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Inhibition of Xanthine Oxidase by Various Aldehydes†

Fraser F. Morpeth‡ and Robert C. Bray*

ABSTRACT: The inactivation of bovine milk xanthine oxidase by various aldehydes has been investigated. For each aldehyde, the inactivation reaction gives rise to a unique molybdenum(V) electron paramagnetic resonance signal from xanthine oxidase (the Inhibited signal). Of the aldehydes tested, only a few (mainly aromatic) failed to undergo this reaction. The g values of the Inhibited signals vary systematically from one aldehyde to another. As the substituents of the α-carbon atom become more electron withdrawing, so the g_{av} increases. The inactivation rate depends on both enzyme and aldehyde concentration. Oxygen or another oxidizing substrate is also required for inhibition by 3-pyridinecarboxaldehyde and butyraldehyde but not formaldehyde. Reactivation of xanthine oxidase inhibited by an aldehyde occurs spontaneously after removal of excess aldehyde. For butyraldehyde or 3-pyridinecarboxaldehyde, greater than 95% recovery of activity was observed. The rate of reactivation is dependent both on the nature of the molecule bearing the aldehyde group and on a pK (6.6) of the complex with the enzyme. Evidence is presented that the modifying aldehyde in the Inhibited signal-giving species has (contrary to earlier assumptions) not been oxidized. These results are discussed in relation to the structure of the molybdenum center, and a mechanism for the inhibiting reaction is suggested.

Booth (1938) carried out the first, and until very recently [see Morpeth (1983)] the most rigorous, examination of the specificity of xanthine oxidase toward aldehyde substrates. In the course of these studies, he found that formaldehyde inactivates xanthine oxidase. This phenomenon was apparently not investigated further until Pick et al. (1971) provided evidence that the inactivation of xanthine oxidase by methanol (Polonovski et al., 1947; Rajagopalan & Handler, 1964; Coughlan et al., 1969; Bray et al., 1968) which results in a unique electron paramagnetic resonance (EPR)[1] signal, due to molybdenum(V) and given the name Inhibited, was in fact due to the enzyme slowly oxidizing methanol to formaldehyde. Since then, several other alcohols and aldehydes have been reported to react similarly with xanthine oxidase, always yielding the Inhibited signal (Tanner & Bray, 1978a,b; Malthouse et al., 1981a). Another carbonyl compound, formamide, will also give the Inhibited signal (F. F. Morpeth, G. N. George, and R. C. Bray, unpublished results). At least with formaldehyde and methanol, this signal is obtainable not only from xanthine oxidase but also from the related enzymes xanthine dehydrogenase (Barber et al., 1976) and aldehyde oxidase (Bray et al., 1982).

The Inhibited signal differs from other molybdenum(V) species from active xanthine oxidase in that it is air stable (Bray et al., 1968). However, little is known about the nature of the inhibitory reaction, though Bray & Gutteridge (1982) have proposed a structure for the Inhibited species on the basis of ^{17}O-substitution studies.

The present work was undertaken to determine the nature of the inhibitory side reaction, occurring during the turnover of aldehyde substrates by xanthine oxidase, which gives rise to the Inhibited signal. The main approach we have used has been to vary the nature of the aldehyde molecule and to study the effect of this, and of other variables such as pH, on the formation and breakdown of the species formed in the inhibitory reaction.

† From the School of Chemistry and Molecular Sciences, University of Sussex, Falmer, Brighton, BN1 9QJ, U.K. *Received August 11, 1983.* The work was supported by grants from the Medical Research Council.
‡ Present address: Department of Food Technology, University of Reading, Reading, U.K.

[1] Abbreviations: EPR, electron paramagnetic resonance; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DPPH, diphenylpicrylhydrazyl; EDTA, ethylenediaminetetraacetic acid.

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Table I: EPR Parameters of Molybdenum(V) Inhibited Signals Obtained by Treating Xanthine Oxidase with Various Aldehydes[a]

substrate	g values (half line widths) 1	2	3	av	ref
formaldehyde	1.9911 (0.20)	1.9772 (0.14)	1.9513 (0.17)	1.9732 (0.17)	Tanner et al. (1978)
acetaldehyde	1.9936 (0.22)	1.9783 (0.15)	1.9546 (0.19)	1.9755 (0.19)	Malthouse et al. (1981a)
propionaldehyde	1.9937 (0.21)	1.9786 (0.16)	1.9565 (0.20)	1.9763 (0.19)	present work
butyraldehyde	1.9945 (0.21)	1.9790 (0.16)	1.9567 (0.20)	0.9767 (0.19)	present work
4-pyridinecarboxaldehyde	1.9960 (0.20)	1.9790 (0.16)	1.9551 (0.16)	1.9767 (0.17)	present work
2-pyridinecarboxaldehyde	1.9957 (0.20)	1.9811 (0.15)	1.9549 (0.17)	1.9772 (0.17)	present work
3-pyridinecarboxaldehyde	1.9976 (0.20)	1.9800 (0.16)	1.9561 (0.16)	1.9779 (0.17)	present work
2-amino-4-hydroxy-6-formylpteridine	1.9970 (0.20)	1.9800 (0.18)	1.9541 (0.20)	1.9770 (0.19)	Malthouse et al. (1981a)
3-indolecarboxaldehyde	1.9947 (0.19)	1.9791 (0.17)	1.9564 (0.17)	1.9767 (0.18)	present work
glycolaldehyde	1.9931 (0.25)	1.9777 (0.18)	1.9547 (0.21)	1.9752 (0.21)	Malthouse et al. (1981a)

[a] Signals were generated by treating the enzyme, aerobically in 50 mM Na^+-Bicine buffer, pH 8.2, with a large excess of the aldehyde for about 45 min at 20 °C and then gel filtering. Parameters of the signals were determined with the help of computer simulations. g values are believed to be accurate to ±0.0003. Half line widths are given in millitesla.

Materials and Methods

Enzymes and Substrates. Xanthine oxidase was prepared and assayed spectrophotometrically with xanthine as substrate, as described previously (Hart et al., 1970; Bray, 1975, 1982); superoxide dismutase was a gift from Professor G. Rotilio (Rome); catalase was obtained from BLC (Lewes, U.K.) and alcohol dehydrogenase from Sigma London Chemical Co. Ltd. (Poole, Dorset, U.K.). All aldehydes were obtained from Sigma or BDH Chemicals Ltd. (Poole, Dorset, U.K.). The simple aliphatic aldehydes were redistilled before use. All other aldehydes were used as supplied.

Inhibition of Xanthine Oxidase by Aldehydes. The enzyme and aldehyde were mixed together under appropriate conditions, as described in the text for individual experiments. The number of turnovers of aldehydes by the enzyme before inhibition was essentially complete was determined by monitoring oxygen consumption from the solution with an oxygen electrode. In experiments where anaerobic conditions were required, this was achieved by alternate evacuation and flushing with highly purified argon. For EPR studies, inhibition was achieved by incubating xanthine oxidase for 45–60 min with a large excess of the appropriate aldehyde. The reaction was carried out in an open vessel with frequent shaking to ensure aerobiosis. To remove excess aldehyde, the inhibited enzyme (about 0.5 mL) was gel filtered on a Sephadex G25 column [from Pharmacia (Great Britain) Ltd., Hounslow, Middlesex, U.K.]. Xanthine oxidase and 3-pyridinecarboxaldehyde were found to be separated on a small column (5 cm × 1.5 cm). However, for quantitative separation of butyraldehyde and xanthine oxidase, a longer (26 cm × 1.5 cm) column was required. On this column, the xanthine oxidase peak eluted at 19 mL and that of butyraldehyde at 29 mL. The peaks were sufficiently well separated that any possible contamination of the enzyme fraction with the aldehyde would be quite insignificant on a stoichiometric basis. (Xanthine oxidase was detected by its absorbance at 450 nm, 3-pyridinecarboxaldehyde by its absorbance at 265 nm, and butyraldehyde by its ability to oxidize NADH in the presence of equine alcohol dehydrogenase.)

EPR Procedures. All spectra were recorded on frozen samples of the enzyme, with a dual sample cavity containing manganese and DPPH standards, on a Varian E9 spectrometer linked to a PDP 11/10 computer (Bray et al., 1978). Running conditions were generally as follows: temperature about 120 K; microwave power 10 mW; microwave frequency 9.3 GHz; modulation amplitude 0.16 mT. Computer-generated difference spectra were obtained as described previously (Bray et al., 1978). All EPR parameters were determined with the help of computer simulation of the experimental spectra (Lowe, 1978; George, 1983). The absolute intensity of the experimental spectra, using a Cu^{2+}-EDTA standard, was determined by integration, with suitable corrections, as described in Bray et al. (1978). When these procedures are used, integrations should be accurate to within about ±10% and g values to within ±0.0003 [cf. Bray & Gutteridge (1982)].

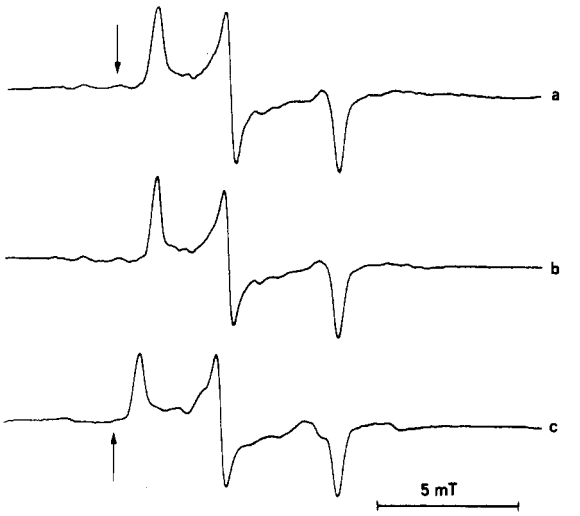


FIGURE 1: Inhibited Mo(V) EPR signal from reaction of various aldehydes with xanthine oxidase. The aldehydes used for spectra a–c were respectively butyraldehyde, indole-5-acetaldehyde, and 3-pyridinecarboxaldehyde. The medium was 50 mM Na^+-Bicine buffer, pH 8.2, and spectra were obtained by treating xanthine oxidase with a large excess of the aldehyde at 4 °C for 30 min and then removing excess reagent by gel filtration. The arrow indicates the resonance of diphenylpicrylhydrazyl (g = 2.0037).

Results

Inhibited Signals from Various Aldehydes. Malthouse et al. (1981a) showed that a number of aldehydes, in addition to formaldehyde, could react with xanthine oxidase to give molybdenum(V) Inhibited signals. For each aldehyde, the parameters of the signal were unique and characteristic of that aldehyde. We have extended the list of aldehydes reacting in this manner, and the EPR parameters of a wide range of aldehyde Inhibited signals are given in Table I. Figure 1 shows three typical and essentially pure Inhibited EPR signals. Of the aldehydes we tested, only the following did not give the Inhibited signal, under the conditions of Table I: salicylaldehyde, benzaldehyde, *o*-fluorobenzaldehyde, *p*-nitrobenzaldehyde, and chloral hydrate.

Inspection of Table I shows that there are definite trends in values of the EPR parameters of the Inhibited signal, depending on the chemical nature of the molecule bearing the aldehyde group. In general, it seems that the more electron

withdrawing the substituents of the α-carbon atom, the higher the g values. Though steric and other factors undoubtedly play a significant part, the importance of electron-withdrawing properties is clearly shown in two cases in Table I. Thus, first, with simple aliphatic aldehydes, g_{av} increases with increasing chain length. The largest increase is seen on going from formaldehyde to acetaldehyde, and the relative increase on adding one carbon to the chain decreases as the chain length increases. This mirrors the relative increase in electron-withdrawing properties of the alkyl side chain. Second, with the pyridinecarboxaldehydes, 3-pyridinecarboxaldehyde has a higher g_{av} value than does 2-pyridinecarboxaldehyde or 4-pyridinecarboxaldehyde, which have parameters quite similar to one another. The 2- and 4-positions in pyridine carry much more electron density than does the 3-position. Thus, the inductive effect on the aldehyde group in 3-pyridinecarboxaldehyde is greater.

Earlier experiments in this laboratory (S. Gutteridge, unpublished results) have shown the EPR parameters of the formaldehyde Inhibited signal to be, in contrast to those of the Rapid and Slow signals (Gutteridge et al., 1978a,b), quite insensitive to variations in the nature of the buffer employed or to the presence in the medium of anions such as nitrate. In agreement with this, for both the 3-pyridinecarboxaldehyde and the butyraldehyde Inhibited signals, the line shapes and g values did not change in zwitterionic buffers between pH 5.5 and 10. These data suggest that conversion of the enzyme to the form giving the Inhibited signal prevents access of anions and buffer molecules to the molybdenum site.

Time Course of the Inhibitory Reaction and the Effect of Oxygen. Pick et al. (1971) found that during reaction of the enzyme with formaldehyde under aerobic conditions, loss of xanthine oxidase activity paralleled closely development of the formaldehyde Inhibited signal.

There has been a tendency in the literature on this signal to assume [by analogy with early work on inhibition of the enzyme by methanol (Rajagopalan & Handler, 1964)] that oxygen is required for the inhibitory reaction. In attempts to check this, we found differing results with different aldehydes. With formaldehyde, although at pH 8.2 maximum formation of the Inhibited signal occurred in the presence of oxygen, nevertheless, some formation of the signal occurred under anaerobic conditions, even in the presence of 1 mM dithionite. On the other hand, incubating xanthine oxidase anaerobically for 2 h with either 3-pyridinecarboxaldehyde or butyraldehyde gave no significant Inhibited EPR signal, though either of these substrates gave the signal readily under aerobic conditions.

We then turned our attention to following the inhibitory reaction, induced by incubation of xanthine oxidase with aldehydes under various conditions, by withdrawing samples of the reaction mixtures at intervals and performing xanthine oxidase activity measurements on them. Preliminary experiments indicated that, aerobically, both the enzyme concentration and the aldehyde concentration were of critical importance. Thus, the average number of enzyme turnovers with 3-pyridinecarboxaldehyde, which had to occur before the enzyme was inactivated, was as low as 15 with dilute enzyme and 100 mM aldehyde. However, this number of turnovers increased 10-fold if the concentration of the aldehyde was reduced to 2 mM or if, alternatively, the enzyme concentration was increased to 5–10 μM.

In Figure 2, the time course of inactivation of xanthine oxidase by 3-pyridinecarboxaldehyde, under standard conditions, but in the presence of varying amounts of oxygen, is shown. The retention of activity under anaerobic conditions

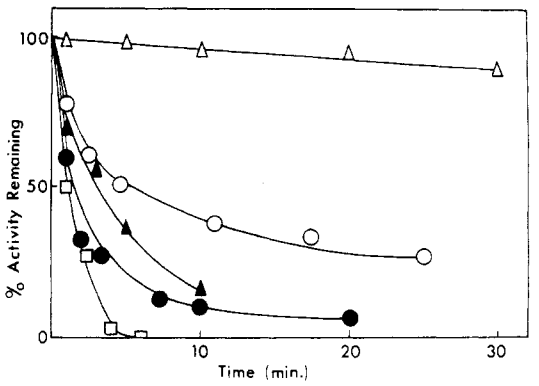


FIGURE 2: Time course of inactivation of xanthine oxidase by 3-pyridinecarboxaldehyde. The reactions were performed at 4 °C in 50 mM Bicine buffer, pH 8.2. The incubations contained 21 μM xanthine oxidase and 40 mM 3-pyridinecarboxaldehyde and were carried out under the following conditions: (△) anaerobic; (●) aerobic; (□) bubbling with oxygen; (○) aerobic in the presence of 100 mM salicylate; (▲) anaerobic in the presence of 10 mM potassium ferricyanide. Xanthine oxidase was assayed on samples withdrawn from the reaction mixtures as described under Materials and Methods.

and the loss of activity in the presence of oxygen are particularly noteworthy and confirm the oxygen requirement for the inhibitory reaction with this aldehyde, as indicated by the EPR experiments.

One possible reason for such an oxygen dependence would be that the aldehyde is not inactivating the xanthine oxidase directly but that hydrogen peroxide or the superoxide anion, both produced in the turnover, is somehow involved in the reaction. Either these might react with the aldehyde to give a more reactive species [cf. Marklund (1972)] or, alternatively, they might first react with the enzyme to activate it in some way. The latter possibility is made unlikely by recent studies in this laboratory (George, 1983) which show [cf. Betcher-Lange et al. (1979)] that reduced active xanthine oxidase is slowly converted by hydrogen peroxide to the inactive desulfo form of the enzyme. In any case, we confirmed directly that such reactions are not involved in aldehyde-induced inhibition. Thus, inclusion of superoxide dismutase (5 μM) and catalase (5 μM), or both enzymes, in aerobic incubation mixtures of xanthine oxidase and the aldehyde had no effect on the rate of loss of enzymic activity or on the rate of appearance of the Inhibited signal. Also, as Figure 2 shows, when xanthine oxidase is incubated anaerobically with 3-pyridinecarboxaldehyde (40 mM) and potassium ferricyanide (10 mM), as an alternative electron acceptor, than activity again rapidly disappears. We found, furthermore, that anaerobic incubation of this aldehyde with the enzyme in the presence of ferricyanide gave rapid appearance of the Inhibited signal (maximum intensity within 5 min).

The data thus confirm that only the enzyme and the aldehyde are involved in the inhibitory reaction, though the mechanism may be complex and more than one oxidation state of the enzyme may participate. It appears to be the aldehyde itself which is involved and not the carboxylic acid produced by its oxidation. This was indicated by our additional finding [cf. Pick et al. (1971)] that incubation of any of several carboxylic acids with xanthine oxidase under a variety of conditions caused no loss of activity and no appearance of the Inhibited signal.

Reversal of the Inhibitory Reaction. The reactivation of formaldehyde- or methanol-inhibited xanthine oxidase has been studied by a number of workers (Rajagopalan & Handler, 1964; Coughlan et al., 1969; Pick et al., 1971). A variety of conditions have been tried, but no more than 40–50% of the

Table II: Variation of the First-Order Rate Constant for Reactivation of Xanthine Oxidase following Inhibition by Various Aldehydes[a]

aldehyde	first-order rate constant (h^{-1})
2-pyridinecarboxaldehyde	5.2
3-pyridinecarboxaldehyde	0.8
4-pyridinecarboxaldehyde	0.7
formaldehyde	~0.01
acetaldehyde	0.4
propionaldehyde	2.0
butyraldehyde	1.7

[a] All experiments were carried out at 25 °C in 50 mM Na^+-Bicine, pH 8.2, by the procedure described in the caption to Figure 3 (inset).

enzymic activity was reported to be recovered, accompanied by complete loss of the Inhibited signal. The irreversible loss of activity may be ascribed (Pick et al., 1971) to formaldehyde's properties as a modifier of amino groups and to its possible role in modifying the flavin site [cf. Ní Fholáin & Coughlan (1977) and Morpeth (1983)] as well as the molybdenum site of the enzyme.

In contrast to these findings with formaldehyde, we were able to achieve essentially quantitative recovery of xanthine oxidase activity by simple aerobic incubation of samples of the enzyme which had been inhibited by treatment with a number of other aldehydes. Thus, when xanthine oxidase (100 μM) in 50 mM Na^+-Bicine, pH 8.2, was inactivated by incubation for 30 min at 25 °C with a large excess of 3-pyridinecarboxaldehyde (40 mM) or butyraldehyde (100 mM), and was subsequently separated from excess aldehyde by gel filtration in the same buffer, then at 25 °C activity rapidly reappeared. With both aldehydes, 95% or more reactivation was achieved.

Steady-state kinetic measurements of K_m and V_{max} in the xanthine oxidase assay confirmed that these two aldehydes had caused no permanent modification of the catalytic properties of the molybdenum center. After treatment with formaldehyde and subsequent reactivation of the enzyme, the K_m for xanthine was found to be unaffected, although the V_{max} was diminished.

Since we were able to form Inhibited species with a large number of different aldehydes, it seemed of interest to study, comparatively, the kinetics of the reactivation process. This was done, by the procedure outlined above, for a number of different aldehydes. Typical reactivation time course data are presented in Figure 3 (inset), and results are summarized in Table II. The linear semilogarithmic plots of activity vs. time obtained with three different aldehydes (Figure 3, inset) show that recovery of activity is a simple first-order process, and indeed this was found to be the case, irrespective of the initial percent functionality of the enzyme and of the nature of the modifying aldehyde.

First-order rate constants for the reappearance of activity for several aldehydes are given in Table II. Particularly striking is the comparatively fast rate of breakdown for 2-pyridinecarboxaldehyde (k = 5.2 h^{-1}) in comparison to 3-pyridinecarboxaldehyde (k = 0.8 h^{-1}) and 4-pyridinecarboxaldehyde (k = 0.7 h^{-1}) (Figure 3, inset). This is presumably due to the close proximity of the nitrogen to the aldehyde group in 2-pyridinecarboxaldehyde. There is also a large increase in the rate of breakdown as the carbon chain length of the simple aliphatic aldehydes is increased, the formaldehyde species breaking down with a first-order rate constant of about 0.01 h^{-1} while with butyraldehyde it was 1.7 h^{-1}. Thus, it would seem that the stability of the inhibited species is strongly

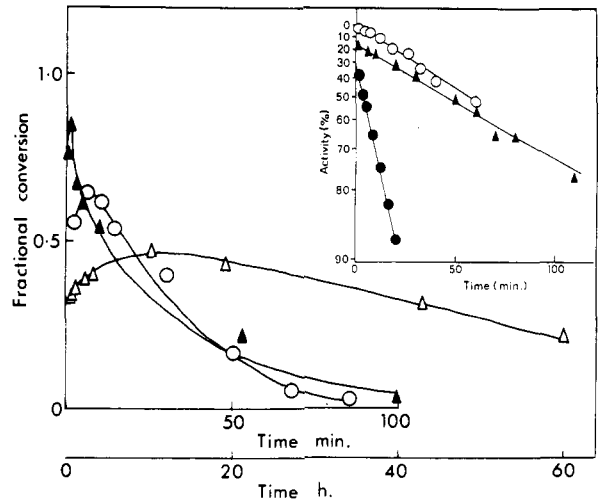


FIGURE 3: Variation of the absolute intensity of the Mo(V) Inhibited signal with time after separation from excess of the aldehyde and (inset) time course of reappearance of xanthine oxidase activity. Xanthine oxidase was modified with an aldehyde, and excess reagent was removed by gel filtration. In each case, 50 mM aldehyde was left to react with 0.5 mM xanthine oxidase (70% functional) at 4 °C for 40 min in 50 mM Na^+-Bicine buffer, pH 8.2. The mixture was then separated on Sephadex G-25. The inhibited enzyme was left to reactivate aerobically at 25 or 4 °C in the same buffer. In the *main graph*, the aldehydes and conditions were as follows: (○) butyraldehyde at 25 °C (these points should be read against the minutes scale); (▲) 3-pyridinecarboxaldehyde at 4 °C (these points should be read against the hours scale); (△) formaldehyde at 4 °C (these points should be read against the hours scale). Time t = 0 was taken as when the aldehyde-free xanthine oxidase was first eluted from the Sephadex G-25 column. Each point represents a separate sample of enzyme. The integrated intensities were determined as described in the text and corrected (at t = 0) for reactivation during gel filtration and for the presence of desulfo xanthine oxidase. The *inset* shows similar experiments in which, at suitable intervals and after appropriate dilution, samples were assayed for xanthine oxidase activity as described under Materials and Methods. The aldehydes used were (○) 3-pyridinecarboxaldehyde, (▲) 4-pyridinecarboxaldehyde, and (●) 2-pyridinecarboxaldehyde, and reactivation was at 25 °C. Activities (as activity/A_{450} ratios) are expressed as a percentage of those before treatment with the aldehyde and are plotted on a logarithmic scale. Some reactivation occurred during the gel filtration.

dependent on the local chemical environment in the molybdenum site.

If this were the case, then the rate of breakdown of the inhibited species could be dependent on the state of ionization of any residues in close proximity to molybdenum. The variation with pH of the first-order rate constant for the breakdown of butyraldehyde-inhibited xanthine oxidase was therefore investigated. Results (not shown) indicated enhanced rates of breakdown at pH values below a pK value of about 6.6. Limiting values of the rate constant above and below this pK were 1.4 and 3.6 h^{-1}, respectively. A corresponding experiment with 3-pyridinecarboxaldehyde indicated a similar pH dependence of the reactivation process. The nature of the ionizing group in the enzyme which is involved is uncertain.

Stability of the Molybdenum(V) Inhibited Signal. Though the original experiments of Pick et al. (1971) showed that the reactivation of formaldehyde-treated enzyme (apart from that induced by hydroxylamine) was invariably accompanied by loss of the Inhibited EPR signal, we thought it important to attempt to correlate activity reappearance and signal disappearance, after inhibition by other aldehydes. We therefore determined, by double integration of the Inhibited EPR signals, the proportion of molybdenum of active xanthine oxidase present as the signal at various times after separation by gel filtration of maximally inactivated enzyme from excess aldehyde. Results for three different aldehydes are presented

in Figure 3 (main graph). In all cases, it appears that signal disappearance is a biphasic process, with a small initial increase in signal intensity followed by a decrease. This is particularly noticeable with formaldehyde-inactivated xanthine oxidase. With 3-pyridinecarboxaldehyde- or butyraldehyde-modified enzyme, the initial increase is much less marked, but the effect has been observed in several experiments. The significance of this and of the variations from one aldehyde to another in maximal conversion of the enzyme to the Inhibited signal (Figure 3) will be considered under Discussion.

We compared the rate of disappearance of the signal, after the initial increases, in experiments of the type illustrated in Figure 3, with activity reappearance rates measured as previously described. The first-order rate constant describing the decrease in the 3-pyridinecarboxaldehyde Inhibited signal, at 4 °C in 50 mM Na^+-Bicine buffer, pH 8.2, was 0.056 h^{-1}. Under identical conditions, the activity reappeared with a first-order rate constant of 0.062 h^{-1}. Thus, within the accuracy of the experiments, the rates are the same. With the butyraldehyde-inhibited enzyme at 25 °C, the discrepancy was slightly larger, the rate constants for Inhibited signal disappearance and reappearance of activity (Table II) being 2.4 and 1.7 h^{-1}, respectively.

Effect of Oxygen on Reversal of the Inhibitory Reaction. All the experiments described so far involved reversal of the inhibitory reaction of aldehydes by simple aerobic incubation. It might have been assumed that oxygen would be important in the reactivation since oxidation of molybdenum(V) to molybdenum(VI) is involved and since, as mentioned previously, oxygen has effects, in some cases dramatic, on the rate of inhibition of xanthine oxidase by aldehydes. However, in contrast, we found in several experiments that the rate of reappearance of activity with 3-pyridinecarboxaldehyde-modified enzyme was independent of the presence of oxygen, as was the time course for the Inhibited signal disappearance. This finding clearly suggests that on breakdown of the Inhibited species, the electron trapped in molybdenum(V) is passed on to the iron–sulfur centers and the flavin (which are normally in the oxidized state in the Inhibited species).

To test this hypothesis, butyraldehyde-inhibited xanthine oxidase, carefully freed from excess aldehyde by gel filtration (see Materials and Methods), was rapidly made anaerobic in a special spectrophotometer cell by alternate flushing with oxygen-free argon and evacuation. The absorption spectrum was immediately recorded, and the spectral changes with time were followed (Figure 4). The optical spectrum at the end of the reaction clearly corresponds to a partially reduced state of xanthine oxidase [cf. Olson et al. (1974)]. The spectrum of fully oxidized xanthine oxidase appeared immediately on opening the cuvette to oxygen. We carried out this experiment a number of times with results similar to those shown. We quantified the extent of reduction of the enzyme occurring in the reactivation process, in each of these experiments, by measuring the percentage decrease of absorbance at 450 nm which was observed (cf. Figure 4). Such changes may be converted into numbers of reducing equivalents accepted per xanthine oxidase half-molecule by reference to reductive titration curves for the enzyme presented by Olson et al. (1974). However, two corrections have to be applied. First, xanthine oxidase is purified as a mixture of active and 20–30% inactive desulfo enzyme. The latter does not react with aldehydes to give the Inhibited species. The amount of desulfo enzyme present is readily estimated from the initial activity/absorbance at 450 nm ratio (Bray, 1975). Second, during the course of gel filtration to remove excess aldehyde, some 10–30% of the

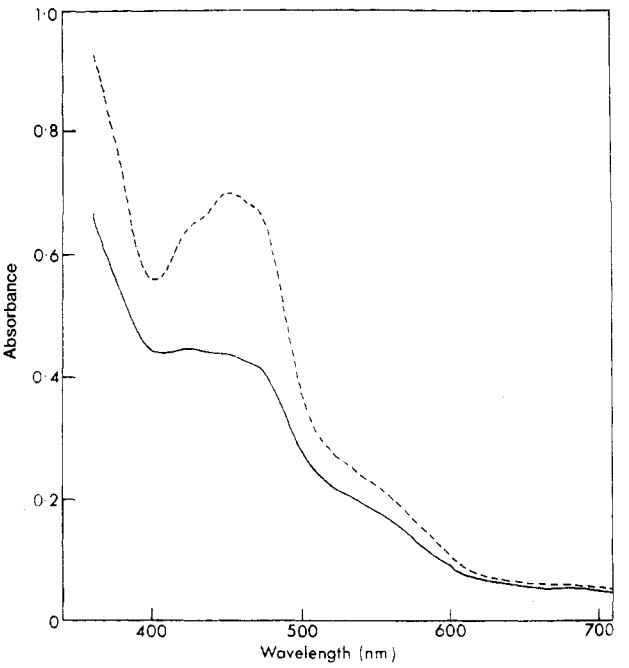
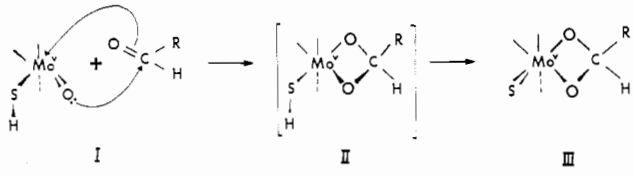


FIGURE 4: Spectra of anaerobic aldehyde-inhibited xanthine oxidase. The enzyme was inactivated by treatment with an excess of butyraldehyde, freed from the excess by gel filtration in 50 mM Na^+-Bicine, pH 8.2, and immediately made anaerobic in a spectrophotometer cell (see Materials and Methods). Spectra were recorded immediately (- - -) and 2 h later (—) when absorption changes had ceased.

inhibited enzyme, depending on the rapidity with which the separation was carried out, will have become reactivated. It is therefore necessary to extrapolate to zero time on the column. After these corrections have been applied, our final value from four experiments was three to four electrons per functional active center liberated in the reactivation process. We will reserve detailed consideration of this value to the Discussion. However, clearly [and allowing for some Mo(IV)] no more than 1-2 reducing equiv can have come from molybdenum. The other electrons, then, are presumably due to the aldehyde molecule being released and oxidized as the inhibited complex breaks down. Thus, contrary to earlier assumptions [e.g., see Bray (1982)], in the Inhibited signal-giving species, the aldehyde residue has not been oxidized.

Effect of Dithionite on Reversal of the Inhibitory Reaction. Pick et al. (1971), in their studies on the reactivation of formaldehyde-inhibited xanthine oxidase, reported that sodium dithionite was one of the most effective and fast-acting reactivating agents they found. The effectiveness of dithionite in eliminating the formaldehyde Inhibited signal has been confirmed in this laboratory (R. T. Pawlik and R. C. Bray, unpublished results), but it was found that only about 50% recovery of activity could be achieved, accompanied always by substantial conversion to the desulfo enzyme. These results emphasize the possibility that a molybdenum(IV) inhibited species may exist and be unstable. We therefore carried out experiments on another aldehyde. Aldehyde-free butyraldehyde-inhibited xanthine oxidase was made anaerobic by shaking the solution under a stream of nitrogen for a few minutes. Sodium dithionite (final concentration 10 mM) was added to the modified enzyme, and the rate of reactivation was followed as described earlier (cf. inset of Figure 3). The first-order rate constant for reactivation at 25 °C was 1.62 h^{-1}, which is in close agreement with the rate constant under aerobic conditions of 1.7 h^{-1} (Table II). Under identical conditions, the Inhibited EPR signal disappeared and was replaced immediately by a mixture of a very small amount

Scheme I: Suggested Reaction Mechanism for Formation of the Mo(V) Inhibited EPR Signal-Giving Species by Reaction of an Aldehyde with Partly Reduced Xanthine Oxidase[a]



I II III

[a] Structure I corresponds to the Rapid type 1 signal-giving species as depicted by Bray & Gutteridge (1982), II is a possible intermediate which is not detected, and III is the Inhibited signal-giving species.

of a Rapid type 1 and a Slow signal. During the reactivation process, no increase in the Rapid signal was seen, but this would hardly be expected, since active enzyme would be predominantly in the molybdenum(IV) state under these conditions (the Slow signal is from the desulfo form of the enzyme). These findings are consistent with the existence of a molybdenum(IV) inhibited species but indicate that its stability may vary from aldehyde to aldehyde and may not be parallel to the stability in the molybdenum(V) state.

Discussion

Structure of the Inhibited Signal-Giving Species. Our work with a variety of aldehydes provides considerable new information on the nature of the inhibitory reaction between aldehydes and the molybdenum center of xanthine oxidase and related enzymes. The structure of the Inhibited EPR signal-giving species, on the basis of the present work and that of Bray & Gutteridge (1982), is most likely that shown in Scheme I (structure III). The particular contribution of the present work is the finding that, in the signal-giving species, oxidation of the aldehyde residue has not taken place. Thus, the aldehyde molecule, shown in its hydrated state and linked to molybdenum through two oxygen atoms, still carries the aldehydic hydrogen. In earlier work [e.g., see Bray (1982)], it was assumed that oxidation of the aldehyde had taken place, so that this hydrogen was replaced by a hydroxyl.

For the formaldehyde (or methanol) Inhibited species (R = H), hyperfine couplings to Mo(V) have been reported for the carbon, the sulfur, the two oxygens, and one of the hydrogens of structure III of Scheme I, and indeed, the basic structured was proposed (Bray & Gutteridge, 1982) on the basis of these data. The lack of coupling to Mo(V) of the proton in structure III for all aldehydes (and of the second proton from formaldehyde) is probably not surprising, since it is the third ligand out from the molybdenum. Rather, it is the coupling of the first proton in the formaldehyde Inhibited signal which is unusual, and which no doubt relates to a particular geometric relationship in the complex of this aldehyde with the enzyme.

Alternative structures and geometries for the Inhibited signal-giving species also have to be considered. In particular, there is a possibility that the sulfur and one of the oxygens in structure III are interchanged [cf. George (1983)], thus making the oxygen a terminal ligand, with one bridging oxygen and one bridging sulfur between molybdenum and carbon. [The ^{17}O data of Bray & Gutteridge (1982) do not exclude this possibility. Note also that since the sulfur is only weakly coupled (Malthouse et al., 1981b) this may be in an axial position.] However, further support for a structure with two bridging oxygen ligands as in III is provided by our observations that benzaldehyde and various derivatives failed to inhibit the enzyme, as tested by activity measurements or by looking for the Inhibited EPR signal. Thus, benzaldehyde, salicylaldehyde, *o*-fluorobenzaldehyde, *p*-nitrobenzaldehyde, and 2,5-dihydroxybenzaldehyde all failed to inhibit. (Though, because of solubility problems some of these compounds had to be tested at relatively low concentrations, the failure of 2,5-dihydroxybenzaldehyde to inhibit is particularly significant, since it was used at 100 mM.) We interpret this failure of benzaldehyde derivatives to inhibit as follows. Whereas simple aliphatic aldehydes and pyridinecarboxaldehydes readily become hydrated, benzaldehyde does not do so (Pocker & Meany, 1967). Thus, formation of structure III, which is analogous to that in the aldehyde hydrates, would be unlikely for the aromatic aldehydes.

Oxidation State of Molybdenum in the Inhibitory Product and in Relation to Its Formation and Decay. The Rapid molybdenum(V) EPR signal is the normal signal from partly reduced functional xanthine oxidase (Bray, 1980), and conversion to the signal-giving species is governed by the redox potentials for the systems Mo(VI)/Mo(V) and Mo(V)/Mo(IV) in the enzyme (Cammack et al., 1976). Since at pH 8.2 the potentials of these two systems are roughly equal, it follows that the maximum yield of the Rapid signal is about 33%. Similar considerations apply to the Slow signal from the desulfo enzyme, though here equilibria among the three oxidation states of molybdenum are only rather slowly established (Cammack et al., 1976). More work would be required to establish whether for the Inhibited signal a similar situation applies. For the 3-pyridinecarboxaldehyde Inhibited signal, maximum conversion to the signal-giving species in a number of experiments was 65–80% (Figure 3), values which are higher than have been reported previously from this laboratory for Mo(V) signals from xanthine oxidase or any other molybdenum-containing enzyme. For other aldehydes, particularly formaldehyde, lower values were obtained. It is noteworthy, e.g., for butyraldehyde, for which a maximum of about 65% conversion to the Mo(V) Inhibited signal was obtained when the enzyme had lost all activity, that this activity loss was fully reversible. This certainly provides strong evidence for the presence of Mo(VI) or Mo(IV) Inhibited species for this aldehyde.

On the whole, the data appear to favor a Mo(IV) species not in rapid equilibrium with the Mo(V) Inhibited species. Thus, in the reactivation experiments of Figure 3, the initial rather small transient increase in intensity of the EPR signal, if this is not due to artifacts, could well be due to oxidation of a Mo(IV) species. Similarly, the finding that somewhat more than 3 reducing equiv is liberated [2 equiv for oxidation of the aldehyde and 1 equiv for Mo(V)] points to some Mo(IV). The activity data indicate that while with butyraldehyde reduction to such a Mo(IV) state does not change the reactivation rate significantly, with formaldehyde reduction speeds up reactivation. Conversely, our finding for some aldehydes of parallelism between the rates of Inhibited signal disappearance and of activity reappearance would suggest that any molybdenum(VI) inhibited species is unstable.

Lastly, the effect of the reaction conditions, and particularly the effect of the presence or absence of oxygen, on the development of inhibition by aldehydes and on the formation of the Inhibited EPR signal has to be considered. The question of interest is whether the aldehyde molecule reacts, to inhibit the enzyme, with molybdenum initially in the Mo(VI), Mo(V), or Mo(IV) state. Unfortunately, we are not able at present to answer this question in a definite way, because the inhibitory reaction is slow compared with enzyme turnover and as soon as turnover starts all three oxidation states are present together. A finding favoring the lower oxidation states is the requirement

for high concentrations of aldehydes to obtain inhibition. However, as with the reactivation process, different aldehydes behave differently, and the finding that with 3-pyridinecarboxaldehyde the enzyme only became inhibited in the presence of oxygen or ferricyanide clearly points, in this case, toward preferential involvement of the higher oxidation states of the metal in the inhibitory reaction. We have to conclude that the inhibitory reaction can probably occur with molybdenum in more than one oxidation state and that the observed reaction rate is characteristic not only of the aldehyde but also of the oxidation state in which the metal is reacting.

A possible clue to the origin of differences in the behavior of different aldehydes in this respect may be given by consideration of the Rapid signals. Active enzyme gives two kinds of Rapid signals, type 1 and type 2, depending on the nature of the substrate or other molecules or ions which are present in the solution (Bray, 1980). The nature of the difference between these two species is not properly understood, but type 2 Rapid signals are associated with an extra oxygen ligand of molybdenum as detected by ^{17}O studies (Bray & Gutteridge, 1982). We found that at pH 8.2, 3-pyridinecarboxaldehyde gives Rapid type 2 signals, whereas formaldehyde gives Rapid type 1 signals (Bray & Meriwether, 1966). Whether the formation of the type 2 signal is related in any way to the failure of the pyridinecarboxaldehyde to inhibit under anaerobic conditions remains to be seen.

Scheme I shows a possible reaction mechanism for formaldehyde reacting with the active center to give the Inhibited species, when starting with molybdenum in the Mo(V) state, in the Rapid type 1 configuration as depicted by Bray & Gutteridge (1982) (structure I). The scheme shows nucleophilic attack by the terminal oxygen ligand of molybdenum, as in the turnover reaction (Gutteridge & Bray, 1980). However, with the sulfur ligand already protonated in structure I, turnover cannot proceed, since there is no acceptor for the aldehyde proton (or for two electrons), and instead the carbonyl group attacks molybdenum to give the Inhibited species (III) via the intermediate (II). With regard to reaction of methanol to give the Inhibited species, we note that its oxidation, according to the mechanism of Gutteridge & Bray (1980), must involve, as an intermediate, a hydrated formaldehyde species already linked to molybdenum through one of its oxygens. Thus, the finding (Pick et al., 1971) that methanol is effective in giving the Inhibited signal without the accumulation of significant concentrations of formaldehyde in the solution is understandable.

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Registry No. Molybdenum, 7439-98-7; propionaldehyde, 123-38-6; butyraldehyde, 123-72-8; 4-pyridinecarboxaldehyde, 872-85-5; 2-pyridinecarboxaldehyde, 1121-60-4; 3-pyridinecarboxaldehyde, 500-22-1; 3-indolecarboxaldehyde, 487-89-8; formaldehyde, 50-00-0; acetaldehyde, 75-07-0; indole-5-acetaldehyde, 88730-27-2; xanthine oxidase, 9002-17-9.

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