pH Dependence of the Cooperative Interactions and Conformation of Tryptophan Oxygenase*

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SUMMARY

Allosteric interactions in the cupro-heme enzyme tryptophan oxygenase (EC 1.13.11.11) of Pseudomonas acidovorans are shown to be pH-dependent. Increasing the assay pH from 6.0 to 8.0 progressively desensitizes the enzyme from both homotropic and heterotropic ligand interactions. This pH-dependent reversible transition has a pK of 6.2. Hill coefficients for the substrate L-tryptophan of 2.0 and 1.4 were measured at pH 6.0 and pH 7.0, respectively. In attempting to identify the enzymatic residue (or residues) responsible for these pH-dependent effects, the enzyme was observed to be irreversibly inactivated by photoinduced oxidation in the presence of the sensitizer, methylene blue. The photoinactivated enzyme showed a loss of one-half its Soret band absorption which accompanied the loss of one-half its heme and histidine contents. This first order photoinactivated inactivation was pH-dependent and corresponded to a requirement for a protonated species with a pK of 6.2. These results suggest that histidine residues may be involved in the catalytic function and in mediating cooperative interactions of tryptophan oxygenase.

Absolute and difference sedimentation velocity analyses indicate that the molecule undergoes a conformational transition when the pH is decreased from pH 8.0 to pH 6.0. This conformational alteration, measured as a 3.9% increase in sedimentation coefficient, can be regarded as an equivalent decrease in the frictional coefficient. If, a more or less spherical shape to the molecule is assumed, then, the 3.9% decrease in the frictional coefficient between pH 8.0 and 6.0 corresponds to a 12% decrease in apparent hydrodynamic volume of the enzyme. Thus, protonation of an enzymatic moiety, possibly histidine, determines both the conformational and functional interactions between enzymatic sites.

Tryptophan oxygenase (L-tryptophan-2,3-dioxygenase, EC 1.13.11.11) purified either from rat liver (1) or from Pseudomonas acidovorans (2) is an allosteric tetrameric cupro-heme enzyme catalyzing the reaction: L-tryptophan + O2 → N-formylkynurenine. In light of the mammalian enzyme's sensitivity to pH (3), it was decided to re-examine the allosteric interactions of the bacterial enzyme to explore whether its regulatory properties are pH-dependent. It was found that cooperative interactions are affected strongly and reversibly by the environmental hydrogen ion concentration. It was observed that the sigmoidal substrate saturation behavior, i.e. the homotropic effect of L-tryptophan, was absent at pH 8.0, weak at pH 7.0, and very much more pronounced at pH 6.0. Furthermore, the heterotropic activator, α-methyltryptophan becomes progressively less effective in stimulating enzymatic activity as the assay pH is raised from 6.0 to 8.0.

Previous studies from this laboratory of the bacterial enzyme had shown that, in the absence of tryptophan, the equilibrium binding of CO to the reduced ferro-enzyme was independent of pH between 6.0 and 8.0 (4). However, in the presence of sub-saturating levels of L-tryptophan, the affinity of the enzyme for CO was markedly pH-dependent. This was shown to be due to a pH dependence of the apparent Ka for L-tryptophan in conjunction with the fact that CO binds with higher affinity to the enzyme-tryptophan complex than to the free enzyme (5, 6). The present study attempts to explore further the functional significance of these pH-dependent processes, to identify the ionizing moiety on the enzyme, and to correlate conformational and functional behavior of the enzyme.

MATERIALS AND METHODS

Enzyme—Homogeneous tryptophan oxygenase was prepared from Pseudomonas acidovorans as previously described (2, 7). The enzyme was assayed and spectra were obtained on a Cary 14 recording spectrophotometer equipped with the sensitive slide attachment. One unit of enzymatic activity is defined conventionally as the catalytic formation of 1 μmol of formylkynurenine/min at 25°C; specific activity denotes enzyme units per mg of protein. In the presence of tryptophan, protein was measured turbidimetrically as previously described (8), otherwise, protein was determined by the method of Lowry et al. (9). Phosphate buffers were used throughout these experiments.

The pH measured with a Radiometer microelectrode. The heme content of the enzyme was measured colorimetrically by the dipyrindine hemochromogen method of Paul et al. (10). An extinction coefficient for the 405 nm Soret band of 1.70 × 10^4 m^-1 cm^-1 per heme was determined previously for the pseudomonad enzyme (11).

Photooxidation—Photoinduced oxidation was carried out at room temperature (25°C ± 1°C) with a 15-watt white fluorescent light
lam placed 15 cm above the sample. Convenient reaction rates were observed with 50 to 100 μm methylene blue.

Amino acid analyses were carried out upon 0.48 mg of enzyme with 12 enzyme units/mg of protein. The protein was hydrolyzed for 24 hours at 110° in 4 n methanesulfonic acid. The automated amino acid analysis was performed according to the procedure of Spackman et al. (12).

Since the enzyme routinely was stored frozen in 0.10 m phosphate buffer, pH 7.0, containing 0.01 m L-tryptophan it was found convenient to free the enzyme from tryptophan by passage through a Sephadex G-25 column equilibrated with 0.1 m sodium phosphate at pH 7.0.

Kinetics—Hill coefficients were evaluated from initial steady state catalytic rates using a calculator programmed for a least squares linear regression of the Hill equation (12):

\[ \log \frac{V}{V_{\text{max}}} = n \log S - \log K \]

where \( n \) is defined as the interaction or Hill coefficient, \( S \) is a function of the active sites occupied, taken from \( V/V_{\text{max}} \), and \( S \) is the L-tryptophan concentration. Activity values used to evaluate \( n \) were averages of three separate determinations.

Sedimentation Analysis—The enzyme used in the ultracentrifugation studies was converted to the oxidized form with 10 mm potassium ferricyanide. The enzyme was freed from tryptophan and excess potassium ferricyanide by chromatography on Sephadex G-25 previously equilibrated with 0.1 m sodium phosphate either at pH 6.0 or pH 8.0. This enzyme preparation showed catalytic activity only in the presence of reductants such as ascorbate.

Velocity sedimentation experiments were performed on a Spinco model E analytical ultracentrifuge equipped with monochrometer and photoelectric scanner accessory units. The measurements were carried out according to the procedures developed by Schachman (14) utilizing 12-mm double-sector cells. Sedimentation coefficients were calculated by least squares analysis from plots of log s versus time and corrected to values in water at 20°. The technique of difference sedimentation was adapted for use with the photoelectric scanner. A four-place rotor was used in all the experiments and during each experiment standard sedimentation velocity cells were prepared for two of the four places, and in the third a difference sedimentation cell was placed.

The partial specific volume of the protein, \( \bar{v} \), was computed from amino acid composition data to be 0.730 at 4° (2). Buffer densities were measured with an analytical balance equipped with the Sartorius specific gravity attachment. The viscosities of the buffers were measured at the appropriate temperature using a Cannon viscometer.

RESULTS

The initial steady state catalytic rate of tryptophan oxygenase as a function of L-tryptophan concentration was measured at pH 6.0 and 7.0 (Fig. 1). At pH 6.0, substrate cooperativity as well as the activating effect of the positive modulator, \( \alpha \)-methyltryptophan are evident. When assayed at 7.0, the substrate saturation curve shifts, is markedly less sigmoidal, and the effect of \( \alpha \)-methyltryptophan is marginal. Table I summarizes kinetic observations made at three pH values. It is clear that as the assay pH is raised, both the Hill coefficient \( n \), as well as the tryptophan level needed to achieve one-half maximal catalytic rate are reduced.

In order to determine the pK of the functional enzymatic entity responsible for cooperativity, steady state enzyme velocities were measured as a function of pH at both subsaturating and saturating levels of L-tryptophan. The upper curve of Fig. 2 represents enzymatic activity at the saturating levels of 10 and 50 μm L-tryptophan, and shows the effect of pH on increasing \( V_{\text{max}} \). It can be seen that from pH 6.0 to pH 7.0 \( V_{\text{max}} \) increases only about 15% whereas at the subsaturating level of 0.3 mm L-tryptophan the catalytic rate increases nearly 6-fold. When plotted as a Dixon plot, this latter pH effect is shown to have a pK of 6.2 (Fig. 3). All of these pH-dependent kinetic data were found reversible.

Since the pK for this transition was found to be 6.2, it was reasoned that a histidine residue (or residues) might be the ionizable group responsible for mediating this pH effect. In order to test this possibility, the enzyme was subjected to a specific chemical modification. In the presence of the photosensitizing dye, methylene blue, the enzyme was found to be inactivated by exposure to light. This type of reaction has been interpreted as reflecting a specific oxidation of histidine in certain proteins (15 17).

The first order irreversible loss of enzymatic activity upon illumination is shown in Fig. 4. Control experiments showed that the simultaneous presence of both the light and the dye were essential for this inactivation. The photoactivated enzyme could not be reactivated by a variety of reducing agents including ascorbate, dithionite, or borohydride. The addition of free hematin to the inactivated enzyme similarly failed to restore catalytic activity.

Presented in Table II are results indicating that photooxidative inactivation of the enzyme is accompanied by the loss of about half the enzymatic heme measured as the dipyridine hemochromogen method of Paul et al. (10). Furthermore, Fig. 5 shows that accompanying the essentially complete loss of enzymatic activity is a loss of nearly 50% of the enzymatic Soret (405 nm) absorption. Spectral analysis (not shown) of the
Fig. 2 (left). Steady state catalytic activity of tryptophan oxygenase as a function of pH. Assay conditions were as described in Fig. 1. The lower curve represents enzyme activity at the subsaturating tryptophan concentration of 0.3 mM, the upper curve shows enzyme activities at 10 mM and at 50 mM tryptophan. The data points were taken from the lower curve of Fig. 2 representing the catalytic activity at the subsaturating tryptophan concentration of 0.3 mM. The arrow indicates the inflection point at pH 6.2. T.O., tryptophan oxygenase.

Fig. 3 (right). A Dixon plot of the substrate saturation data. The data points were taken from the lower curve of Fig. 2 representing the catalytic activity at the subsaturating tryptophan concentration of 0.3 mM. The arrow indicates the inflection point at pH 6.2. T.O., tryptophan oxygenase.

Fig. 4. Photoinduced inactivation of tryptophan oxygenase at 50 μM and at 83 μM methylene blue. The enzyme was illuminated as described under “Materials and Methods” for the times indicated in 0.1 M sodium phosphate, pH 7.0, buffer and subsequently diluted 300-fold for the assay in 10 mM tryptophan containing 3 mM ascorbate in 0.1 M sodium phosphate, pH 7.0, buffer equilibrated with atmospheric oxygen. Data are expressed as per cent initial enzyme activity when compared with enzyme which was incubated with methylene blue but not subjected to illumination.

Fully inactivated enzyme, after passage through Sephadex G-25 to remove excess methylene blue, established that the inactivated enzyme had the same ultraviolet spectrum at 280 nm as the unmodified control. This observation renders unlikely the possibility that inactivation resulted from modification of either enzymatic tyrosine or tryptophan residues.

Table II

<table>
<thead>
<tr>
<th></th>
<th>Heme content by dipyridine hemochromogen</th>
<th>Per cent heme content</th>
<th>Per cent enzymatic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.02</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Photooxidized</td>
<td>1.28</td>
<td>44</td>
<td>6</td>
</tr>
</tbody>
</table>

Fig. 5. Effect of photooxidation on tryptophan oxygenase activity and on the Soret (405 nm) absorption band. The lower curve represents enzyme activity following illumination in the presence of 50 μM methylene blue for the times indicated. At each time point the enzyme was removed from the direct illumination and kept in the dark for subsequent absorption measurements at 405 nm as depicted in the upper curve. Data are plotted as per cent of initial activity or absorption when compared with samples not subjected to illumination.

It was unfortunately not possible to determine whether the substrate L-tryptophan could protect specifically against photoinduced inactivation. This was precluded since at the high substrate levels required to saturate the enzyme, L-tryptophan probably absorbed a significant amount of the incident light and may be photooxidized. The 1 mM D-tryptophan, which is neither a substrate nor an inhibitor of the enzyme, nevertheless protected partially against photoinduction, presumably due to its absorption of incident light and its photooxidizability (17).

Amino acid analysis, carried out on control and 95% inactivated enzyme showed a loss of histidine content (Table III). The only other residues which were diminished somewhat upon oxidation were methionine and proline. Methionine, however, was considered unlikely to be responsible for inactivation since the photoinduced effect was found to be irreversible, a result not expected for methionine oxidation (18).
Tryptophan oxygenase was illuminated in the presence of 50 μM methylene blue for 20 min which suffices to destroy 95% of its catalytic activity. After passage through Sephadex G-25 to remove the methylene blue, the enzyme was hydrolyzed for 24 hours at 110° in 4 N methanesulfonic acid and its amino acid content determined (12). Control samples, free of methylene blue, were treated similarly. The amino acids depicted are normalized to 71 glycines per 122,000 molecular weight (2).

### Table III

**Effect of photooxidation upon amino acids of tryptophan oxygenase**

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Residues/122,000 molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>71.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>49.6</td>
</tr>
<tr>
<td>Serine</td>
<td>45.1</td>
</tr>
<tr>
<td>Glutamine</td>
<td>94.6</td>
</tr>
<tr>
<td>Proline</td>
<td>49.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>71.0</td>
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<tr>
<td>Alanine</td>
<td>128.4</td>
</tr>
<tr>
<td>Cystine</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>52.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>23.8</td>
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<tr>
<td>Isoleucine</td>
<td>32.4</td>
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<tr>
<td>Leucine</td>
<td>98.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>23.9</td>
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<tr>
<td>Phenylalanine</td>
<td>25.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>22.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>22.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>63.7</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
</tr>
</tbody>
</table>

Since photooxidation resulted in both a reduction of the Soret (405 nm) absorption as well as a loss of apparent heme content, it was decided to determine whether free heme was released from the protein. Fully inhibited enzyme was passed through Sephadex G-25 and the optical densities at 280, 405 and 668 nm were measured (Fig. 6). The material eluting after the protein peak contained methylene blue, but no species absorbing at 405 nm, therefore indicating that free heme was not released from the protein.

The first order rate of photoinduced inactivation was observed to be pH-dependent. Fig. 7 depicts the catalytic activity remaining after a fixed 10-min illumination of enzyme in the presence of 50 μM methylene blue in 0.10 M phosphate at the indicated pH values. Also on this figure is the theoretical ionization curve for a moiety with an assumed pK of 6.2. The homology of these two functions suggests that oxidation of histidine (pK 6.0) (19) may be responsible for enzymatic inactivation. Furthermore, the photooxidation of histidine in the presence of methylene blue has been previously shown to be pH-dependent, the protonated form being more resistant to oxidation (20).

The sedimentation of tryptophan oxygenase at pH 6.0 and 8.0 shows a small but consistent difference of 3.9% in the $s_{20,w}$ values calculated from the slope differences as shown in Fig. 8, and taking into consideration the solvent density and viscosities at the two pH values. Also shown is a control experiment with bovine serum albumin sedimented at the same two pH values.

1 The inability to measure the pH dependence below pH 6 was due to the irreversible precipitation of the enzyme below this pH. The isoelectric point for this enzyme has been determined to be 5.2 (19).
The desensitization of both homotropic and heterotropic interactions caused by increasing the assay pH from 6.0 to 8.0 is consistent with a two-state concerted transition model as originally proposed by Monod et al. (23); the protonated form being the "T" state which is in reversible equilibrium with the ionized genuine S difference, namely a time-dependent, progressively increasing area under the curve that denotes the difference between the boundaries of the sedimenting species. In addition this figure includes another control in which ovalbumin is compared with bovine serum albumin at pH 7.1. The results are consistent with literature values in that bovine serum albumin was observed to sediment faster than ovalbumin. This control difference sedimentation showed a pattern characteristic of a real difference in sedimentation values for these two proteins. Furthermore, included in this same run were sedimentation velocity measurements which when analyzed as described under "Materials and Methods," gave s\textsubscript{0} values of 3.98 for bovine serum albumin and 3.48 for ovalbumin.

Presented in Table IV are the results of four sedimentation velocity analyses of tryptophan oxygenase carried out at pH 6.0 and at pH 8.0. In the fourth column are the calculated differences in 5s\textsubscript{0}, at the two pH values the average difference being 0.25 ± 0.11 s or expressed as percentage, a 3.9% difference.

**DISCUSSION**

Previous studies of microbial tryptophan oxygenase (11), led to the conclusion that saturation of an allosteric site by L-tryptophan or α-methyltryptophan brings about a strengthening of intersubunit interactions. Employing the nomenclature of the allosteric model proposed by Monod et al., the fully catalytically active high affinity isomer, or "R" state is actually the higher sedimenting, more tightly coupled quaternary arrangement and the "T" state the more open or relaxed conformation (23). The present study focuses upon the effects of pH on the cooperative interactions, primarily the loss of sigmoidal substrate saturation kinetics with increasing pH. It remains an interesting possibility that occupation of an allosteric site may alter the pK of a group (or groups) which potentiates intersubunit interactions, as expressed by homotropic and heterotropic ligand effects.

It appears that deprotonation of one or more enzymatic histidine residues is supported by the consideration of the Hill coefficients evaluated from steady state kinetics measured at different pH values. This inference is supported by the consideration of the Hill coefficients.
enzyme or "R" state. However, our data are equally compatible with the induced fit model of Koshland et al. (24). In this sequential transition model, saturation of effector sites would lead to progressive changes in intersubunit bonding domains, proportional to the degree of saturated effector sites. In tryptophan oxygenase one could assume that ionizable functions may mediate, or transmit, intersubunit bonding signals which are manifested as either homotropic and heterotropic cooperative interactions, or both.

In the case of the pseudomonad enzyme catalytic activity is shown to be sensitive to photooxidation in the presence of the sensitizer, methylene blue. Both light and dye are required for inactivation of catalytic activity. The loss of enzymatic activity is irreversible and obeys a first order decay rate. Inactivation results in a 50% loss in both enzymatic heme and histidine residues. It remains an interesting possibility that a heme-linked histidine may be essential for catalytic activity, or mediating cooperative interactions, or both. It is, of course, possible that the ionizable group (or groups) at pH 6.2 may be a different residue (or residues) than that which is oxidized during photooxidation. This possibility is, however, rendered less likely by the correlation between the pH dependence of allosteric behavior (Figs. 2 and 3), and the pH dependence of the loss of catalytic activity during photooxidation (Fig. 7).

In addition to the kinetic and chemical observations discussed above, we also sought evidence of physical differences in the enzyme at the two pH values. Results of sedimentation velocity analysis establish that the molecule undergoes a conformational transition when the pH is decreased from pH 8.0 to pH 6.0. This conformational alteration, detected as a 3.9% increase in $\theta_{12}$, can be regarded as a 3.9% decrease in the frictional coefficient if a more or less spherical shape to the molecule is assumed. A 3.9% decrease in the frictional coefficient would result from a 12% increase in apparent hydrodynamic volume as the pH shifts from pH 8.0 to pH 6.0. It is of considerable interest that this conformational transition occurs over the same pH range where kinetically the enzyme becomes desensitized from all allosteric effects, both homotropic as well as heterotropic.

The fact that the sigmoidicity in the catalytic activity versus tryptophan concentration curve of pH 6.0 is converted to a hyperbolic curve with a markedly lowered $K_{d}$ by either raising the pH or by adding the positive effector, $\alpha$-methyltryptophan, indicates that at the low pH a restraint is operative at the catalytic site. Under the conditions where tryptophan oxygenase behaves allosterically, i.e., at low pH, the molecule is apparently in a more compact configuration, presumably the conformational state in which the intersubunit domains are operational in mediating cooperative interactions. As the pH is increased the molecule expands resulting in allosteric desensitization. Accompanying the apparent molecular expansion at high pH, the enzymatic histidine residues become deprotonated and sensitive to photooxidation. It is reasonable to propose that these three pH-dependent observations may be linked, i.e., that with increased pH the molecule expands into a conformational state in which allosteric interactions are hindered, and that these events are linked to functionally significant histidine moieties.

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