ($k_{on} = \frac{k_{on}}{[CO]}$) was determined to be $6.8 \times 10^3$ (2) s$^{-1}$. The ratio of the rapid and slow phase second order rate constants ($k_{on}/k_{on}$) indicates that the presence of L-tryptophan increases the "on" rate of carboxyferroheme-enzyme complex formation by 19-fold. The assumption that a pseudo-first order process is being observed for the rapid species is shown to be valid by the data in Table I. Flash photolysis was performed as described under "Materials and Methods."  

*Average of (n) experiments; reproducibility was ±8%.

Fluorotryptophan is an inhibitor of tryptophan oxygenase which specifically binds to the catalytic site (4); α-methyltryptophan on the other hand is a positive effector, which at the concentrations employed specifically binds to the allosteric site (11). Use of these two analogues makes it possible to discern the relative contribution of saturation of the catalytic and allosteric sites to the altered reactivity of the enzyme with CO. The data in Table II indicates that progressive saturation of the catalytic and allosteric sites by L-tryptophan increases the proportion of the species of ferroheme enzyme which rapidly combines with CO. Saturation of the allosteric site alone with α-methyltryptophan does not convert any of the slow reacting species to the rapidly reacting species. Saturating levels of 5-fluorotryptophan in the presence or absence of α-methyltryptophan causes the transition to the rapid phase indicating that saturation of the catalytic site is the determining factor for the slow phase-rapid phase transition. In the presence of a nonsaturating level of 5-fluorotryptophan (0.2 mM) saturation of the allosteric site with α-methyltryptophan (1.0 mM) causes an increase in the proportion of the ferroheme enzyme which rapidly reacts with CO (50% to 86%). Thus, it seems that the consequence of saturation of the allosteric site is to bring about a conformational change which increases the binding constant for L-tryptophan or its analogue 5-fluorotryptophan at the catalytic site.

**Effect of saturation of the catalytic and allosteric sites on the proportion of the rapid species present during carboxyferroheme complex formation**

Pseudomonad tryptophan oxygenase (specific activity, 11) was used as in Table I. Flash photolysis was performed as described under "Materials and Methods."  

<table>
<thead>
<tr>
<th>Ligand (concentration)</th>
<th>$k_{on}$</th>
<th>Percentage of rapid species $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0 x 10^4 (2)</td>
<td>None 0</td>
</tr>
<tr>
<td>L-Tryptophan (0.03 mM)</td>
<td>1.0 x 10^4 (2)</td>
<td>4.2 x 10^4 (1) 31</td>
</tr>
<tr>
<td>L-Tryptophan (0.09 mM)</td>
<td>1.0 x 10^4 (1)</td>
<td>7.5 x 10^4 (1) 63</td>
</tr>
<tr>
<td>L-Tryptophan (0.3 mM)</td>
<td>1.0 x 10^4 (1)</td>
<td>1.2 x 10^4 (1) 95</td>
</tr>
<tr>
<td>L-Tryptophan (3.0 mM)</td>
<td>1.0 x 10^4 (3)</td>
<td>1.8 x 10^4 (3) 96</td>
</tr>
<tr>
<td>DL-5-Fluorotryptophan (3.6 mM)</td>
<td>1.0 x 10^4 (2)</td>
<td>1.0 x 10^4 (2) 92</td>
</tr>
<tr>
<td>DL-α-Methyltryptophan (1.0 mM)</td>
<td>1.1 x 10^4 (4)</td>
<td>None 0</td>
</tr>
<tr>
<td>DL-5-Fluorotryptophan (3.6 mM) plus DL-α-methyltryptophan (1.0 mM)</td>
<td>1.0 x 10^4 (2)</td>
<td>1.7 x 10^4 (2) 95</td>
</tr>
<tr>
<td>DL-5-Fluorotryptophan (0.2 mM)</td>
<td>1.0 x 10^4 (4)</td>
<td>6.1 x 10^4 (4) 50</td>
</tr>
<tr>
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<td>7.4 x 10^4 (2) 86</td>
</tr>
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</table>

*The percentage of rapid species was calculated as $\frac{\Delta A_{fast}/(\Delta A_{fast} + \Delta A_{slow})}{100}$, where ΔA is the initial change in absorbance for the rapid species and ΔA is the initial change in absorbance for the slow species. ΔA and ΔA were determined as described under "Materials and Methods."  

*Average of (n) experiments; reproducibility was ±10%.

Fig. 3 (left). Same as Fig. 1. Transmission change in the presence of L-tryptophan at a faster time scale. Rapid phase. Fig. 4 (right). Logarithm delta absorbance plot of the transmission change shown in Fig. 3. Upper trace (□□□□□) is the rapid phase and the lower trace (■■■■■) is the slow phase.

**Effect of Saturation of Catalytic and Allosteric Sites on $k_{on}$.**

**Table I**

**Effect of [CO] and [enzyme] on $k_{on}$ for carboxyferroheme complex formation**

Pseudomonad tryptophan oxygenase (specific activity, 13.7) in 0.1 M potassium phosphate, pH 7.0, was reduced with solid sodium dithionite. After the addition of L-tryptophan, the mixture was equilibrated with 100% CO (950 PM at 23-24°C) or diluted with a saturated CO solution. Flash photolysis was then performed as described under "Materials and Methods."  

**Table II**

**Effect of saturation of the catalytic and allosteric sites on the proportion of the rapid species present during carboxyferroheme complex formation**

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<tr>
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<td>1.0 x 10^4 (2) 92</td>
</tr>
<tr>
<td>DL-α-Methyltryptophan (1.0 mM)</td>
<td>1.1 x 10^4 (4)</td>
<td>None 0</td>
</tr>
<tr>
<td>DL-5-Fluorotryptophan (3.6 mM) plus DL-α-methyltryptophan (1.0 mM)</td>
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Fluorotryptophan is an inhibitor of tryptophan oxygenase which specifically binds to the catalytic site (4); α-methyltryptophan on the other hand is a positive effector, which at the concentrations employed specifically binds to the allosteric site (11). Use of these two analogues makes it possible to discern the relative contribution of saturation of the catalytic and allosteric sites to the altered reactivity of the enzyme with CO. The data in Table II indicates that progressive saturation of the catalytic and allosteric sites by L-tryptophan increases the proportion of the species of ferroheme enzyme which rapidly combines with CO. Saturation of the allosteric site alone with α-methyltryptophan does not convert any of the slow reacting species to the rapidly reacting species. Saturating levels of 5-fluorotryptophan in the presence or absence of α-methyltryptophan causes the transition to the rapid phase indicating that saturation of the catalytic site is the determining factor for the slow phase-rapid phase transition. In the presence of a nonsaturating level of 5-fluorotryptophan (0.2 mM) saturation of the allosteric site with α-methyltryptophan (1.0 mM) causes an increase in the proportion of the ferroheme enzyme which rapidly reacts with CO (50% to 86%). Thus, it seems that the consequence of saturation of the allosteric site is to bring about a conformational change which increases the binding constant for L-tryptophan or its analogue 5-fluorotryptophan at the catalytic site.
DISCUSSION

L-Tryptophan and oxygen are the substrates of tryptophan oxygenase. In addition to binding at the catalytic site L-tryptophan binds to an allosteric site on the enzyme (1). The $K_{eq}$ for O$_2$ decreases as the enzyme becomes saturated with L-tryptophan (11). Experimental attempts to elucidate the molecular events underlying this allosteric transition have employed the O$_2$ analogue, CO, which binds as does oxygen to the enzymic heme yielding a spectrophotometrically identifiable carboxyenzyme complex. These earlier spectrophotometric studies (3) of the binding of CO to pseudomonad tryptophan oxygenase were of necessity equilibrium measurements. The equilibrium constant for this reaction increased 28-fold from $1.9 \times 10^6$ $M^{-1}$ to $5.3 \times 10^6$ $M^{-1}$ as the enzyme was saturated with L-tryptophan. A detailed understanding of the molecular processes of carboxyenzyme complex formation was not possible at that time, and it was somewhat uncertain whether this was a consequence of the saturation by L-tryptophan of the catalytic allostery. or both, sites. Some insight was provided by the observation that $\alpha$-methyltryptophan, which binds only to the allosteric site (11), did not bring about any change in $K_{eq}$ (3), whereas saturation of the catalytic site with 5-fluorotryptophan, which does not bind to the allosteric site, did increase $K_{eq}$ (4). As the equilibrium constant, $K_{eq}$ is the ratio of the rate constants ($k_{on}$ and $k_{off}$) for the reaction, it remained indeterminate whether the absence of an effect on $K_{eq}$ when the allosteric site was saturated represented no change or parallel changes in these rate constants. Similarly, the increase in $K_{eq}$ caused by saturation of the catalytic site could have been due to an increase in $k_{on}$ or a decrease in $k_{off}$ or a combination of both. In the present studies direct determination of the proportion of the enzyme with a rapid $k_{on}$ enabled us to distinguish among these various alternate hypotheses.

As shown in Fig. 1 saturation of tryptophan oxygenase with L-tryptophan leads to a conversion of essentially all the enzyme to the species with a rapid $k_{on}$. This 18- to 20-fold increase in $k_{on}$ for CO accounts for almost all of the effect of L-tryptophan upon $K_{eq}$ (Table III). Furthermore, $\alpha$-methyltryptophan alone does not influence $k_{on}$ (Table II) indicating that the lack of effect of $\alpha$-methyltryptophan on $K_{eq}$ is paralleled by no change in $k_{on}$ or $k_{off}$. The conclusion that saturation of the catalytic site alone by L-tryptophan is the necessary and sufficient event converting pseudomonad tryptophan oxygenase from the species with a slow $k_{on}$ to the species with a rapid $k_{on}$ for CO binding is supported by the effect of 5-fluorotryptophan (Table II) which similarly converts the enzyme to the species with the rapid $k_{on}$. Essentially all of the change in free energy ($-6.4$ Cal $M^{-1}$) resulting from the augmentation in the equilibrium binding of CO to the ferroheme moiety of the L-tryptophan-saturated chemically reduced enzyme is derived from intramolecular events evoked by saturation of the catalytic site. No major stabilization of the ternary L-tryptophan carboxyenzyme complex is evident, i.e. $k_{off}$ is little changed. The allosteric effector, $\alpha$-methyltryptophan, has no effect upon $K_{eq}$ or $k_{on}$ either in the complete absence of compounds which bind to the catalytic site or in the presence of saturating levels of such compounds. Only in the presence of subsaturating levels of catalytic site ligands does $\alpha$-methyltryptophan exert an effect upon both $K_{eq}$ and $k_{on}$. Thus, the functional significance of binding of the effector $\alpha$-methyltryptophan to the allosteric site is to augment the affinity of the enzyme’s catalytic site for L-tryptophan or 5-fluorotryptophan.

It is of interest to note that earlier studies (22) established that saturation of the allosteric site of reduced pseudomonad tryptophan oxygenase evoked major conformational changes in the enzyme with an increase in $\epsilon_{280}$ and increased resistance to denaturation by sodium dodecyl sulfate but did not lead to a change in $K_{eq}$ for carboxyenzyme complex formation (3). As presently shown, saturation of the allosteric site alone does not lead to an increase in the proportion of the enzyme with rapid $k_{on}$. In contrast saturation of the catalytic site with 5-fluorotryptophan did not lead to any gross conformational effect (22) but does convert the enzyme to the species with an increased $K_{eq}$ (3) and increased $k_{on}$ for carboxyenzyme complex formation. The drift of the $k_{on}$ for the fast species (Table II, $0.42 \times 10^6$ to $1.9 \times 10^6$ $M^{-1}$ sec$^{-1}$) as a function of the concentration of L-tryptophan or 5-fluorotryptophan may reflect that our two-state analysis of the data may be an oversimplification. There may be intermediate states of tryptophan oxygenase which exist during the transformation of the enzyme from the slow to the rapid CO-binding species.

Tryptophan oxygenase has been shown to contain 2 moles of heme and 2 g atoms of copper per tetrameric enzyme molecule (12). The number of catalytic and allosteric sites has not yet been established and it is conceivable that two catalytic sites

### Table III

<table>
<thead>
<tr>
<th>Protein (addition)</th>
<th>$K_{eq}$ $M^{-1}$</th>
<th>Ratio$^a$</th>
<th>$k_{on}$ $s^{-1}$</th>
<th>Ratio$^b$</th>
<th>$k_{off}$ $s^{-1}$</th>
<th>Ratio$^c$</th>
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<tr>
<td>Pseudomonad tryptophan oxygenase</td>
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<td>$1.0 \times 10^4$</td>
<td>18</td>
<td>5.3</td>
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<td>$1.8 \times 10^4$</td>
<td></td>
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<td>Myoglobin</td>
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<td>$4.0 \times 10^4$</td>
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<td>20</td>
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<tr>
<td>Human hemoglobin A, rapid species</td>
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<td>Escherichia coli NADPH-sulfite reductase</td>
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<td>$4.5 \times 10^4$</td>
<td>21</td>
<td></td>
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</table>

$^a$ Ratio of $K_{eq}$, $k_{on}$, or $k_{off}$ plus organic substrate to that in the absence of organic substrate. In the case of hemoglobin it is the ratio of the rapid species to the slow species.
negative responses are intrinsic properties of these respective substrate, camphor, is present (Table III). Thus, positive and negative responses are intrinsic properties of these respective enzyme molecules. It remains unknown whether the enhanced reactivity of the l-tryptophan-tryptophan oxygenase complex for CO is a consequence of subtle electronic alterations in the enzyme heme, or to an l-tryptophan-mediated interaction of CO with the enzyme copper moieties, thus increasing its rate of interaction with CO and stabilizing the carboxyensemble complex.

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Allosteric Modulation of the Transient Kinetics of Carboxytryptophan Oxygenase Complex Formation after Laser Flash Photolysis
Philip Feigelson, Frank O. Brady and James A. McCray


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