Beef Kidney 3-Hydroxyanthranilic Acid Oxygenase

PURIFICATION, CHARACTERIZATION, AND ANALYSIS OF THE ASSAY

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Beef kidney 3-hydroxyanthranilic acid oxygenase has been purified to homogeneity. It is a single subunit protein of \( M_r = 34,000 + 2,000 \) with a frictional coefficient \( (f/f_0) \) of about 1.1. The enzyme readily aggregates to form, apparently inactive, higher molecular weight oligomers. The very rapid loss of enzyme activity during the assay was analyzed extensively. It was found to be due to inactivation of the enzyme by the substrate, 3-hydroxyanthranilate, and unrelated to enzyme turnover or oxidation of bound iron. The loss of activity was shown to be a first order decay process, and methods are given for obtaining accurate initial reaction rates under all conditions.

Evidence was presented that the enzyme assumes a catalytically inactive conformation at pH 3.4, which only relatively slowly rearranges to an active form at pH 6.5; the rearrangement can be blocked by the presence of substrate. We have found that \( \text{Fe}^{2+} \), which is required for enzymatic activity, can equilibrate freely, albeit slowly, with the enzyme during the course of the enzyme reaction even in the presence of saturating 3-hydroxanthranilate. Under assay conditions, the \( \text{Fe}^{2+} \) has an apparent dissociation constant of 0.04 mM.

The kinetic properties of the enzyme were found to be dramatically different in \( \beta,\beta \) dimethylglutarate buffer and collidine buffer; both the rate of loss of activity during the assay and the substrate \( K_m \) and \( V_{max} \) were affected.

Tryptophan is utilized in the formation of the nicotinic acid ring of NAD in many species of animals. In 1948, Mitchell et al. (1) showed that in the rat 3-hydroxyanthranilic acid is an intermediate in this conversion. The product of the enzymatic reaction involving 3-hydroxyanthranilic acid was identified ultimately by Wiss and Bettendorf (2) as \( \alpha \)-amino-\( \beta \)-carboxymuconate-\( \epsilon \)-semialdehyde, which is unstable and spontaneously rearranges to quinolinic acid (3). The enzyme responsible for the catalysis was shown to be a dioxygenase (4). It has been named 3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6; 3-hydroxyanthranilate:oxygen oxidoreductase).

Early experiments indicated that the enzyme is found primarily in the liver and kidney (5). Most of the attempts at purification of the oxygenase have been made with beef liver (6-9), calf liver (10, 11), or beef kidney (12). These attempts met with limited success because of the great instability of the enzyme (6, 9, 13, 14). The extent of purification was often difficult to assess because of considerable increases in apparent enzyme activity which occurred during various steps in the purification schemes (7, 11).

The existing studies of the oxygenase have been complicated by a very rapid inactivation of the enzyme during its reaction, resulting in a nonlinearity of the assay with time (6) and making a determination of the true initial velocity of the enzyme-catalyzed reaction very difficult. In studies with partially purified beef liver enzyme, of the loss of activity during catalysis, Mitchell et al. (8) found that, although there was no product inhibition, there was a relationship between product formed and the extent of inactivation. They concluded from their studies that the inactivation during the enzymatic reaction resulted from the oxidation of bound ferrous iron (8) which Long et al. (13) had shown to be required for enzymatic activity. It should be noted that the nonlinearity of the enzyme assay has also been described for the beef kidney enzyme (12).

This work describes the purification to homogeneity of 3-hydroxyanthranilic acid oxygenase from beef kidney and some of the physical properties of the purified enzyme. Experiments are presented analyzing the nonlinearity of the enzyme assay and other properties of the enzyme reaction.

EXPERIMENTAL PROCEDURES

3-Hydroxyanthranilic acid was obtained from Mann Research Laboratories as ammonium sulfate ("Enzyme Grade"). Where indicated, the 3-hydroxyanthranilic acid was recrystallized from ethanol/HCl, 4/1. Crystallized and lyophilized bovine serum albumin, crystallized and lyophilized horse skeletal muscle myoglobin, and six times crystallized acetylmutase from A. were obtained from Sigma. Pancreatic ribonuclease and yeast alcohol dehydrogenase were from Boehringer Mannheim Corp. Carboxymethylcellulose (Whatman
CM-52) and diethylaminoethylcellulose (Whatman DE52) were obtained from H. Reeve Angel. Sephadex G-75 Superfine was defined by a modification (absolute ethanol was substituted for anhydrous ether) of the procedure of Kawata and Chase (15). β, β-Dimethylglycine was obtained from Eastman, was recrystallized from water.

Methods

Enzyme Assay

3-Hydroxyanthranilic acid oxygenase was assayed by a modification of the procedure of Ogasawara et al. (12); the procedure used is as follows.

Activation—The iron-free enzyme was activated by incubation for 3 min in 1 ml of a solution containing 1.2 mM formate, 10 mM ferrous ammonium sulfate, and 0.1 mM cysteine, at a final pH of 6.5. (Formic acid was used rather than HCl, the medium previously reported (12), because of its better buffering capacity at this pH.)

Assay—Following the 3-min activation incubation, the mixture was neutralized with 2 ml of 0.1 M collidine-chloride buffer, pH 6.5. (The final concentration of reactants was 0.067 M collidine, 0.016 M acetate, 0.4 mM formate, 0.38 mM ferrous ammonium sulfate, and 0.05 mm cysteine at a final pH of 3.4. (Formic acid was used rather than HCl, the medium previously reported (12), because of its better buffering capacity at this pH.)

Purification of 3-Hydroxyanthranilate Oxygenase from Beef Kidney

Table I is a summary of the procedure used in the purification of beef kidney 3-hydroxyanthranilate oxygenase. All operations were carried out at 4°C unless otherwise specified.

Step 1—Two fresh beef kidneys were cut into 0.1-inch cubes and homogenized in three times the volume of 0.01 M acetic acid in a large Waring Blender: 30 s at low speed, followed by 60 s at high speed. The homogenate was then centrifuged at 12,3000 x g for 1 hour at 4°C to remove the debris.

Step 2—The clear supernatant was added ammonium sulfate to 45% of saturation (25.8 g/100 ml) by the slow addition of the solid salt. The resulting suspension was centrifuged at 12,300 x g for 30 min at 4°C. The pellet, which contained essentially no activity, was discarded. Solid ammonium sulfate was added to the supernatant to 60% of saturation (62.7 g/100 ml), followed by centrifugation at 12,300 x g for 1 hour at 4°C. The supernatant was concentrated and desalted as above and in the same collidine-chloride buffer.

Step 3—The supernatant obtained was chromatographed on a carboxymethylcellulose column (5 x 10 cm) (Whatman CM52) equilibrated with 0.05 M potassium acetate (pH 5.0) containing 10% glycerin. Protein was eluted from the column by a linear salt gradient of 0 to 0.15 M potassium phosphate buffer (pH 6.5), and dialyzed overnight at 4°C against 85 times the volume of 0.05 M potassium phosphate buffer (pH 5.0) containing 10% glycerin. The precipitate which formed during dialysis was removed by centrifugation at 16,300 x g for 20 min at 4°C.

Step 4—The sample was then applied to a diethylaminoethylcellulose column (1.5 x 18 cm) (Whatman DE52) equilibrated with 0.01 M collidine-chloride (pH 6.5) containing 0.1 M potassium chloride. Protein was eluted from the column by a linear salt gradient of 0 to 0.15 M potassium chloride, 1500 x 1500 ml in the equilibration buffer. Fractions of specific activity greater than 100 were pooled and concentrated using an Amicon ultrafiltration apparatus equipped with a PM-10 membrane. The concentrate was decolorized by gel filtration over Sephadex G-50 (fine) column (2.2 cm x 43 cm), equilibrated with 0.01 M collidine-chloride buffer.

RESULTS

Purification of 3-Hydroxyanthranilic Acid Oxygenase from Beef Kidney

Table I is a summary of the procedure used in the purification of beef kidney 3-hydroxyanthranilic acid oxygenase. All operations were carried out at 4°C unless otherwise specified.

Step 1—Two fresh beef kidneys were cut into 0.1-inch cubes and homogenized in three times the volume of 0.01 M acetic acid in a large Waring Blender: 30 s at low speed, followed by 60 s at high speed. The homogenate was then centrifuged at 12,3000 x g for 1 hour at 4°C to remove the debris.

Details of the instrument available on request.
Step 5—The desalted enzyme was then applied to DEAE-cellulose column (1.5 x 18 cm) equilibrated with 0.01 M collidine chloride (pH 6.5) containing 0.01 M potassium chloride and also containing 1 mM m-hydroxybenzoic acid, an inhibitor of the enzyme (II). (Normally, the column from the preceding step is reused by washing with 3 column volumes of equilibration buffer made 2 M in potassium chloride and then 3 volumes of equilibration buffer without the additional potassium chloride, both washes containing 1 mM m-hydroxybenzoic acid.) The enzyme is eluted by a linear gradient of 0 to 0.15 M potassium chloride, 100 x 100 ml, made up in the equilibration buffer. Fractions comprising 90% of the recovered activity were pooled and concentrated, on the Amicon ultrafilter, as above.

Step 6—Finally, the concentrated fractions were chromatographed over a Sephadex G-75 Superfine column (2.5 x 56 cm) equilibrated with 0.01 M collidine chloride (pH 6.5) containing 0.01 M potassium chloride. Elution of the enzyme was accomplished with the same buffer. The enzyme, as it elutes from the Sephadex column, appears to be homogeneous (Fig. 1). The purified enzyme can be stored at 0°C without further treatment.

Stability of Enzyme

The stability of the oxygenase varies depending upon the stage of purification. The enzyme following ammonium sulfate fractionation and CM-cellulose chromatography (Steps 2 and 3) is maximally stable at pH 5.0 in 0.05 M potassium acetate containing 10% glycerol. However, at all stages following the first DEAE-cellulose column (Step 4), the enzyme is most stable in 0.01 M collidine-chloride buffer containing 0.01 M potassium chloride at pH 6.5.

Up to and including the first DEAE-cellulose column the enzyme obtained can be frozen in a dry ice-ethanol bath and stored at -90°C for at least 1 month with no loss of activity; however, the enzyme from the last step in the purification is unstable to this treatment. Instead, the pure enzyme was stored at 0°C in 0.01 M collidine chloride containing 0.01 M potassium chloride at pH 6.5 with 85% of the activity remaining after storage for 1 month under these conditions.

Activation of Enzyme for Assay

The activation of the enzyme with ferrous iron has a pH optimum of 3.4. When the enzyme was activated at more acid pH, the activity obtained decreased to a value at pH 3.0 which was only 40% of that at pH 3.4. The effect of increasing the pH of activation is not as severe; and the activity obtained by activation at pH 6.5 is normally about 50% of the activity found when the enzyme is activated at pH 3.4. However, the activation of the enzyme at pH 6.5 is different with different batches of enzyme. We have not investigated this observation; although, by analogy with phosphoglucomutase (23), it may be that different batches of enzyme contain different amounts of bound metals which prevent complete activation at pH 6.5, but are easily removed at pH 3.4.

With the pure enzyme, maximum activity is found after activation for 3 min. Increasing or decreasing the activation time causes a decline in the resulting activity.

Purity and Size of Enzyme

Evidence that the purified oxygenase (after Step 6 in the purification) is homogeneous was obtained by several methods. Fig. 1A shows that in the final step of the purification procedure, the enzyme elutes as an apparently homogeneous protein from the Sephadex G-75 Superfine column. It can be seen that the protein peak is coincidental with the peak of enzyme activity, and the enzyme specific activity is constant (within 5%) across the peak. When this enzyme was rechromatographed over a Sephadex G-75 Superfine column in the presence of internal molecular weight standards (cytochrome c, diisopropyl fluorophosphate chymotrypsin, ovalbumin, and serum albumin), a plot of log (molecular weight) vs. fraction number shows a single peak consistent with the size of the enzyme.

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** A, Sephadex G-75 Superfine filtration of 3-hydroxyanthranilate oxygenase. The pooled, concentrated enzyme fractions from Step 6 in the purification procedure were chromatographed over a Sephadex G-75 Superfine column (2.5 x 56 cm) equilibrated with 0.01 M collidine chloride (pH 6.5) containing 0.01 M potassium chloride and eluted with the same buffer; each fraction contained 1.9 ml (see "Purification of 3-Hydroxyanthranilic Acid Oxygenase from Beef Kidney"). Plotted are total enzyme activity in units (O--O), specific activity in units per mg (A--A), protein concentration in milligrams per ml (O--O), and specific activity in units per mg (A--A). B, photograph of a polyacrylamide gel obtained by electrophoresis of Fraction 96 from the Sephadex column in the presence of sodium dodecyl sulfate. Fifty micrograms of protein were applied to the gel. After electrophoresis, the gel was stained with Coomassie blue according to the method of Weber et al. (17).
versus elution volume yielded an apparent maximum molecular weight for 3-hydroxyanthranilic acid oxygenase of 34,000 ± 1,700.

As another criterion of purity, the apparently homogeneous enzyme obtained from the Sephadex chromatography (Step 6, Table I) was subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (see "Methods"). Only one protein band was observed (Fig. 1A), indicating that the enzyme is pure by this criterion as well. Identical results were obtained with enzyme which had been oxidized with performic acid (17), and then subjected to electrophoresis as above. The molecular weight of the enzyme was also determined by this technique, with ovalbumin, α-chymotrypsinogen A, and myoglobin as internal standards. A plot of log (molecular weight) versus mobility yielded a M_r of 32,000 ± 2,000 for 3-hydroxyanthranilic acid oxygenase. This value is in good agreement with the value from Sephadex chromatography and indicates that the active enzyme is a single peptide chain.

The enzyme was also subjected to discontinuous gel electrophoresis under both acidic and basic conditions (see "Methods"). In each case, the apparently homogeneous protein seen in Fig. 1 displayed three well separated bands. Taking into consideration the information from the Sephadex chromatography and the sodium dodecyl sulfate gel electrophoresis, there appeared two possible explanations for these multiple bands: the pure protein could oligomerize under the electrophoresis conditions, or else there were several proteins present of the same size but different charge. These two cases can be distinguished using the method of Hedrick and Smith (24). In their method, replicate protein samples are subjected to discontinuous gel electrophoresis in polyacrylamide gels containing different percentages of acrylamide; the mobility of each band at each acrylamide concentration relative to the dye front is determined and the results plotted as the log R_e versus acrylamide concentration as is shown in Fig. 2 for the kidney enzyme. If the protein species represented by the bands were similar in size but of different charge, the three lines would be parallel, i.e. have identical slopes; however, if the protein species were the result of oligomerization of one protein, then the lines would have different slopes and would converge to a single point (24). Fig. 2 shows that the three protein bands do appear to arise from the oligomerization of one protein species. From the slopes of the lines, the three protein bands have electrophoretic mobilities consistent with the presence of monomer, dimer, and tetramer species of 3-hydroxyanthranilic acid oxygenase. It should be mentioned that the intersection of the lines at 2% acrylamide concentration is the same as that found by Hedrick and Smith in their experiments with oligomers of bovine serum albumin (24).

Analytical ultracentrifugation experiments also indicated that the enzyme readily polymerized. The apparently homogeneous enzyme, which Sephadex chromatography indicated had a maximum molecular weight of 34,000, developed species ranging up to about 180,000 when analyzed by high speed sedimentation equilibrium centrifugation by the method of Yphantis (25) and Roark and Yphantis (26). The enzyme was analyzed in a three-sector centrifuge cell at protein concentrations of 3.0, 1.0 and 0.3 mg/ml, and centrifuged at 28,000 rpm at 20°C in a Beckman model E analytical ultracentrifuge equipped with Rayleigh interference optics. The solution required 5 days to attain equilibrium at this temperature, while at 0°C, equilibrium was not attained within 7 days. The data obtained at 20°C were analyzed by the method of Roark and Yphantis (26) and gave the results indicated above. These results support very well the evidence from discontinuous gel electrophoresis that 3-hydroxyanthranilic acid oxygenase can oligomerize under some conditions. No species of molecular weight greater than 34,000 has been found to have enzymatic activity.

Physical Characterization

A sedimentation coefficient for the enzyme was obtained by centrifugation in a 5 to 20% sucrose gradient as described under "Methods." The proteins used as standards were yeast alcohol dehydrogenase, with a sedimentation coefficient of 7.61 S (27), and bovine pancreatic ribonuclease, 1.78 S (28). By this method, the enzymatically active oxygenase was found to have a sedimentation coefficient of 3.24 ± 0.15 S.

The Sephadex gel chromatography of the enzyme, using the four internal protein standards mentioned above, gave a Stokes radius of 25.4 ± 1.3 Å for the oxygenase (29). A diffusion coefficient, D, of 8.5 ± 0.5 x 10^{-10} cm^2/s was calculated from these same data. The sedimentation coefficient and the diffusion coefficient can be substituted into the Svedberg equation to yield a calculated molecular weight. Using a v of 0.759, calculated from the amino acid composition (30), the molecular weight determined from the Svedberg equation is 36,000 ± 2,400. The frictional ratio for the oxygenase was calculated (31) to be 1.08 ± 0.06, which indicated that the enzyme is nearly spherical in shape.

The size parameters of beef kidney 3-hydroxyanthranilic acid oxygenase are summarized in Table II.

Nonlinearity of Enzyme Assay

As was discussed in the introduction, it has been reported that the oxygenase from both kidney and liver undergoes very

*William Koontz and Ross Shiman, manuscript in preparation.
rapid inactivation during the enzyme reaction as evidenced by the nonlinearity of the plot of absorbance (at 360 nm) versus time (1, 12). Before undertaking further studies, we wished to find a way of obtaining true initial velocities for the enzyme reaction and we then wanted to determine both the nature and the cause of this assay nonlinearity.

In the experiment shown in Fig. 3, the enzyme from kidney was assayed under normal assay conditions and the absorbance at 360 nm recorded at 1 s intervals using the integrating DVM (see "Methods"). As is seen in Fig. 3, the plot of $A_{360}$ versus time (closed circles) is indeed nonlinear and is visibly curving by the substrate. These possibilities will be considered in sequence.

If the inactivation of the enzyme during the reaction were the result of a first order decay process, then the instantaneous reaction velocity should obey an equation of the form $v = Ae^{-kt}$ and a plot of log $(v)$ versus time should be linear with a negative slope. The successive 4-s absorbance values (plotted as closed circles in Fig. 3) were subtracted yielding an average velocity over each 4-s period (i.e. $\Delta A_{360}/4$ s) throughout the reaction. These velocities were then plotted against time on the logarithmic scale shown in Fig. 3. The points clearly fit a straight line, and therefore imply a first order process. The slope of the resulting line reflects the rate of decrease of the absorbance and, therefore, the rate of inactivation of the enzyme. From this slope, the point at which the velocity has decreased by 50% of the initial velocity, i.e. the half-life of the enzyme and of the reaction, can be determined. Under the normal assay conditions employed here, the reaction has a half-life of about 120 s.

If the inactivation of the enzyme during the reaction were the result of a first order decay process, then the instantaneous reaction velocity should obey an equation of the form $v = Ae^{-kt}$ and a plot of log $(v)$ versus time should be linear with a negative slope. The successive 4-s absorbance values (plotted as closed circles in Fig. 3) were subtracted yielding an average velocity over each 4-s period (i.e. $\Delta A_{360}/4$ s) throughout the reaction. These velocities were then plotted against time on the logarithmic scale shown in Fig. 3. The points clearly fit a straight line, and therefore imply a first order process. The slope of the resulting line reflects the rate of decrease of the absorbance and, therefore, the rate of inactivation of the enzyme. From this slope, the point at which the velocity has decreased by 50% of the initial velocity, i.e. the half-life of the enzyme and of the reaction, can be determined. Under the normal assay conditions employed here, the reaction has a half-life of about 1.8 min.

The true initial velocity of the reaction can also be calculated by extrapolation of the plot of log $(\Delta A_{360}/4$ s) versus time back to zero time, that is, to the time of addition of the substrate. Such an extrapolation is justified by the fact that the plot is linear over the long time period examined. This now allowed us to determine, for the first time, accurate initial reaction velocities for 3-hydroxyanthranilic acid oxygenase.

The assay nonlinearity (to be used interchangeably with "curvature") seen in Fig. 3 could arise from several sources: the production and accumulation of an inhibitor, either the main product or a side product of the reaction; dissociation of Fe$^{3+}$ from the enzyme; inhibition by Fe$^{3+}$ arising from oxidation of enzyme-bound Fe$^{2+}$ or by oxidation of the Fe$^{3+}$ in solution; or inactivation of the enzyme, either reversibly or irreversibly, or by the substrate. Such possibilities will be considered in sequence.

### Inhibitor Accumulation

If the assay curvature were the result of the accumulation of an inhibiting product, a proportionality would exist between enzyme concentration and the degree of curvature. The most suitable conditions for observing this would be a saturating substrate concentration, where an increase in the concentration of enzyme would result in a proportional increase in the concentration of the product (or products) of the reaction.

The results of such an experiment are shown in Fig. 4. From the plots of log $(\Delta A_{360}/4$ s) versus time the initial velocity ($v_i$) and the velocity at 1 min ($v_{1\text{ min}}$) were obtained. On this graph the assay curvature is expressed as the ratio of the initial velocity, and the velocity after 1 min of enzymatic reaction ($v/v_{1\text{ min}}$). Two conclusions can be drawn from this figure: that the initial velocity is related linearly to enzyme concentration, and that the ratio of $v/v_{1\text{ min}}$ shows little change over the 40-fold range of enzyme concentrations employed. It would also

### Table II

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedimentation coefficient, S</td>
<td>3.24 ± 0.15 S</td>
<td>Sucrose gradient centrifugation$^a$</td>
</tr>
<tr>
<td>Stokes radius, $a$</td>
<td>25.4 ± 1.3 Å</td>
<td>Sephadex gel filtration$^b$</td>
</tr>
<tr>
<td>Diffusion coefficient, $D$</td>
<td>8.5 ± 0.5 $\times 10^{-7}$ cm$^2$/s</td>
<td>Sephadex gel filtration$^b$</td>
</tr>
<tr>
<td>Frictional ratio, $f/f_0$</td>
<td>1.06 ± 0.06</td>
<td>Sephadex gel filtration$^b$</td>
</tr>
<tr>
<td>Molecular weight, $M_r$</td>
<td>36,000 ± 2400</td>
<td>Svedberg equation$^c$</td>
</tr>
<tr>
<td></td>
<td>34,000 ± 1700</td>
<td>Sephadex gel filtration$^b$</td>
</tr>
<tr>
<td></td>
<td>32,000 ± 2000</td>
<td>Sodium dodecyl sulfate gel electrophoresis$^c$</td>
</tr>
</tbody>
</table>

$^a$ Calculated from the results of sucrose gradient centrifugation as described in the text.

$^b$ Calculated as indicated in the text.

$^c$ Calculated from the equation, $M_r = RZ'/D(1 - t^2P)$, as described in the text.

$^d$ Determined from a plot of log $M_r$ versus elution volume as described in the text.

$^e$ Calculated from the results of sodium dodecyl sulfate gel electrophoresis as described in the text.
be expected that if the nonlinearity were a result of inhibition by some product of the reaction, the curvature would increase with time. The fact that the slope of log (ΔA₄₆₀/₄₄ s) versus time is constant (Fig. 3) also argues against this possibility. Therefore, it would appear that the accumulation of product (or products) resulting in inhibition of the enzyme is not responsible for the nonlinearity of the assay.

It should be noted that the spontaneous decay of the reaction product to quinolinic acid, which does not absorb light at this wavelength, has a half-life of approximately 33 min under our assay conditions while the half-life of the reaction itself is only 1.8 min.

**Effect of Fe³⁺**

Experiments performed to determine whether inhibition by Fe³⁺ was the cause of the assay curvature are shown in Table III. These suggest that not only is it unlikely that such inhibition is occurring, but that Fe³⁺ does not seem to bind appreciably to the enzyme. In the experiment shown in Table III A, ferric ion equal to the concentration of ferrous iron normally used, i.e. the maximum concentration of Fe³⁺ which could possibly be produced, was added to the assay mixture. As the table shows, even this concentration of Fe³⁺ results in only a 20% inhibition of the reaction rate. In Table III B results are presented from experiments in which the enzyme was incubated with Fe³⁺, but no Fe³⁺, under conditions normally used for activation, i.e. at pH 3.4. An aliquot of the Fe³⁺-incubated enzyme was then added to whole reaction mixture containing the normal concentration of Fe³⁺. This is shown in Line 3 in Table III B. It can be seen that the initial velocity obtained does not differ from the control. Shown in Line 2, where the enzyme was incubated at pH 3.4 in the absence of either Fe²⁺ or Fe³⁺. If ferric bound tightly to the enzyme to any significant extent, then the rate after incubation with Fe³⁺ should be lower than that found after incubation with no Fe³⁺.

**Inactivation of Enzyme by Substrate**

Experiments were carried out to determine the effect of the substrate concentration on the curvature of the assay. In the experiments shown in Fig. 5, the curvature was determined at several different concentrations of substrate and at a constant enzyme concentration; the enzyme level was low enough that no more than 10% of the substrate was consumed during the measurement period. It is clear that the effect of substrate concentration is quite pronounced; at the lowest substrate concentration little or no curvature is seen, while at the highest the reaction velocity decreases by 50% after 1 min of reaction. A plot of ln vᵢ/vᵣ versus substrate concentration (open circles) appears linear. (This particular method of presentation was chosen since in the simplest case, an inactivation reaction can be represented by E + S k₁ E (inactive) or k₁ S E - dE/dt. Integrating and rearranging yields t·k₁·S = ln vᵢ/vᵣ, where vᵢ is the instantaneous velocity t seconds after the reaction was initiated.) The line obtained in Fig. 5 intersects the ordinate very near to a ratio of 1.0 implying that the substrate
concentration is mainly, and possibly totally, responsible for the assay curvature.

Also shown in the same figure is the saturation curve for the enzyme (Fig. 5, open triangles). It can be seen that even when the initial velocity has become constant (i.e. the enzyme is turning over at a maximum rate) the curvature is still increasing. This makes the additional point that the curvature is unrelated to enzyme turnover.

Experiments were performed which demonstrate that the inactivation of the enzyme during the reaction cannot be reversed easily. In this experiment enzyme was activated in the usual manner and then allowed to react until the reaction rate was about 80% of the initial rate. At this time an aliquot of the mixture was taken and placed into new activation medium. The initial velocity obtained with this reactivated enzyme had, within experimental error, the same velocity as it had when removed from the initial reaction mixture.

**Effect of Activation pH on Enzyme**

Up to this point all enzyme assays shown were done in the same manner and with the same order of addition of the reactants; that is, after activation at pH 3.4, the enzyme was adjusted to pH 6.5 by the addition of collidine-chloride buffer, and the reaction was then initiated by the addition of substrate. However, if the reverse order of addition of enzyme and substrate was used (i.e. the activated enzyme was added to the collidine-chloride buffer which already contained substrate), quite different results were obtained. In this case, the velocity was very low initially and gradually increased with time (Table IV). The magnitude of the effect can be seen by comparing Line 2 of Table IV (the reverse order of addition) with Line 1 or Table IV (the normal order of addition).

When a similar experiment was performed using enzyme which had been activated at pH 6.5, rather than 3.4, the reaction rate was now independent of the order of addition of reactants and showed the normal curvature (Lines 3 and 4 of Table IV). (As indicated earlier, the enzyme can always be activated to some extent at pH 6.5, and this particular enzyme preparation could be activated unusually well at this pH.) In a separate experiment (not shown) the enzyme was first activated at pH 3.4, then preincubated at pH 6.5 for 2 min and an aliquot of this solution taken for assay, the assay was then allowed to proceed as described in Table IV where the enzyme was activated at pH 6.5. Thus, the only condition for observing the anomalous behavior is when enzyme at pH 3.4 is added to a solution at pH 6.5 which already contains substrate.

These observations suggest that the enzyme at pH 3.4 is in a form which can bind substrate but is enzymatically inactive. When the enzyme is placed into buffer in the absence of substrate (i.e. the normal order of addition) at pH 6.5, it can rearrange to its normal, active form; however, if substrate is present, it apparently can "trap" the enzyme in its pH 3.4 form and prevent any significant enzymatic reaction. The slow increase in reaction velocity under these conditions would be due to the slow "escape" of the enzyme from the pH 3.4 form. These conclusions are summarized in the following scheme.

$$E_{3.4} \rightarrow E_{3.4} + \text{substrate} \rightarrow E_{3.4} + \text{products}$$

where $E_{3.4}$ and $E_{3.4}$ denote enzyme conformations at pH 3.4 and 6.5, respectively.

**Equilibration of Enzyme with Ferrous Iron**

In the normal assay, the enzyme is activated at pH 3.4 in the presence of 1.0 mM Fe$^{2+}$, but the Fe$^{2+}$ concentration present during the enzyme reaction is only 0.33 mM. When the enzyme, activated with 1.0 mM Fe$^{2+}$, was assayed at a final concentration of 0.0067 mM Fe$^{2+}$, the apparent initial velocity of the reaction was decreased and the initial curvature greatly increased (Table V). The plot of log ($\Delta A_{260}/4$ s) versus time was no longer linear and showed an initial steep slope with an apparent half-life of 0.8 min; by about 2 min, the slope was shallower and appeared linear with an apparent half-life of 1.77 min. This latter half-life is the same as that obtained under normal conditions. Lines 3 to 6 of Table V show that if enzyme activated with 1.0 mM Fe$^{2+}$ is allowed to preincubate in 0.0067 mM Fe$^{2+}$ for varying lengths of time before the addition of substrate, the apparent initial velocity (extrapolated from the initial, rapidly sloping portions of the curves) decreases to a constant value. When the preincubation time is long enough, the plot of log ($\Delta A_{260}/4$ s) versus time becomes linear over the whole time course and has the same slope as that found in a normal assay. These results strongly suggest that in the absence of substrate the activated enzyme is able to re-equilibrate over a period of several minutes with the iron in the pH 6.5 buffer.

By preincubating the enzyme for 6 min at pH 6.5, as described in Table V, but at several different final Fe$^{2+}$ concentrations, a series of initial velocities was obtained. The data (not shown) fit an expression of the form

$$v = \frac{V'_{e}}{K_{s} + [Fe]}$$

where $V'_{e}$ and $K_{s}$ represent the initial velocity at infinite substrate concentration and the Michaelis constant for the enzyme, respectively.
TABLE V

Equilibration of Fe**+ with enzyme during reaction

In all experiments the enzyme was activated at pH 3.4 as usual with 1.0 mm Fe**+ (see "Methods"). An aliquot of the activated enzyme was then added to 3.0 ml of assay solution at pH 6.5 containing 0.067 M collidine, 0.033 mM cysteine, 0.4 mM formic acid, with the indicated final concentration of Fe**+. After preincubation for the indicated length of time, the reaction was initiated by the addition of 1.0 mmol of 3-hydroxyanthranilic acid. Initial velocity and half-life were calculated by a least squares analysis of log (ΔAA/min) versus time obtained with the integrating DVM (see "Methods").

<table>
<thead>
<tr>
<th>Final [Fe**+] (mM)</th>
<th>Preincubation time (min)</th>
<th>Initial velocity (ΔAA/min)</th>
<th>Initial half-life (min)</th>
<th>2 min half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0.3</td>
<td>0.78</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td>0.33</td>
<td>6.0</td>
<td>0.80</td>
<td>0.39</td>
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<td>0.53</td>
<td>0.16</td>
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<tr>
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<td>0.44</td>
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<tr>
<td>0.0067</td>
<td>6.0</td>
<td>0.35</td>
<td>0.16</td>
<td></td>
</tr>
</tbody>
</table>

where \( v \) is the velocity of the reaction, \( V \) is the apparent maximum velocity, and \( K_d \) is the apparent dissociation constant for the iron. This expression suggested that there was one Fe**+ per active site with a dissociation constant of about 0.04 mM involved in the equilibrium; a range of final Fe**+ concentrations of 0.33 to 0.0067 mM was used. (One Fe**+ per active site has been found for other oxygenases of this type (32).)

The data in Table V also imply that the enzyme can equilibrate freely with iron in the presence of substrate, since the velocities at 2 min and the one-half lives at 2 min, shown in Lines 3 to 6 of Table V are the same and independent of the preincubation time. Thus, when enzyme equilibrated with "high" (1.0 mM) iron is placed in a solution containing "low" (0.0067 mM) iron, the active enzyme-iron complex dissociates until a new equilibrium is reached, which the presence of saturating substrate does not effect, and a decreased reaction velocity is seen.

Behavior of Enzyme in Different Buffers

Because of the resemblance of collidine to known inhibitors of the enzyme (11), other buffers were examined to determine whether the behavior of the enzyme so far observed was unique to collidine. Two buffers tried were piperazine-N,N'-bis(2-ethanesulfonic acid), and 2-(N-morpholino)ethanesulfonic acid, both of which gave results very similar to those found with collidine. These two buffers do not chelate metals and bear no structural resemblance to collidine (33).

However, the enzyme does behave quite differently in sodium \( \beta,\beta \)-dimethylglutarate buffer. In 0.067 M dimethylglutarate, the initial velocity is only about 20% of that obtained in 0.067 M collidine and the half-life of the reaction is much longer. Further, the surprising observation was made that by decreasing the concentration of dimethylglutarate 10-fold, to 0.0067 M, the initial velocity is greatly increased and the half-life is decreased; i.e. by reducing the dimethylglutarate buffer concentration, results are obtained which more closely resemble those found with the collidine buffer. Experiments were also performed (data not shown) which showed that the effects obtained by decreasing the dimethylglutarate concentration are not merely the result of a decrease in salt or carboxyl group concentration. The differences between dimethylglutarate and collidine are obtained only when the different buffer species are used in the reaction mixture; the results of the assay are independent of the buffer used in the preincubation or activation mixture. In addition, no appreciable differences are observed when the concentration of either the collidine, piperazine-N,N'-bis(2-ethanesulfonic acid), 2-(N-morpholino)ethanesulfonic acid buffers are decreased 10-fold in the reaction mixture.

Fig. 6 shows substrate saturation curves plotted according to the method of Lineweaver and Burk (34) of data obtained from assay of the enzyme in 0.067 M collidine, 0.067 M dimethylglutarate, and 0.0067 M dimethylglutarate. As can be seen in the figure, the apparent \( K_m \) in 0.067 M dimethylglutarate is 24 times that in 0.067 M collidine and the apparent \( V_{max} \) is one-seventh as big as that found with the collidine buffer. The effect of decreasing the concentration of dimethylglutarate 10-fold is to increase the apparent \( V_{max} \) to the value obtained with collidine, while reducing the apparent \( K_m \). Thus, the
effects observed result from drastic changes in the apparent \( K_m \) and \( V_{\text{max}} \) of the enzyme in the presence of dimethylglutarate buffer. The origin of these effects is still under investigation, but the results clearly indicate that considerable caution should be exercised in changing the buffer conditions in the assay of this enzyme.

**DISCUSSION**

In this communication, we have described the purification to homogeneity of 3-hydroxyanthranilic acid oxygenase. From the evidence we have obtained, the enzyme is a single subunit protein of 34,000 ± 2,000 Mₚ. It has a frictional ratio (f/fo) near 1.1, and oligomerizes readily. Thus far, no protein species of more than 34,000 Mₚ have been found which possess enzymatic activity, and, in fact, the oligomerization may be partially responsible for the known lability of the enzyme. There are two observations which support this: pure enzyme, which has lost activity on standing, has been restored to nearly its initial specific activity by subjecting it to chromatography on a Sephadex G-75 column; and the specific activity of the homogeneous protein remains fairly constant despite a variable loss of enzymatic activity during the purification procedure. Both these observations indicate that the inactivated enzyme does not co-purify with the active enzyme, and in fact any aggregated form would certainly be removed by the Sephadex gel chromatography (the final step of the purification, Table I).

The results reported here of experiments considering the nonlinearity of the enzyme assay indicate that the substrate itself is the cause of the irreversible inactivation of the enzyme during catalysis. Although the mechanism by which this substrate-induced inactivation occurs remains to be elucidated, certain points can be made about this effect. The data from Fig. 5 make it clear that the turnover of the enzyme per se is not involved in the inactivation process. However, the results obtained using the dimethylglutarate buffer do suggest that there might be a relationship between the enzyme activity and the rate of inactivation during the assay. The effect of this buffer is to increase the half-life of the enzyme during the assay with a concurrent lowering of the apparent \( V_{\text{max}} \). A dilution of the dimethylglutarate buffer yields a decreased half-life and an increased apparent \( V_{\text{max}} \) (Fig. 6). (The possibility that the observed effect is caused by some impurity present in the substrate is unrelated to the rate of enzyme turnover (Fig. 5).) In any kinetic studies of this enzyme, the fact that 3-hydroxyanthranilate oxygenase is a good cheator of Fe³⁺ (37) must be taken into account. Although we have not studied this problem systematically, preliminary data suggest that the ferrous hydroxyanthranilate complex is not a substrate for this enzyme. To avoid any problems resulting from this, the substrate saturation curves shown in Fig. 6 have been obtained at low Fe³⁺ concentrations. Under these conditions the saturation curves do appear hyperbolic over a very wide range of substrate concentrations.

With the complete purification of 3-hydroxyanthranilic acid oxygenase, its physical characterization and a method for obtaining true initial velocities for the enzymatic reaction, it is now possible to undertake systematic studies of the biochemistry and biology of this enzyme.

**REFERENCES**

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