BBA 65684

# STUDIES ON BACTERIAL URATE:OXYGEN OXIDOREDUCTASE

## II. OBSERVATIONS CONCERNING THE PROPERTIES AND COMPONENTS OF THE ACTIVE SITE

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(Received July 25th, 1967)

SUMMARY

1. Some properties of *Arthrobacter pascens* urate oxidase (urate:oxygen oxidoreductase, EC 1.7.3.3) were investigated in regard to its active site.

The addition of $Fe^{3+}$ can partially protect the enzyme from inactivation at low pH or at low ionic strength and stimulate reactivation.

2. The addition of $Cu^{2+}$ inactivated the enzyme, but this inactivation was counteracted by the presence of uric acid, the substrate.

3. The inhibitory and inactivating actions of $Cu^{2+}$ on the enzyme were counteracted by the addition of $Fe^{3+}$.

4. Cyanide inhibited the enzyme activity. The binding site of cyanide to the enzyme was the site to which the oxygen was supposed to bind.

5. The iron and copper contents of the enzyme were less than 0.2 mole per mole of enzyme protein. But many observations made it possible to consider that the iron, not the copper, may act as one of the prosthetic groups of the enzyme.

6. The kinetic data suggested the existence of a ternary complex consisting of the enzyme and the two substrates, uric acid and oxygen.

## INTRODUCTION

With urate oxidase (urate:oxygen oxidoreductase, EC 1.7.3.3.), studies on the enzyme chemistry of the active site have progressed following purification of the enzyme[1–7]. In 1955, MAHLER, HÜBSCHER AND BAUM[6,7] proposed a cuproprotein theory of pork liver urate oxidase from the following observations. The copper content per specific activity of the enzyme was approximately constant in various enzyme preparations at different stages of purification and, moreover, 1 mole copper per mole of the enzyme protein was found. Nonenzymatic oxidation of uric acid catalysed by in-

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organic copper ion supported this cuproprotein theory[7]. Contrary to our expectation, in the bacterial urate oxidase, we found that it might have $Fe^{3+}$ rather than $Cu^{2+}$ as a prosthetic metal.

In this report some properties and components of the active site of the enzyme were investigated.

## METHODS

### 1. *Enzyme assay*

Two methods were followed. The first was the spectrophotometric measurement of the disappearance of uric acid described in the previous paper. The second was the polarographic measurement of the disappearance of oxygen. The dissolved oxygen in the semi-closed reaction mixture vessel was measured by oxygen electrode (Yanagimoto Co. Ltd., Japan). The oxygen concentration was calculated from Bunsen's absorption coefficient at 20° to be 0.0313 (for details see Figs. 6 and 7).

### 2. *Metal analysis*

Quantitative determination of metals was carried out by atomic absorption spectrophotometry (Hitachi-Perkin Elmer, Tokyo, Japan), Reference experiments showed that 0.035 $\mu$g/ml of iron and 0.015 $\mu$g/ml of copper could be detected as the lowest limit levels, and no interference of buffer ions was confirmed in our experiment systems. Wavelengths of light sources were 372 m$\mu$ for iron and 325 m$\mu$ for copper, respectively. In all experiments, water deionized by ion-exchange resin was used which was ascertained not to contain detectable amounts of metals by the method mentioned above.

## RESULTS AND DISCUSSION

### 1. *Effect of ionic strength and $Fe^{3+}$ on the inactivation and the reactivation of the enzyme*

As shown in Fig. 1, at low pH the enzyme was inactivated as mentioned previously, but the addition of ammonium sulfate protected the enzyme from inactivation and the addition of $Fe^{3+}$ also partially protected it. The inactivated enzyme was reactivated by incubation with 0.5 M ammonium sulfate in borate buffer (Fig. 2). In this process, $Fe^{3+}$ by itself had no ability to reactivate the enzyme but could stimulate the reactivation process which was caused by ammonium sulfate. During this inactivation process, chelating agents showed little or no protective effect and had no interacting effect on the protection by ammonium sulfate (Fig. 3). On the inactivation of the enzyme at low ionic strength, the protective effect of $Fe^{3+}$ was observed too.

From these results it is considered that $Fe^{3+}$ assists in reversible conformational change of the enzyme protein.

### 2. *Effect of $Cu^{2+}$ and $Fe^{3+}$ on the stability and activity of the enzyme*

With $Cu^{2+}$ the enzyme was not only inhibited but also gradually inactivated at low temperature. But if uric acid (the substrate of the enzyme) were present together with $Cu^{2+}$, the inactivation process was completely prevented (Fig. 4).

These results seemed to show the following: (1) Inhibitory $Cu^{2+}$ interacts with the enzyme protein, not with the substrate. (2) The site of interaction between the

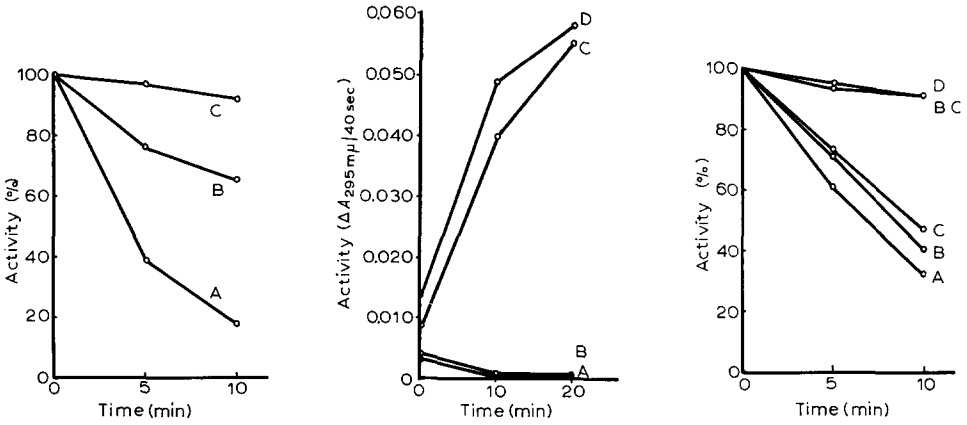



Fig. 1. Protective effect of ammonium sulfate and $Fe^{3+}$ against inactivation of *A. pascens* urate oxidase by treatment at low pH. The enzyme preparations were dissolved in the solutions described below and incubated at 30°. At intervals, 0.2 ml was taken and the enzyme activity was measured and expressed as percent of the initial activity (incubation time 0). Curve A, 0.05 M phosphate buffer (pH 6.7); Curve B, the same buffer containing $1.8 \cdot 10^{-4}$ M $Fe^{3+}$; Curve C, the same buffer containing 0.2 M ammonium sulfate (pH 6.7, adjusted).

Fig. 2. Reactivation of *A. pascens* urate oxidase by ammonium sulfate and $Fe^{3+}$. The enzyme solution partially inactivated at low pH (see Fig. 1) was dissolved in the solutions described below and incubated at 30°. Periodically the enzyme activity was assayed as described in the legend to Fig. 1. Curve A, 0.2 M borate buffer (pH 9.0); Curve B, the same buffer containing $1.8 \cdot 10^{-4}$ M $Fe^{3+}$; Curve C, the same buffer containing 0.5 M ammonium sulfate (pH 9.0); Curve D, the same buffer containing $1.8 \cdot 10^{-4}$ M $Fe^{3+}$ and 0.5 M ammonium sulfate (pH 9.0).

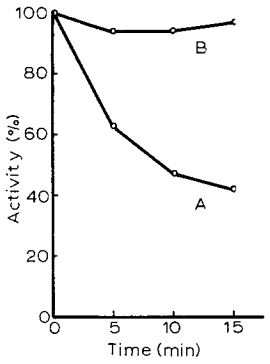
Fig. 3. Effect of chelating agents on the inactivation of the *A. pascens* urate oxidase at low pH. The enzyme preparations which were dissolved in the solutions described below were incubated at 30°. See Fig. 1 for conditions of periodical enzyme assay. Solutions were 0.05 M phosphate buffer (pH 6.9) which contained: no additions (Curve A); $10^{-3}$ M *o*-phenanthroline (Curve B); $10^{-3}$ M diethyldithiocarbamate (Curve C); 0.2 M ammonium sulfate (Curve D); $10^{-3}$ M *o*-phenanthroline *plus* 0.2 M ammonium sulfate (Curve B'); and $10^{-3}$ M diethyldithiocarbamate *plus* 0.2 M ammonium sulfate (Curve C').

enzyme and $Cu^{2+}$ is the active site (or sites) of the enzyme which is influenced during enzyme catalysis. We then examined the relationship between the inhibitory $Cu^{2+}$ and $Fe^{3+}$. As shown in Fig. 5, the inhibitory action of $Cu^{2+}$ on the enzyme competed with $Fe^{3+}$, and the inactivating effect of $Cu^{2+}$ on the enzyme was observed to be decreased by the addition of $Fe^{3+}$.

From these results, it is supposed that $Fe^{3+}$ can interact with or relate to the active sites of the enzyme. This idea seemed to be supported by the following observations. The stimulative action of $Fe^{3+}$ on the enzyme became larger at low pH or at low ionic strength, that is, by adding $1.2 \cdot 10^{-5}$ M $Fe^{3+}$, the activity increased 100% at pH 8.0 and 6% at pH 9.0, and 120% at 6.7 mM borate buffer concentration and 5% at 133 mM of the same buffer. That is, when the pH value or the ionic strength of the enzyme solution decreased, the enzyme protein seemed to change its conformation or its components and the extent of the effects of $Fe^{3+}$ seemed to parallel these changes.

*3. Effects of cyanide and chelating agents on the enzyme activity*

It was reported that the liver urate oxidase was inhibited sharply by cyanide.



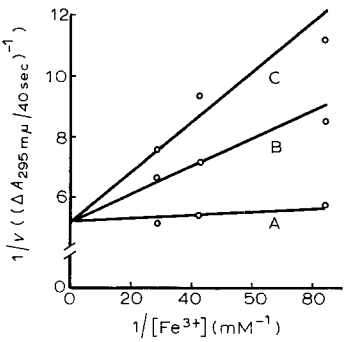




Fig. 4. Effect of $Cu^{2+}$ and uric acid on the inactivation of *A. pascens* urate oxidase. The enzyme preparations, which were dissolved in the solutions mentioned below, were incubated at 0–2°. Periodical enzyme assays were made as described in the legend of Fig. 1. Solutions were 0.1 M borate buffers (pH 9.0) containing $4.6 \cdot 10^{-6}$ M $Cu^{2-}$ (Curve A) and $4.6 \cdot 10^{-6}$ M $Cu^{2+}$ *plus* $4 \cdot 10^{-4}$ M uric acid (Curve B).

Fig. 5. Protection of *A. pascens* urate oxidase from inhibition by $Cu^{2+}$ by $Fe^{3+}$. The enzyme activity with the various concentrations of $Cu^{2+}$ and $Fe^{3+}$ was measured in 0.02 M borate buffer (pH 9.0). The Lineweaver–Burk plots ($1/v$ against $1/[Fe^{3+}]$ )were drawn at various concentrations of $Cu^{2+}$: none (Curve A); $2.6 \cdot 10^{-7}$ M (Curve B) and $5.1 \cdot 10^{-7}$ M (Curve C).

The *Arthrobacter pascens* urate oxidase was inhibited by cyanide, too (Table I). In liver enzyme[6,9], the synergistic effect of the inhibition of a reducing agent and a chelating agent was observed, and MAHLER, HÜBSCHER AND BAUM concluded that cyanide inhibited the liver urate oxidase by acting as both a reducing and a chelating agent. But in the *A. pascens* enzyme, that effect was not observed. (Table I).

TABLE I

EFFECT OF CYANIDE, CHELATING AGENTS, CARBONYL REAGENTS AND REDUCING REAGENTS ON *A. pascens* URATE OXIDASE ACTIVITY

The enzyme activity when some reagents were added was measured in 0.13 M borate buffer (pH 8.6) with or without some preincubation (enzyme + reagents) at 30° and expressed as the percent inhibition. The additive effect of chelating agents is shown in parentheses.

| *Reagent* | *Concentration (M)* | *Preincubation time (min)* | *Inhibtion (%)* |
|---|---|---|---|
| Cyanide | $3.3 \cdot 10^{-6}$ | 0 | 70 |
| | $1.7 \cdot 10^{-6}$ | 0 | 55 |
| | $1.7 \cdot 10^{-6}$ | 10 | 40 |
| Dithionite | $1.0 \cdot 10^{-3}$ | 0 | 17 |
| Sulfide | $3.3 \cdot 10^{-3}$ | 0 | 65 |
| | $3.3 \cdot 10^{-3}$ | 10 | 45 |
| Sulfide + neocuproin | $3.3 \cdot 10^{-3} + 3.3 \cdot 10^{-4}$ | 5 | (17) |
| Sulfide + *o*-phenanthroline | $3.3 \cdot 10^{-3} + 3.3 \cdot 10^{-4}$ | 5 | (14) |
| Sulfide + diethyldithiocarbamate | $3.3 \cdot 10^{-3} + 3.3 \cdot 10^{-4}$ | 5 | (11) |
| Ascorbate | $1.0 \cdot 10^{-3}$ | 0 | 2 |
| | $1.0 \cdot 10^{-3}$ | 30 | 27 |
| Ascorbate + fluoride | $1.0 \cdot 10^{-3} + 1.0 \cdot 10^{-3}$ | 30 | (8) |
| Hydroxylamine | $1.0 \cdot 10^{-3}$ | 0 | 36 |
| | $1.0 \cdot 10^{-3}$ | 10 | 33 |
| Hydroxylamine + fluoride | $1.0 \cdot 10^{-3} + 1.0 \cdot 10^{-3}$ | 10 | (8) |
| Semicarbazide | $1.0 \cdot 10^{-3}$ | 10 | 0 |

TABLE II

EFFECT OF URIC ACID CONCENTRATION ON THE INHIBITION OF *A. pascens* URATE OXIDASE BY CYANIDE AND *o*-PHENANTHROLINE

The enzyme activities with or without cyanide ($1.7 \cdot 10^{-6}$ M) or *o*-phenanthroline ($3.3 \cdot 10^{-4}$ M) were measured at three concentrations of uric acid and expressed as the percent inhibition.

| *Reagent* | *Substrate concn. (M)* | *Inhibition (%)* |
|---|---|---|
| Cyanide | $3.3 \cdot 10^{-5}$ | 22 |
| | $6.6 \cdot 10^{-5}$ | 30 |
| | $13.2 \cdot 10^{-5}$ | 44 |
| *o*-Phenanthroline | $3.3 \cdot 10^{-5}$ | −7 |
| | $6.6 \cdot 10^{-5}$ | 11 |
| | $13.2 \cdot 10^{-5}$ | 25 |

We examined the binding site of cyanide on the enzyme, together with the chelating agents. As seen in Table II, an inhibition percent of the enzyme activity at a given concentration of cyanide became larger paralleling the concentration of uric acid in the reaction mixture. For *o*-phenanthroline the same tendency was observed. These results drew our attention to the relationship between the inhibition by cyanide and the concentration of oxygen, another substrate. The reaction velocities of the urate oxidase at various concentrations of oxygen were checked. As seen in the Lineweaver–Burk plot in Fig. 6, the line was not simple or straight, but curved upward which

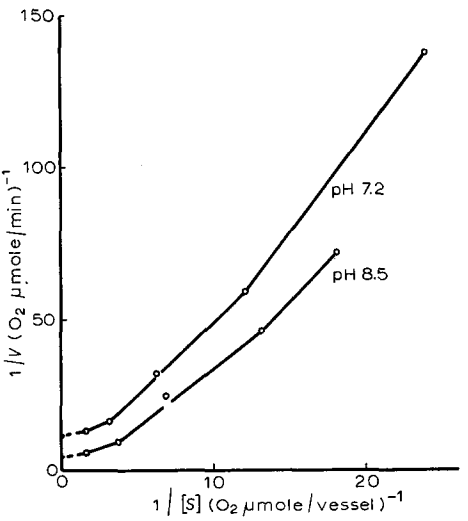



Fig. 6. Effect of oxygen concentrations on *A. pascens* urate oxidase. The reaction mixture consisted of $2 \cdot 10^{-3}$ M uric acid solution 1 ml, enzyme solution 0.1 ml (0.16 unit) and 0.2 M borate buffer containing 0.15 M ammonium sulfate 1.6 ml (pH 7.2 and 8.5). The reaction was started by the addition of uric acid and incubated at room temperature (16–18°). The oxygen content of the reaction vessels was set by bubbling the nitrogen gas into the reaction mixture. The enzyme activity at various oxygen concentrations was represented as the initial velocities of oxygen consumption and plotted in the Lineweaver–Burk plot.

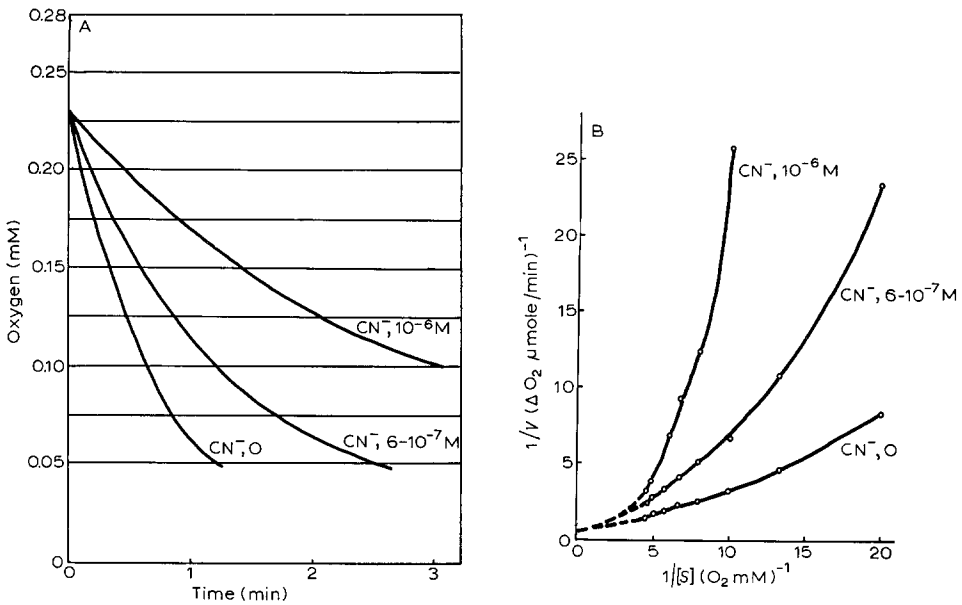



Fig. 7. Effect of cyanide on the *A. pascens* urate oxidase. The reaction mixture consisted of $2 \cdot 10^{-3}$ M uric acid solution, 1 ml, enzyme solution, 0.1 ml (0.16 unit), and 0.2 M borate buffer containing 0.15 M ammonium sulfate (pH 9.0), 1.4 ml, with or without cyanide. The oxygen consumption of each reaction mixture by time was shown (A), and from this chart, $1/v$ was plotted against $1/[O_2]$ (Lineweaver–Burk plot) (B). Dissolved oxygen was 0.28 mM, which was equilibrated with oxygen in air at 20°.

seemed to show the existence of ternary complex consisting of the enzyme, uric acid and oxygen (*cf.* ref. 10). Then the reaction of the enzyme in the presence or absence of cyanide in a semi-closed reaction vessel, in which only the oxygen content was a limiting factor, was observed (Fig. 7a). This result was converted to a Lineweaver–Burk plot (Fig. 7b). It was supposed that cyanide was competing with one substrate, oxygen, on the enzyme.

As mentioned above, some chelating agents have the same action as cyanide, so it is considered that, for example, a metal which can combine with chelating agents acts as a cofactor in relation to binding site of oxygen to the enzyme.

*4. Quantitative determination of iron and copper in the urate oxidase*

In contrast to the liver enzyme, in *A. pascens* urate oxidase the possibility that the enzyme contained iron was considered. Therefore four samples of the urate oxidase each of which was at a different stage of the purification procedure, were subjected to quantitative atomic absorption spectrophotometric analyses of iron and copper.

In order to separate the contaminating metals, all samples were dialysed against the buffer containing EDTA and their specific activity was rechecked just before metal analysis. As shown in Table III, iron and copper contents of the urate oxidase, which was ascertained to have the same specific activity as that before dialysis, were very low, contrary to our expectations. Assuming the molecular weight of the *A. pascens* enzyme to be approx. 100 000, then, by analogy with the sedimentation constant of the liver enzyme, there are 0.1 mole iron and 0.2 mole copper per mole enzyme protein.

TABLE III

IRON AND COPPER ANALYSIS OF THE *A. pascens* URATE OXIDASE

Four enzyme preparations (0.5–2 ml) were dialysed against 0.1 M borate buffer containing 0.1 M ammonium sulfate and $3.3 \cdot 10^{-4}$ M EDTA (pH 8.0) for 48 h at 4°. An aliquot of each dialysate was taken and the activity measured. The remainder was subjected to metal analysis by atomic absorption spectrophotometry (see METHODS).

| *Preparation* | *Activity (units/ml)* | *Specific activity (units/g protein)* | *Fe content* | | *Cu content* | |
|---|---|---|---|---|---|---|
| | | | *(μg/ml)* | *(μg/units)* | *(μg/ml)* | *(μg/units)* |
| A | 66.3 | 21 100 | 0.175 | $2.6 \cdot 10^{-3}$ | 0.33 | $5.0 \cdot 10^{-3}$ |
| B | 181 | 18 100 | 0.490 | $2.7 \cdot 10^{-3}$ | 0.25 | $1.4 \cdot 10^{-3}$ |
| C | 42.3 | 11 100 | 0.105 | $2.5 \cdot 10^{-3}$ | 0.035 | $0.8 \cdot 10^{-3}$ |
| D | 72.2 | 6 900 | 1.015 | $14.1 \cdot 10^{-3}$ | 0.13 | $1.8 \cdot 10^{-3}$ |

From these metal contents *A. pascens* urate oxidase is clearly seen to be different from the liver enzyme. While iron content per specific activity was approx. constant in the four samples, this was not true of the copper content. These results seem to support the hypothesis that iron, which binds loosely to the enzyme, plays some part in catalysis of *A. pascens* urate oxidase.

In summary, the inhibitory action of chelating agents or cyanide makes us suppose that there must be a metal in the catalytic site. The metal seems to be iron from the iron content per enzyme and other behavior of $Fe^{3+}$ on the enzyme. However, the discrepancy remains that chelating agents, except neo-cuproin, do not have strong inhibitory or inactivating effects on the enzyme.

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