Hepatic Aldehyde Oxidase

III. THE SUBSTRATE-BINDING SITE*

K. V. RAJAGOPALAN AND PHILIP HANDLER

From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina

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Previous reports on rabbit liver aldehyde oxidase described the purification and some properties of the enzyme (1) and the differential inhibition of electron transfer to various acceptors (2). The latter study furnished evidence for the participation of several electron carriers in the internal electron transport sequence of the enzyme. The present communication summarizes the results of studies designed to elucidate the nature of the substrate-binding site of hepatic aldehyde oxidase and the manner of interaction with substrate.

In 1946, Knox (3) presented evidence for the identity of the quinone oxidase of rabbit liver with the aldehyde oxidase of the same tissue. The ability of a crude preparation of the enzyme to catalyze oxidation of a group of aldehydes as well as that of diverse nitrogen-containing aromatic heterocyclic compounds was evident from these studies. The same dual specificity is also exhibited by the highly purified enzyme (1). Subsequently, the ability of the enzyme to oxidize phenazine methosulfate was also demonstrated (4).

In the present studies, the substrate specificity of the enzyme has been further investigated. In addition, the inhibition, by several compounds, of enzyme-substrate interaction has been studied. The results suggest the presence of a reactive sulfhydryl group and of molybdenum at the substrate-binding site of the enzyme. Comparative studies have also been made with the somewhat similar xanthine oxidase.

EXPERIMENTAL PROCEDURE

Materials—Many reagents were obtained from the sources cited earlier (1, 2). 7-Methylhypoxanthine, 9-methylhypoxanthine, and 7-methylxanthine were supplied by Cyclo Chemical Corporation, Inc.; 1-(4'-pyridyl)pyridinium chloride hydrochloride, N-methylquinolinium chloride, and N-ethylquinolinium iodide were products of Eastman Organic Chemicals. The latter was converted into the chloride form by passage over a column of Dowex 1-chloride. Mercaptoethanol was supplied by Matheson, Coleman, and Bell. 2,3-Dimercaptopropanol was a product of Mann Research Laboratories. p-Chloromercuribenzoate and N-ethylmaleimide were obtained from Gallard-Schlesinger Manufacturing Corporation.

Rabbit liver aldehyde oxidase was purified as described earlier (1). Milk xanthine oxidase, obtained from Worthington Biochemical Corporation, was further purified on a commercial preparation of hydroxyapatite (2); the specific activity of the final preparation was about 30% greater than that reported for the crystalline enzyme (5).

Methods—The routine assay of aldehyde oxidase activity with N1-methylnicotinamide as substrate has previously been described (1). Possible substrates were generally tested by their ability to effect the enzyme-catalyzed aerobic reduction of horse heart cytochrome c, as determined spectrophotometrically at 550 mμ. When necessary, identification of the product of enzyme action on a substrate was achieved by comparison of the absorption spectrum of the product with the reported spectra of known derivatives.

Xanthine oxidase activity was routinely determined by the increase in absorbance at 295 mμ with 5 x 10-5 m of xanthine as substrate in 0.05 m potassium phosphate containing 0.005% Versene Fe-3. When DPNH was used as substrate, activity was measured with cytochrome c as acceptor, correction being applied for any slight nonenzymic reduction of cytochrome c. Absorption spectra were recorded with a Cary model 14 recording spectrophotometer. Initial velocities in spectrophotometric assays were measured either with the Zeiss PMG II spectrophotometer equipped with a sensitive recorder or in the Cary spectrophotometer with the 0 to 0.1 optical density slide wire.

RESULTS

The ability of rabbit liver aldehyde oxidase to oxidize a wide variety of substrates has already been documented by Knox (3), who observed that the presence of a quaternary nitrogen greatly facilitated the rate of oxidation of heterocyclic substrates. He further reported that the enzyme did not oxidize any of several purine compounds which were tested. We had earlier observed (1) that at levels of 5 x 10-5 m, sufficient to give experimental maximal velocities with milk xanthine oxidase, the oxidation of neither xanthine nor hypoxanthine by purified aldehyde oxidase was demonstrable.

Table I summarizes the results obtained with a number of different groups of compounds tested as substrates for aldehyde oxidase. The Kₐ values, obtained from Lineweaver-Burk data (6), are also included for many of the compounds. Some of the salient features of these results may be noted.

Oxidation of Aromatic Quaternary Nitrogen Compounds—The product of oxidation of N1-methylnicotinamide by aldehyde oxidase is N1-methyl-2-pyridone-5-carboxamide (7). The absorption spectrum of a reference sample of N-methyl-2-quinolone was identical with that of the enzymic oxidation product of N-methylquinolinium, indicating that the site of attack by the
enzyme was the \( \alpha \) carbon atom (position 2) with respect to the ring nitrogen. The absorption spectrum of the enzymic oxidation product of \( N \)-ethylquinolinium chloride was not distinguishable from that of \( N \)-methyl-2-quinoline; hence it was inferred that the product was \( N \)-ethyl-2-quinoline. The oxidation product of phenazine methosulfate has been identified earlier (4). Comparison of the absorption spectrum of oxidized 1-(4-pyridyl)pyridinium chloride with the reported spectrum of 1-(4-pyridyl)-4'-pyridone (8) showed that they were identical. Surprisingly, \( N \)-methylquinolinium bromide did not serve as substrate for the enzyme, but competitively inhibited the oxidation of \( N \)-methylnicotinamide, with a \( K_i \) of 0.019 M. \( N \)-Methylnicotinic acid (trigonelline) did not act as a substrate for the enzyme.

**Purines**

- \( N \)-Methylquinolinium bromide served as substrate for the enzyme.
- \( N \)-Methylquinolinium chloride did not serve as substrate for the enzyme.
- \( N \)-1-Methyl-2-quinoline did not serve as substrate for the enzyme.
- \( N \)-methylquinolinium chloride did not serve as substrate for the enzyme.
- \( N \)-methylquinolinium chloride did not serve as substrate for the enzyme.
- \( N \)-methylquinolinium chloride did not serve as substrate for the enzyme.
- \( N \)-methylquinolinium chloride did not serve as substrate for the enzyme.

**Other**

- Quinoline
- Acetaldehyde
- Salicylaldehyde

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Product</th>
<th>( K_m )</th>
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<tbody>
<tr>
<td>Quaternary compounds</td>
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<tr>
<td>( N )-Methylquinolinium</td>
<td>( N )-1-Methyl-2-pyridine-5-carboxamide</td>
<td>3 ( \times ) 10^4</td>
</tr>
<tr>
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<td>( N )-Methyl-2-quinoline</td>
<td>1 ( \times ) 10^4</td>
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<td>Phenazine methosulfate</td>
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<tr>
<td>1-(4-Pyridyl)pyridinium</td>
<td>10-Methylphenazin-3-one</td>
<td>1.9 ( \times ) 10^4</td>
</tr>
<tr>
<td>( N )-Methylpyridinium</td>
<td>1-(4-Pyridyl)-4'-pyridone</td>
<td>1.9 ( \times ) 10^4</td>
</tr>
<tr>
<td>Purines</td>
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<td></td>
</tr>
<tr>
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</tr>
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</tr>
<tr>
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<tr>
<td>7-Methylxanthine</td>
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</tr>
<tr>
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<td>2-Quinolinol</td>
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<td>Acetic acid</td>
<td>1 ( \times ) 10^2</td>
</tr>
<tr>
<td>Salicylaldehyde</td>
<td>Salicylic acid</td>
<td>1.5 ( \times ) 10^4</td>
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* \( K_i \) as competitive inhibitor.

**Effect of pH on Substrate Specificity**—The oxidation of aromatic quaternary compounds proceeded as rapidly at pH 10 (0.05 M carbonate buffer) as at pH 7.8. \( K_m \) for \( N \)-methylnicotinamide at pH 10 was only 8 \( \times \) 10^{-5} M as compared to 3 \( \times \) 10^{-4} M at pH 7.8. Conversely, \( K_m \) values for aldehyde and nonquaternary aromatic substrates increased markedly with increasing pH. The similarity in the behavior of aldehyde oxidase to that of milk xanthine oxidase in this respect is discussed below.

**Excess Substrate Inhibition**—At concentrations 200 times the \( K_m \) value or greater, \( N \)-methylnicotinamide, \( N \)-methylquinolinium chloride, \( N \)-ethylquinolinium chloride, and phenazine methosulfate did not elicit excess substrate inhibition of aldehyde oxidase. In contrast, uncharged substrates, such as purine, salicylaldehyde, acetaldehyde, and quinoline, exhibited marked inhibition of activity when present at a concentration 50 to 100 times the \( K_m \) value. As an example, the inhibition caused by excess purine will be evident from the Lineweaver-Burk plot in Fig. 1. The products formed by oxidation of these compounds were not inhibitory in any instance.

**Inhibition by Quinacrine**—Earlier studies (1) indicated that quinaene is an effective inhibitor of aldehyde oxidase. Fig. 2 shows that quinaene is formally competitive with substrate, in this case \( N \)-methylnicotinamide. The \( K_i \) for quinaene, 1.5 \( \times \) 10^{-4} M, is lower than \( K_m \) for any substrate so far tested. The formally competitive character of the inhibition was evident when either oxygen or cytochrome \( c \) was the acceptor, and with
either N1-methylnicotinamide or salicylaldehyde as substrate; the $K_i$ values were essentially the same in all cases. In contrast, the inhibition of xanthine oxidase by quinacrine was noncompetitive in character, as seen in Fig. 3.

Inhibition by Cyanide—The susceptibility of aldehyde oxidase to irreversible inhibition by cyanide was reported previously (1). In the present studies it has been observed that the presence of quinacrine during incubation of enzyme and cyanide, in the absence of substrate, resulted in marked protection of the enzyme against inactivation by cyanide (Fig. 4). Clearly related to this phenomenon is the observation that those substrates which induce excess substrate inhibition, e.g. quinoline, also protect the enzyme against cyanide inhibition, whereas those substrates, such as N1-methylnicotinamide, which are not inhibitory at high concentration and which bear a positive charge on the ring nitrogen adjacent to the carbon atom hydroxylated by the enzyme, are relatively ineffective in this regard (Fig. 5). In sum, these results suggest that cyanide attacks the enzyme at or near the substrate-binding site. Were this the case, after treatment with cyanide, most of the components of the electron transport sequence of the enzyme (2) should not be reducible by substrate. Indeed, no reduction of either the flavin or iron components was detected spectrophotometrically (22) when cyanide-treated enzyme was incubated with N1-methylnicotinamide.

**Fig. 1.** The oxidation of purine by hepatic aldehyde oxidase. Formation of 8-hydroxypurine was followed by the increase in optical density at 290 nm in 0.05 m phosphate, pH 7.8, containing 0.005% Versene Fe-3.

**Fig. 2.** Competitive inhibition by quinacrine of the oxidation of N'-methylnicotinamide by aldehyde oxidase.

**Fig. 3.** Noncompetitive inhibition by quinacrine of the oxidation of xanthine by milk xanthine oxidase. O—O, control; △—△, plus 5 x 10^{-4} M quinacrine.

**Fig. 4.** Effect of quinacrine on inactivation of aldehyde oxidase by cyanide. Enzyme and cyanide were preincubated with and without quinacrine in 0.05 m phosphate, pH 7.8, for the time intervals shown. Activity was then determined by 30-fold dilution into the assay medium, which contained 5 x 10^{-3} M N1-methylnicotinamide. The activity of the enzyme pretreated with cyanide and quinacrine was compared with a control containing 2 x 10^{-6} M quinacrine in the assay medium.
FIG. 5. Relative effects of N\(^1\)-methyl nicotinamide (A) and quinoline (B) on cyanide inactivation of aldehyde oxidase. Activity was assayed by measuring the increase in optical density at 330 m\(\mu\). N\(^1\)MN, N\(^1\)-methyl nicotinamide.

FIG. 6. Competitive inhibition by arsenite of oxidation of N\(^1\)-methyl nicotinamide by aldehyde oxidase. ○, control; △, 3.3 × 10\(^{-6}\) M arsenite; □, 6.7 × 10\(^{-6}\) M arsenite; ○, 1 × 10\(^{-5}\) M arsenite.

Inhibition by Arsenite—Arsenite inhibition of hog liver aldehyde oxidase has been shown to be competitive with substrate (13). Analysis of the effect of arsenite on rabbit liver aldehyde oxidase showed that arsenite is also a competitive inhibitor of this enzyme (Fig. 6). The \(K_i\) values for arsenite, at levels of 3.3 × 10\(^{-6}\), 6.7 × 10\(^{-6}\), and 1 × 10\(^{-5}\) M, were calculated to be 6.2 × 10\(^{-6}\), 6.3 × 10\(^{-6}\), and 6.3 × 10\(^{-6}\) M, respectively, indicating the purely competitive nature of the inhibition. Preincubation of enzyme with arsenite for 10 minutes did not inactivate the enzyme. In contrast, preincubation with arsenite readily inactivated milk xanthine oxidase, in agreement with the findings of Peters and Sanadi (14). When xanthine oxidase was assayed in the presence of 1 × 10\(^{-3}\) M arsenite, which was added with the substrate, the inhibition was weak and uncompetitive in nature, as seen in Fig. 7.

Combined Effect of Arsenite and Mercaptoethanol—Addition of mercaptoethanol not only failed to reverse the effect of arsenite on aldehyde oxidase, but actually potentiated the inhibition, as seen in Fig. 8. Whereas the degree of inhibition by arsenite...
alone was constant throughout the time course of an incubation with enzyme and substrate and reversible by dilution, inhibition by arsenite plus mercaptoethanol was progressively more severe with time and irreversible. At the level used, mercaptoethanol did not significantly affect enzyme activity. However, when present in a concentration in excess of $5 \times 10^{-5} \text{ M}$, mercaptoethanol by itself effected a progressive inhibition of enzyme activity. $2,3 \text{ Dimercapto-1-propanol}$ failed to reverse arsenite inhibition, even when present at concentration equimolar with that of arsenite. Like mercaptoethanol, BAL also accentuated arsenite inhibition and caused progressive inactivation of the enzyme. Again, considerably higher levels of BAL alone led to similar inactivation of the enzyme. Preincubation of aldehyde oxidase and BAL before addition of substrate led to lowered enzyme activity, indicating that inactivation by BAL was not dependent on the presence of substrate.

As reported by Hurwitz (15), oxidized BAL, prepared by titrating BAL with potassium ferricyanide, also inhibited aldehyde oxidase. However, incubation of oxidized BAL with enzyme in the absence of substrate did not inactivate the enzyme. Thus, inhibition by oxidized BAL was dependent upon the enzyme reaction and was progressive. Cysteine neither reversed arsenite inhibition nor accentuated it. Cd$^{2+}$, which, like arsenite, is known to react with vicinal dithiol groups (14), failed to inhibit aldehyde oxidase when used at a level of 1 mM.

**Inhibition by p-Mercuribenzoate—p-Mercuribenzoate is a potent inhibitor of rabbit liver aldehyde oxidase (1). Kinetic analysis of the effect of p-mercuribenzoate showed that the inhibition was of the competitive type (Fig. 9). The $K_i$ values, calculated from data obtained at $3.3 \times 10^{-7}$, $6.7 \times 10^{-7}$, and $1 \times 10^{-6} \text{ M}$, were $4.2 \times 10^{-7}$, $2.2 \times 10^{-7}$, and $1.5 \times 10^{-7} \text{ M}$, respectively. The variation in $K_i$ values might have been occasioned by the presence, in the assay mixture, of $10^{-4} \text{ M}$ Versene Fe-3, which can, conceivably, bind to p-mercuribenzoate (16). The highest concentration of p-mercuribenzoate used, $10^{-5} \text{ M}$, was about 10 times in excess of the concentration of enzyme in the assay system. In contrast to the extreme effectiveness of p-mercuribenzoate as an inhibitor, $10^{-3} \text{ M}$ N-ethylmaleimide was without effect on aldehyde oxidase.

**Inhibition by Methanol—**Both milk xanthine oxidase and hepatic aldehyde oxidase undergo progressive inactivation when assayed in the presence of a large concentration of methanol. The effect of varying levels of methanol on the oxidation of xanthine by milk xanthine oxidase is shown in Fig. 10. Essentially identical results were obtained with aldehyde oxidase. When a second aliquot of enzyme was added after the initial material had been inactivated, oxidation of substrate resumed at the expected rate and declined as the second lot of enzyme was inactivated; hence, the inhibition was not due to accumulation of an inhibitory product in the medium. Higher homologues of methanol were without effect, as were low concentrations of formaldehyde, formate, and dimethyl ether, tested as possible impurities in the redistilled reagent grade methanol.

As seen in Fig. 10, xanthine oxidase was almost completely inactivated in about 8 minutes by 1.5 M methanol. When the enzyme was preincubated with 1.5 M methanol at 25° for 30 minutes and then assayed by addition of substrate, the same pattern of activity was obtained as shown in Fig. 10. Thus, methanol did not react with the oxidized enzyme; inhibition was dependent on turnover of the enzyme. When xanthine oxidase inactivated in presence of methanol, was precipitated with ammonium sulfate, redissolved, and then assayed, it was incapable of catalyzing the oxidation of xanthine. When similarly prepared, inactive enzyme was dialyzed overnight against 0.05 M phosphate, pH 7.8, about 30% of the original activity reappeared.

**Methanol inhibition of both aldehyde oxidase and xanthine oxidase was independent of the nature of the electron acceptor, viz. $O_2$, cytochrome c, ferricyanide, or dichlorophenindophenol.** Inhibition of xanthine oxidase by methanol was not reversed by ferric pyrophosphate, potassium molybdate, Versene, o-phenanthroline, Tiron (4,5-dihydroxy-m-benzenedisulfonic acid), catalase, uricase, mannitol, cysteine, or FAD.

Under the conditions used in these studies, maximal velocity for xanthine oxidase activity is experimentally attained at a xanthine concentration of about $13.5 \times 10^{-6} \text{ M}$. As has long been known, higher levels of xanthine result in "excess substrate inhibition." In the presence of 1 M methanol, the initial rate of oxidation as well as total amount of xanthine oxidized before ultimate inactivation continued to increase even at $5 \times 10^{-4} \text{ M}$ xanthine (Fig. 11). However, the absolute rate of xanthine oxidation under these circumstances is less than the maximum attainable in the absence of methanol. Thus, methanol must compete with xanthine at the binding site, with a consequent marked increase in the apparent $K_m$ for xanthine. In accordance with this finding, borate (17) and urea (18), also known to be reversible, competitive inhibitors of xanthine oxidase, mimicked the effect of high levels of xanthine in retarding the rate of methanol inhibition of xanthine oxidase.

High concentrations of methanol affected equally the oxidation of diverse aldehyde and aromatic heterocyclic substrates by both aldehyde oxidase and xanthine oxidase. The only exception...
FIG. 10 (left). Inactivation of milk xanthine oxidase by methanol. Curve 1, control (xanthine, 5 $\times$ 10$^{-5}$ M); Curves 2, 3, and 4, plus 0.5 M, 1.0 M, and 1.5 M methanol, respectively.

FIG. 11 (right). Effect of substrate concentration on the rate of inactivation of xanthine oxidase by 1 M methanol. Xanthine was present at 1.55 $\times$ 10$^{-5}$, 5 $\times$ 10$^{-5}$, 1.5 $\times$ 10$^{-4}$, and 5 $\times$ 10$^{-4}$ M in Curves 1, 2, 3, and 4, respectively.

was the oxidation of DPNH by xanthine oxidase, as seen in Fig. 12, indicating that DPNH, a relatively poor substrate, is not oxidized at the binding site common to other substrates of xanthine oxidase. It does, however, appear to reduce some member of a common internal electron transport sequence since, with DPNH as substrate as with oxidation of purines and aldehydes, there is an obligatory requirement for O$_2$ in order to effect the reduction of cytochrome $c$ (19), which is inhibited by the globin of horse heart myoglobin per ml.

None of the following enzymes were inhibited by 1 M methanol: mitochondrial DPNH-cytochrome $c$ reductase, uricase, snake venom L-amino acid oxidase, beef heart cytochrome $c$ oxidase, horseradish peroxidase, rat liver sulfite-cytochrome $c$ reductase, and dihydroorotic acid dehydrogenase from Zymobacterium oroticum. Only one other enzyme was found to be inhibited by methanol, the molybdenum-containing nitrate reductase of Neurospora crassa (20). The rate of TPNH oxidation by a partially purified preparation of this enzyme, assayed by the procedure of Nason and Evans (20), was reduced 32% by 1 M methanol. However, the extent of inhibition was immediate and constant throughout the reaction; the enzyme was not progressively inactivated in the manner described for aldehyde and xanthine oxidases. Moreover, ethanol was even more effective than methanol, 1 M ethanol resulting in 44% inhibition of the catalytic reaction. As reported by Kinsky and McElroy (21), the activity of this enzyme was markedly greater in phosphate than in pyrophosphate buffer; under these conditions, 1 M methanol reduced activity only by 17%.

In view of the marked increase in $K_m$ values for purine and aldehyde substrates at elevated pH in the case of xanthine oxidase, it was of interest to study the relative rates of inactivation of xanthine oxidase by methanol at pH 7.8 and at pH 10.8. The inability of methanol to inactivate this enzyme in the absence of substrate necessitated that the effect be studied on enzyme which was functioning catalytically. Hence, to study the rate of inactivation of xanthine oxidase by methanol at pH 10.8, it was also necessary to employ a concentration of xanthine which would elicit excess substrate inhibition at pH 7.8. In the presence of 5 $\times$ 10$^{-4}$ M xanthine at pH 10.8, the rate of methanol inactivation of xanthine oxidase was much slower than that observed at pH 7.8 in the presence of 5 $\times$ 10$^{-4}$ M xanthine, but was identical with the rate of methanol inhibition at pH 7.8 in the presence of 5 $\times$ 10$^{-4}$ M xanthine. These results indicate that methanol does not "compete" with hydroxyl ions for a locus on the enzyme, and suggest that the groups on the enzyme which are responsible for the increase in $K_m$ of uncharged substrates with increase in pH are not the site of methanol attack.

Inhibition by Dinitrofluorobenzene—When aldehyde oxidase was assayed at pH 7.8 in the presence of low levels of DFB, oxidation of N$'$$'$$'$-methyl nicotinamide rapidly declined (Fig. 13). Inactivation of the enzyme was complete in 2 minutes with 3.3 $\times$ 10$^{-4}$ M DFB, in 5 minutes with 2 $\times$ 10$^{-5}$ M DFB, and in 10 minutes with 2 $\times$ 10$^{-6}$ M DFB. Since preincubation of concentrated enzyme for 10 minutes with 3.3 $\times$ 10$^{-4}$ M DFB followed
by dilution and assay resulted in the same degree of inhibition as would have been produced if the diluted level of DFB had been added at zero time, it was evident that inactivation by DFB was dependent on catalytic turnover of the enzyme. The pattern of inhibition by DFB was similar whether O2 or 2,6-dichlorophenolindophenol was the acceptor or when N7-methyl

Greenelee and Handler (22) have observed that milk xanthine oxidase is not inhibited by DFB at pH 7.8, whereas incubation of this enzyme in the absence of substrate with DFB at pH 10 for increasing time intervals resulted in progressive, irreversible inhibition of activity. In contrast, whereas no reaction-dependent inactivation of aldehyde oxidase was observed at pH 10, incubation of the enzyme at pH 10 with 3.3 × 10^{-4} \text{M} DFB for 10 minutes at 25°C prior to assay resulted in about 30% loss of activity. When aldehyde oxidase was inactivated by incubation with substrate and 3.3 × 10^{-4} \text{M} DFB for 3 minutes at pH 7.8 and reisolated by precipitation with ammonium sulfate, the resultant preparation was inactive both at pH 7.8 and at pH 10 when assayed with N7-methylxanthine as substrate. Enzyme treated similarly but in the absence of substrate retained full activity. Although not quantitatively assayed, the greater intensity of color of the inactive, reisolated enzyme was indicative of dinitrophenylation of the protein.

Trinitrobenzenesulfonic acid, which has been suggested as a reagent specific for alkylation of e-amino groups of lysine (23), failed to inhibit aldehyde oxidase, but proved to be an efficient electron acceptor for the enzyme (2).

**DISCUSSION**

Concurrent studies of the substrate-binding site of aldehyde oxidase and milk xanthine oxidase have been in progress in this laboratory. Greenelee and Handler (24) reported that several aromatic heterocyclic compounds which bear quaternary nitrogen atoms, e.g. N7-methylxanthinamide, become substrates for xanthine oxidase as pH is increased above 9. \( K_w \) values for these compounds decrease with increasing pH whereas \( K_m \) values for uncharged aldehydes and purines increase with increasing pH. Although somewhat less marked, the same general trend is apparent in the present study of rabbit liver aldehyde oxidase, suggesting a general similarity in the character of the binding sites of the two enzymes.

It has been suggested (25) that the substrate specificity and the manner in which individual purines are oxidized by xanthine oxidase reflect the electronic structures of the purines themselves, rather than the nature of the substrate-enzyme interaction. However, from the present study, it is apparent that the environment of the substrate-binding site of the enzyme is paramount in determining the oxidative fate of the purine substrate. Thus, whereas purine is initially oxidized to hypoxanthine by xanthine oxidase, it is converted to 8-hydroxyxpin by aldehyde oxidase as well as by the xanthine dehydrogenase of \textit{C. cylindrosporum} (10). Xanthine, which is perhaps the most efficient substrate for milk xanthine oxidase and for the bacterial enzyme, is not a substrate for aldehyde oxidase. Hypoxanthine is oxidized to xanthine by aldehyde oxidase, but to 6,8-dihydroxypurine by the bacterial enzyme, whereas 7-methylhypoxanthine is oxidized to 7-methylxanthine by both enzymes. These examples indicate that the manner of attack and ultimate product of enzymic purine oxidation are largely determined by the functional arrangement at the enzymic binding site and the manner in which this interacts with substrate. The only instance in which the structure of the substrate appears to be primarily determinant is the oxidation of phenazin methosulfate by aldehyde oxidase (4); the quaternary ring nitrogen is fixed between 2 carbon atoms, each of which is part of the fused ring structure and, hence, unavailable for oxidation. Oxidation then occurs at the next carbon atom which would be expected to be relatively deficient in electrons, much as if it were itself directly linked to the strongly positive quaternary nitrogen.

**Nature of Substrate-Enzyme Interaction**—An abundance of evidence now indicates that aldehydes and aromatic heterocyclic compounds are attacked at common sites on each of these enzymes, e.g. identical \( K_w \) values with respect to both types of substrate for such diverse competitive inhibitors as quinacrine, urea, guanidine, \( p \)-mercuribenzoate, and borate. Accordingly, any tentative description of these enzymic binding sites and their mode of interaction with substrate must account for this dual specificity. Both aldehyde and xanthine oxidases may properly be regarded as hydroxylases rather than dehydrogenases. Perhaps the simplest visualization of such reactions is concurrent removal of a hydride ion from the carbon atom to be "oxidized" with replacement by a hydroxyl ion from the medium. Such an attack would be facilitated if the enzyme were to provide a group which could serve as a Lewis acid, equally capable of ligating both the oxygen function of aldehydic carbonyl groups and the ring nitrogen immediately adjacent to the carbon which is to be hydroxylated in aromatic heterocyclic compounds. Such reactions are depicted in Fig. 14. Requirement for the Lewis acid is obviated for those substrates bearing a quaternary nitrogen adjacent to the carbon which is attacked; the strongly positive nitrogen withdraws electrons from the adjacent carbon, thereby facilitating attack by a hydroxyl ion with departure of a hydride ion. With respect to the various purines, in each instance a carbon atom adjacent to a ring nitrogen is hydroxylated and it may be presumed that it is the fit of the substrate at the enzyme-binding site which is then determinant among the several possibilities.

Although definitive evidence is still required, several observations strongly suggest that the molybdenum atom at the functional site of each of these enzymes may serve as the Lewis acid considered here, and perhaps also as the initial acceptor of electrons from the substrate. These observations are summarized below.

Both enzymes are irreversibly inactivated by cyanide; approximately one cyanide is bound per flavin (or molybdenum) of xanthine oxidase (26). That cyanide binding occurs at the substrate-binding site is evident from the protection against cyanide afforded by a competitive inhibitor, quinacrine, and the fact that neither the flavin nor iron moieties of the cyanide-inactivated enzyme can be reduced by substrate. That the postulated Lewis acid may well be the site of cyanide binding...
to the substrate-binding site is suggested by the protection against inactivation afforded by uncharged substrates, whereas \( N^1 \)-methylhydantoinamide, which could not be bound by a Lewis acid, is relatively ineffective in this regard. Further, various complexes of cyanide and molybdate have been described.

Aldehyde and xanthine oxidases appear uniquely to be sensitive to inactivation by methanol. The fact that excess substrate and competitive inhibitors retard such inactivation suggests that methanol exerts its effect at the substrate-binding site. The requirement for reducing substrate and the absence of any accumulated inhibitory product in the medium indicate that methanol probably reacts with a reduced form of these enzymes. Although the character of the inhibition is different, the fact that, of all the enzymes studied, only the molybdenum-containing "Neurospora" nitrate reductase is also significantly inhibited by methanol gives some support to the suggestion that the molybdenum component may be the site of methanol action. Formation of methanol-metal complexes during an oxidation-reduction reaction has been observed in nonenzymic systems. Thus, Fraser, Sebera, and Taube (27) observed that when \( \text{Cr}^{3+} \) reduces the \( \text{Co}^{3+} \) complex of monomethylformate, ester hydrolysis accompanies the reduction and the methanol so formed is bound as a complex of the resultant \( \text{Cr}^{3+} \). It has also been noted that methanol molecules are more strongly bound to metal ions than are water molecules under the same circumstances (28), but will exchange with water in the medium. The slow recovery of activity of methanol-inhibited aldehyde oxidase upon prolonged dialysis might then reflect slow replacement of methanol by water from the medium, a process which seemingly occurs more rapidly in the case of nitrate reductase. This concept of the mode of activity of methanol is compatible with the fact that the flavin and iron components of methanol-inhibited aldehyde and xanthine oxidase are not reduced by substrate. For such studies, the reaction was followed spectrophotometrically (29).

The suggestion that it is the molybdenum components of these enzymes which are responsible for their hydroxylation activity is also strengthened by the following considerations. All preparations of aldehyde oxidase contain flavin and molybdenum in the ratio 1:1 (I), and anaerobic incubation with substrate results in immediate, complete reduction of the flavin and iron components of the enzyme, i.e., to the same degree as that obtainable with dithionite as judged spectrophotometrically (29). In contrast, the molybdenum content of milk xanthine oxidase varies from preparation to preparation; the highest content again approaches a ratio of 1:1 with the flavin component (30).

Significantly, the extent of instantaneous reduction of the flavin and iron components of the enzyme also varies and in a general way reflects the molybdenum content of the individual preparation. Further, milk xanthine oxidase slowly catalyzes oxidation of DPNH to DPN\(^+\), a reaction which is a dehydrogenation and clearly different in character from the hydroxylation of the other substrates of this enzyme. Bergel and Bray (31) have shown that relatively inactive preparations of xanthine oxidase, known to be deficient in molybdenum, oxidize DPNH as efficiently as does the "native" enzyme. The insensitivity to methanol of the DPNH oxidase activity of xanthine oxidase, therefore, constitutes an additional argument that the molybdenum is specifically required for the hydroxylation activity of this enzyme.

Although the \( \text{Fe}^{3+} \) ion serves well as a Lewis acid in many nonenzymic systems, e.g., as a catalyst for aldol condensations, it seems unlikely that the iron atoms of these two enzymes so serve. Considerable evidence has been marshaled to indicate that the iron atoms are the components of the electron transport sequences of these enzymes which are most remote from the substrate and the immediate site of oxygen reduction (32). Cyanide inactivation was found to be complete when the amount of cyanide bound to xanthine oxidase was only 25% of that required were it bound stoichiometrically to the iron components of xanthine oxidase (26). Moreover, the lack of influence of 1 m methanol on the catalytic activity of dihydroorotic acid dehydrogenase, the iron of which is bound to the protein and functions in a manner similar to that of both xanthine and aldehyde oxidases (29), again indicates that the iron components are not likely to serve the postulated role of a Lewis acid at the substrate-binding sites of the latter enzymes.

Although the role of the molybdenum components of aldehyde and xanthine oxidases as the Lewis acid here postulated remains to be definitively established, there can be little doubt that this enzymic component is reduced and reoxidized during the normal operation of these two enzymes. Electron paramagnetic spectroscopic studies have indicated that the molybdenum, presumably \( \text{Mo}^{6+} \), is reduced to the \( \text{Mo}^{5+} \) condition in the presence of substrate (32). Moreover, studies performed with a stopped flow apparatus indicate that the molybdenum component of aldehyde oxidase is reduced before the appearance of signals reflecting the generation of a free radical or reduction of the iron (32). Conversely, when the fully reduced enzyme is reoxidized in air, the molybdenum is the last component of the sequence to be reoxidized. These observations indicate that the molybdenum is that member of the electron transport sequence of the enzyme in closest proximity to the substrate.

**Sulfhydryl Groups**—The behavior of aldehyde oxidase with both arsenite and \( p \)-mercurobenzoate indicates that a sulfhydryl group participates at an early stage of the total enzymic process. Both reagents serve as formally competitive inhibitors, presumably indicating that the sulfhydryl group to be attacked is situated extremely close to the substrate-binding site. Although reducing substrate appears to be required for the action of arsenite and to accelerate the action of \( p \)-mercurobenzoate, it is not certain whether the sensitive sulfhydryl group which appears in the presence of substrate derives from a disulfide or from some other combination. The failure of arsenite to inactivate aldehyde oxidase argues against the presence of closely situated sulfhydryl groups which might be expected to arise from a disulfide, as in the case of lipoyl dehydrogenase (33, 34). Inactivation of aldehyde oxidase is achieved in the simultaneous presence of arsenite and an added mercaptan. This requirement is not unique to aldehyde oxidase. Several aldehyde dehydrogenases (35) have been shown to be inactivated by arsenite only in the presence of an additional mercaptan; this inhibition was reversed by dimercaptans. Mitochondrial \( \delta \)-hydroxybutyric dehydrogenase and several other mitochondrial oxidative enzymes were found to be inhibited by arsenite, but inactivated only in the presence of both arsenite and BAL (36). Presumably, inactivation reflects the formation of a combination between arsenite and two sulfhydryls, but it is not clear whether, in addition to the functional sulfhydryl of the enzyme, the second sulfhydryl is generated from a neighboring disulfide by the action of the added mercaptan or is that of the added mercaptan itself.

Inhibition of aldehyde oxidase by dinitrofluorobenzene at pH 7.8 also appears to be the consequence of participation of a
sulfhydryl group in the operation of this enzyme. This inactivation is dependent on turnover of the enzyme, since inactivation was complete in 2 minutes when the enzyme was assayed in the presence of 3.3 × 10⁻³ M DFB plus substrate, whereas preincubation of the enzyme with this concentration of DFB for 10 minutes was without effect in the absence of substrate. In view of the brief period required for inactivation in the presence of substrate and the fact that the inactivated enzyme remained soluble, it would appear that inactivation is a consequence of alkylation of a relatively small number of groups on the enzyme. Since, at the pH at which DFB is inhibitory, the exposed lysine and arginine residues are positively charged and unreactive with this reagent, and, in view of the fact that a reducing substrate is required, it would seem likely that the group which is alkylated is a sulfhydryl which appears in consequence of reduction of a group on the enzyme by the reducing substrate. In this sense the enzyme differs significantly from xanthine oxidase, which is not inactivated at pH 7.8 by DFB, even in the presence of its substrate, although the latter is required in order to induce sensitivity to p-mercuribenzoate.

Within, the initial phenomena in oxidation of the substrate remain obscure. It is clear that molybdenum reduction is one of the earliest, if not the first, event in the electron transfer sequence. Since there is no real evidence for a pre-existing disulfide in the enzyme, nor any information concerning the mode of molybdenum binding by the enzyme, and since only a minimal number of groups may reasonably be expected to be present at the substrate-binding site, it seems attractive to consider the possibility of a molybdenum-mercaptide at the active site. In this event the molybdenum could serve as a Lewis acid in the manner postulated and reduction would expose a sulfhydryl group, but the immediate acceptor and subsequent disposition of the hydride ion removed from the substrate are uncertain.

SUMMARY

1. Rabbit liver aldehyde oxidase, known to catalyze oxidation of aldehydes and various nitrogen-containing aromatic heterocyclic compounds, also catalyzes oxidation of purine and hypoxanthine when these are present in high concentration but does not affect xanthine.

2. With increasing pH, 

Kₘ values for substrates bearing a quaternary nitrogen atom decrease while 

Kₘ values for uncharged substrates increase.

3. The irreversible inactivation of aldehyde oxidase by cyanide is significantly retarded by quinacrine, which is a competitive inhibitor against all substrates, and by uncharged substrates, but not by substrates bearing a positive charge.

4. Arsenite, p-mercuribenzoate, and dinitrofluorobenzene were found to be competitive inhibitors of aldehyde oxidase; each of these compounds reacts with sulfhydryl groups which are exposed only in the presence of reducing substrate. In the presence of an exogenous mercaptan, arsenite irreversibly inactivates the enzyme.

5. When incubated with substrate in the presence of 1 M methanol, both aldehyde and xanthine oxidases are rapidly inactivated. This process is retarded by high concentrations of substrate or of various competitive inhibitors. Of a series of other enzymes similarly studied, only the molybdenum-containing nitrate reductase of Neuspora crassa was found to be inhibited by methanol

6. Attention is directed to the fact that the reactions catalyzed by xanthine and aldehyde oxidases are best characterized as hydroxylation of the substrate. A mechanism is proposed for such reactions, and it is suggested that, in both enzymes, the molybdenum compound is present at the substrate-binding site, participates in the hydroxylation event, and is the first component of these enzymes to be reduced.

REFERENCES


