The Inhibition of Xanthine Oxidase by 8-Bromoxanthine*

Russ Hille$ and Richard C. Stewart$  
From the Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109  

(Received for publication, August 24, 1983)

The interaction of xanthine oxidase with the substrate analog 8-bromoxanthine has been examined in an effort to determine the nature of interaction of purines with the active site of the enzyme. It is found that 8-bromoxanthine is an inhibitor of xanthine oxidase with a $K_i$ of approximately 400 $\mu$M; inhibition is uncompetitive with respect to xanthine and noncompetitive with respect to molecular oxygen. While 8-bromoxanthine has only a slight effect on the reaction of reduced enzyme with oxygen, it dramatically slows the rate of enzyme reduction by xanthine, suggesting that inhibition does involve the interaction of 8-bromoxanthine with the molybdenum center of the enzyme. $K_i$ determinations for binding of 8-bromoxanthine to oxidized and reduced xanthine oxidase indicate that the inhibitor binds preferentially to the fully reduced form of the molybdenum center (Mo$^{5+}$), with dissociation constants of 1.5 mM and 18 $\mu$M for oxidized and reduced enzyme, respectively. This preferential binding to the reduced form of the enzyme is manifested in a significant increase in the oxidation-reduction potentials of the molybdenum center as determined by potentiometric titrations with 8-bromoxanthine complexed with xanthine oxidase. The shape of the Mo$^V$ EPR signal observed in the course of these titrations as well as a comparison with results of reductive titrations and $K_i$ determinations with uric acid and xanthine indicate that 8-bromoxanthine interacts with the molybdenum center of xanthine oxidase in a way that is typical of purine substrates and products, despite the presence of the bulky Br group. The inhibitor thus has a potential as a probe of enzyme-substrate interactions, particularly using the technique of x-ray absorption spectroscopy.

Xanthine oxidase is a complex molybdoflavoprotein that catalyzes the hydroxylation of xanthine to form uric acid. This reaction takes place at the molybdenum center of the enzyme and formally involves the removal of a hydride ion from position 8 of the purine ring followed by its replacement with hydroxide from water. A great deal of research effort has focused on the molybdenum center of xanthine oxidase, primarily using electron paramagnetic resonance spectroscopy (for a comprehensive review, see Ref. 1) but also more recently x-ray absorption spectroscopy (2-4). The precise manner in which substrate interacts with the molybdenum center of the enzyme and the sequence of events that result in conversion of substrate to product remain only poorly understood, however, and a number of alternative mechanisms have been proposed (5-8). In an effort to gain further insight into the nature of the molybdenum center of xanthine oxidase, the interaction of enzyme with the substrate analog 8-bromoxanthine has been investigated. This purine derivative, containing a bromine atom at the position normally hydroxylated by enzyme, is found to be an effective inhibitor of xanthine oxidase. By several criteria, however, it is found to interact with enzyme in substantially the same manner as xanthine and uric acid and is thus potentially of use in establishing the nature of the enzyme-substrate interaction at the molybdenum center.

MATERIALS AND METHODS

Xanthine oxidase was isolated from fresh unpasteurized milk by the method of Massey et al. (9) with the addition of the folate affinity chromatography procedure described by Nishino et al. (10) as a final step. Enzyme thus obtained was approximately 70% active, having an AFR value in the range of 140-150. (AFR is defined as the ratio of the absorbance change per min at 295 nm monitoring the conversion of xanthine to uric acid divided by the enzyme absorbance at 450 nm in the assay mix. Under the conditions of the standard assay, 111 $\mu$M xanthine, 200 $\mu$M oxygen, 25 °C, in 0.1 M pyrophosphate, 0.3 mM EDTA, pH 8.5, fully active enzyme has an AFR of 210; see ref. 11.) When necessary, active xanthine oxidase was separated from the inactive form of the enzyme as described by Nishino et al. (10). Using this procedure, enzyme exhibiting an AFR in excess of 200 (i.e. was greater than 95% active) could routinely be obtained.

8-Bromoxanthine was prepared by refluxing 1 g of xanthine in 4 g of bromine in a sealed glass tube for 24 h at 90 °C as described by Fischer and Reese (12). After recrystallization from dilute NH$_4$OH a white powder was obtained that exhibited the spectral properties of 8-bromoxanthine in both 0.1 M KOH ($\lambda_{max}=274$ nm) and 10$^{-5}$ M KOH ($\lambda_{max}=287$ nm). The purity of the reaction product was confirmed by thin layer chromatography using butanol, acetic acid, and water in the ratio 15:3:5 as the developing solvent. In this system, 8-bromoxanthine exhibited an $R_f$ of 0.65 while xanthine and uric acid exhibited the same $R_f$ value of 0.49.

Optical absorption spectra were recorded with a Cary model 118 or 219 spectrophotometer. Room temperature electron paramagnetic resonance (EPR) spectra were recorded with a Varian 112 X-band spectrometer equipped with a TE 102 cavity. Spectra were recorded at ambient temperature with the sample in a quartz EPR flat cell in order to minimize absorption of the microwave radiation by solvent water. Room temperature potentiometric EPR titrations were performed using an apparatus similar to that described by Porras and Palmer (13) using sodium dithionite as reductant. Operating conditions were: field set 3450 G; scan range, 400 G; time constant, 0.25 s; modulation amplitude, 8 G; modulation frequency, 100 kHz; microwave power, 50 milliwatts; microwave frequency, 9.5 GHz. EPR signals were integrated using the method described by Fee (14) to determine the concentration of paramagnetic species. Spectra at 100 K were recorded using standard 4-mm (inner diameter) quartz tubes under the same instrument settings except the microwave power was 27 milliwatts. In the potentiometric titrations, the battery of communication dyes used by Hille et al. (15) were employed. When necessary to determine the concentration of reductant used in the reductive titrations, the stock sodium dithionite solution was stand-
ardized by titration against lumiflavin 3-acetate (16).

Kinetic experiments were performed using a stopped flow spectrophotometer designed and built by Dr. David P. Ballou, Department of Biological Chemistry, The University of Michigan. This instrument was placed on-line to a Data General Nova 2 minicomputer, as described by Entsch et al. (17).

Xanthine (Grade V) was purchased from Sigma and uric acid from Eastman. Unless otherwise stated experiments were performed in 0.1 M sodium pyrophosphate, 0.3 mM EDTA, pH 8.5, at 25 °C.

RESULTS AND DISCUSSION

Steady State Inhibition of Xanthine Oxidase by 8-Bromoxanthine—In order to evaluate the inhibitory effect, if any, of 8-bromoxanthine on the activity of xanthine oxidase, two sets of steady state experiments were performed. In the first, the oxygen concentration was held constant at 900 μM while the concentrations of xanthine and 8-bromoxanthine were systematically varied. In the second, xanthine was held constant at 150 μM and the concentrations of oxygen and 8-bromoxanthine varied. In both experiments, the concentration of the substrate held constant was greater than 10-fold its Km (50 μM for oxygen, 10 μM for xanthine; see Refs. 9 and 18), so that the results reflected to a good approximation the extrapolation to infinite substrate concentration. The results of these experiments are shown in Fig. 1. Somewhat surprisingly, it is found that 8-bromoxanthine, an analog of xanthine, is an uncompetitive rather than competitive inhibitor with respect to reducing substrate; the lines at different concentrations of 8-bromoxanthine in the Lineweaver-Burk plot are parallel (Fig. 1A). A plot of the y axis intercept from the primary plot versus the concentration of 8-bromoxanthine (Fig. 1A, inset) indicates a K of 420 μM. On the other hand, 8-bromoxanthine is a noncompetitive inhibitor with respect to oxygen, as the lines at different inhibitor concentrations converge very near the x axis (Fig. 1B). A plot of the y axis intercept versus the inhibitor concentration (Fig. 1B, inset) gives a value for K of 380 μM, in reasonable agreement with the results from Fig. 1A. The noncompetitive nature of 8-bromoxanthine inhibition with respect to oxygen is to be expected, given that the two interact independently of one another at different sites of the enzyme: the oxygen at the FAD (18) and the 8-bromoxanthine at the molybdenum center (see below). It should be pointed out that the Km values for xanthine and oxygen of 11 and 45 μM, respectively, obtained from the steady state data at zero inhibitor concentration are in good agreement with the accepted literature values reported above.

Xanthine oxidase acts via a ping-pong mechanism, with enzyme alternating between oxidized and reduced forms during turnover (9, 19). The parallel lines observed in Lineeweaver-Burk plots of 1/v versus 1/(xanthine) at a series of oxygen concentrations (or vice versa) are to be expected, however, as xanthine and oxygen interact with enzyme at different sites: the molybdenum center and FAD, respectively. Furthermore, the molybdenum center must be oxidized to MoV and the FAD site reduced to FADH2 for the reductive and oxidative half-reactions, respectively, to take place. A plausible explanation for the noncompetitive inhibition for 8-bromoxanthine with respect to xanthine and its noncompetitive inhibition with respect to oxygen is that 8-bromoxanthine interacts with the molybdenum center of xanthine oxidase but binds to the reduced form of the site, not its oxidized form as does xanthine. Precedent exists for this type of inhibition pattern in the inhibition of xanthine oxidase by alloxanthine (another substrate analog, which forms an extremely tight complex with MoV; see Ref. 20).

Inhibition of the Reductive Half-reaction by 8-Bromoxan-

Fig. 1. Steady state analysis of the inhibition of xanthine oxidase by 8-bromoxanthine. Enzyme concentration was 6.5 x 10^-5 M and was 75% active. In the experiment shown in A, the oxygen concentration was held constant at 600 μM and the xanthine concentration systematically varied at a series of fixed concentrations of 8-bromoxanthine. The inhibitor concentrations used were (from bottom to top): 0, 62, 124, and 310 μM. In the experiment shown in B, the xanthine concentration was held constant 150 μM while the oxygen concentration was systematically varied at a series of fixed concentrations of 8-bromoxanthine. The concentrations of inhibitor used were (again from bottom to top): 0, 100, 200, and 400 μM. In both sets of experiments, the substrate held constant was present at a concentration greater than 10-fold its Km and was, therefore, a reasonable approximation to infinite substrate concentration. The Km values obtained from secondary plots of the y intercept versus the inhibitor concentration in A and B (insets) were 420 and 380 μM, respectively, and in reasonable agreement. The Km values of 11 and 45 μM for xanthine and oxygen, respectively (obtained from the lines in the absence of inhibitor) are in good agreement with the literature values of 10 and 50 μM, respectively (50). T/N, turnover number.
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**Fig. 2.** The inhibition of the reductive half-reaction of xanthine oxidase by 8-bromoxanthine. Enzyme concentration was 5.7 μM. The concentrations of 8-bromoxanthine employed were: 0 μM, 100 μM, 200 μM, 400 μM, and 2.0 mM. From the secondary plot (inset) a K_inhibitor of 400 μM was determined, in good agreement with the steady state results.

75% of the absorbance increase at 450 nm, and its dependence on oxygen concentration were unaffected by the presence of 1.0 mM 8-bromoxanthine (data not shown). The remaining 25% of the reaction was slower in the presence of 8-bromoxanthine, especially above 250 μM oxygen, and multiphasic. This effect is likely a secondary one due to the higher potential at the molybdenum center in the presence of 8-bromoxanthine (see below), rather than a direct effect of 8-bromoxanthine on the rate at which reduced flavin reacts with O_2. The net result of the increase in molybdenum potential would be to remove reducing equivalents from the flavin center, slowing the reaction with oxygen with partially reoxidized enzyme species. Because of the complexity of the slow phase, with effects due to inhibitor dissociation from the molybdenum center, the slow phase was not analyzed further to point out that the observations were qualitatively consistent with inhibitor binding only at the molybdenum center of the enzyme. Taken together with the kinetic results from the reductive half-reaction and the steady state analysis, it is concluded that 8-bromoxanthine inhibits xanthine oxidase by slowing the rate at which enzyme in reduced by xanthine, but in a way that is noncompetitive with respect to xanthine.

**Fig. 3.** Difference titrations of oxidized (A) and reduced (B) xanthine oxidase with 8-bromoxanthine. The enzyme concentrations used were 20.6 and 51.9 μM, respectively. In the former titration, the enzyme was 75% active, in the latter, 98%. In the titration with oxidized enzyme (A) the 8-bromoxanthine concentrations were: 0 μM (base-line), 24.5 μM, 41.3 μM, 82.6 μM, 165 μM, 558 μM, 868 μM, 1.48 mM, and 4.11 mM. The plot of ΔAinal/ΔA versus 1/(8-bromoxanthine) (A, inset) indicates a K_D of 1.5 mM. This spectral change is dependent on the flavin site of the enzyme (see text). In the titration with reduced enzyme (B) the total 8-bromoxanthine concentrations were: 0 (base-line), 19.5, 39, 58.5, 78, 117, 156, and 195 μM. The spectra shown were corrected for dilution after addition of the 8-bromoxanthine. The plot of ΔAinal/ΔA versus 1/(8-bromoxanthine)_inh (B, inset) indicates a K_D of 18 μM. Because the enzyme concentration was on the order of the K_D, it was necessary to determine the concentration of unbound inhibitor explicitly. This was calculated from the total inhibitor concentration minus the amount bound to enzyme (determined from the fractional absorbance change times the total enzyme concentration). This spectral change is dependent on the molybdenum center of the enzyme (see text).

With that shown in Fig. 3A on binding to native or cyanolyzed (inactivated) enzyme (data not shown). Furthermore, binding of ligands to many simple flavoproteins exhibits the same or a very similar spectral change. Examples include the binding of aromatic amino acids to D-amino acid oxidase (21), p-methoxybenzaldehyde to old yellow enzyme (23), benzoate to D-amino acid oxidase (24), and tartronate to lactate oxidase (25). None of these enzymes are known to contain a cofactor other than flavin. It appears likely, therefore, that the ligand-binding phenomena associated with spectral changes similar to that observed with 8-bromoxanthine and oxidized xanthine oxidase reflect a change in the environment of the flavin cofactor and in the present case is not related to the molybdenum center of the enzyme. That this is the case is supported by two further pieces of evidence. First, uric acid, the product of enzymic action on xanthine and a compound known to interact at the molybdenum center (26, 27) does not produce a spectral change similar to that shown in Fig. 3A (this work, data not shown). Second, inactivation of the molybdenum center by cyanide (11) does not alter the ability of 8-bromo-
xanthine to elicit the spectral change, whereas removal of the FAD to generate the deflavox form of xanthine oxidase (18) completely abolishes the ability of the enzyme to produce the spectral change on addition of 8-bromoxanthine. Because inhibitor appears to be interacting with the flavin site of oxidized enzyme and does so with a $K_D$ several-fold greater than the $K_e$ determined from the steady state and reductive half-reaction experiments, it is concluded that the interaction of 8-bromoxanthine with oxidized xanthine oxidase is not the interaction responsible for inhibition.

Binding of 8-bromoxanthine to dithionite-reduced xanthine oxidase, on the other hand, produces a completely different spectral change than that observed with oxidized enzyme, as shown in Fig. 3B. The $K_D$ associated with this spectral change is 18 $\mu$M (Fig. 3B, inset), two orders of magnitude lower than the $K_e$ observed for binding to oxidized enzyme. The extinction change at 360 nm for 8-bromoxanthine binding to reduced xanthine oxidase is 1.55 mm$^{-1}$ cm$^{-1}$. This value may be compared with the extinction change for alloxanthine binding to the reduced molybdenum center, which is approximately 4 mm$^{-1}$ cm$^{-1}$ at 380 nm (20). In contrast to the case with oxidized enzyme, the spectral change associated with the binding of 8-bromoxanthine to reduced xanthine oxidase is unperturbed on removal of the flavin but is abolished by pretreatment of the enzyme with cyanide (data not shown). The binding interaction associated with the spectral change shown in Fig. 3B thus appears to involve the molybdenum center of the enzyme, not the flavin, and is, therefore, the more likely candidate for the interaction giving rise to inhibition.

Kinetics of 8-Bromoxanthine Binding to Reduced Xanthine Oxidase—Given the convenient spectral change associated with the binding of 8-bromoxanthine to xanthine oxidase, the kinetic behavior of inhibitor binding could be examined with a stopped flow apparatus by monitoring the absorption increase at 360 nm. At all concentrations of 8-bromoxanthine employed, the reaction time course was accurately represented as a single exponential process. A plot of $k_{obs}$ versus the 8-bromoxanthine concentration was apparently hyperbolic (not shown), but when replotted as $1/(k_{obs} - k_e)$ versus 1/(8-bromoxanthine) the data did not yield a straight line but rather was bowed, as shown in Fig. 4A. The most straightforward interpretation for this type of behavior is that inhibitor binding is a two-step equilibrium process, the spectral change being associated with the second step, with a finite rate for the reverse rate of the second step, $k_i$ (28).

$$ E + I \rightleftharpoons E.I \rightleftharpoons E.I^* \quad k_i \neq 0 $$

In such a mechanism the double reciprocal plot will asymptote at high 1/(I) values to give 1/$k_e$. A replot of 1/(1/$k_{obs} - k_e$) versus 1/(I) should give a straight line whose x and y intercepts accurately reflect $-1/K_D$ and 1/$k_e$, respectively (28). In the absence of a well defined asymptote (in this case a result of the vanishing amount of spectral change observed at low concentrations of 8-bromoxanthine), $k_i$ may be obtained in the following way. Estimates for $K_D$ and $k_e$ can be obtained from the x and y intercepts of the limiting slope of the data at low 1/(I) values. From the fact that $K_D \times k_e/k_i$ must equal the overall $K_D$ of 18 $\mu$M, $k_e$ can be calculated from limiting slope in Fig. 4A, $K_D$ and $k_i$ can be estimated at 160 $\mu$M and 135 s$^{-1}$, respectively. The value for $k_i$ obtained as just described is 15 s$^{-1}$. Fig. 4B shows a replot of 1/(1/$k_{obs} - 15$ s$^{-1}$) versus 1/(8-bromoxanthine), and it can be seen that the data now give a straight line. That the same values for $K_D$ and $k_i$ are obtained in the replot as in the original double reciprocal plot indicates that the line drawn in Fig. 4A does in fact accurately represent the limiting slope of the data at low values of 1/(I). The equilibrium constant for the second step in the binding scheme, $k_i/k_e$, can be calculated and is found to be 0.11, i.e. the equilibrium position lies to the right as drawn above.

Interaction of Uric Acid and Xanthine with Reduced Xanthine Oxidase—In order to establish whether the interaction of 8-bromoxanthine is relevant to catalysis, titrations of reduced xanthine oxidase were performed with uric acid and xanthine. Uric acid has been reported to bind to reduced enzyme and raise the oxidation-reduction potential of the molybdenum MoV/MoIV couple significantly (28). Xanthine also appears to raise the potentials of both MoV'/MoV and MoV'/MoIV couples, at least in the special case of enzyme that has been complexed with the inhibitor arsenite (15, 29). Reduction of the arsenite-complexed molybdenum center to the MoV valence state (as observed by EPR) occurs earlier in the course of reductive titrations when xanthine is present than when it is absent. If the effect of 8-bromoxanthine on reduced enzyme could be correlated with observations using uric acid and xanthine, results with the inhibitor could prove relevant to the catalytic sequence. Titrations of reduced xanthine oxidase with uric acid and xanthine are shown in Fig. 5, A and B, respectively. In both cases the spectral change observed on binding purine is very similar to that observed with 8-bromoxanthine (Fig. 3B). Furthermore, the $K_D$ values observed for urate and xanthine (18 and 25 $\mu$M; Fig. 5, insets) are comparable to those observed for 8-bromoxanthine (i.e. 18 $\mu$M). The kinetics of urate and xanthine binding to dithionite-reduced enzyme is also similar to the results with 8-bromoxanthine, giving linear double reciprocal plots with non-zero y axis intercepts (data not shown) to suggest again a pre-equilibrium step. The $K_D$ and $k_i$ determined for urate were 115 $\mu$M and 130 s$^{-1}$, respectively, and 125 $\mu$M and 230 s$^{-1}$, respectively, for xanthine.
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The above results suggest that 8-bromoxanthine, uric acid and xanthine interact with the reduced molybdenum center in fundamentally the same way. Further evidence that this is the case comes from EPR work monitoring the paramagnetic MoV state at liquid nitrogen temperatures. The molybdenum EPR signal typically observed from the (functional) active site in enzyme partially reduced by sodium dithionite is shown in Fig. 6A. When the sample is reduced in the presence of 1.0 mM 8-bromoxanthine, the spectrum shown in Fig. 6B is observed. This spectrum is very similar to the so-called Rapid Type 2 signals observed with partially reduced enzyme in the presence of either xanthine or uric acid (28), indicating that 8-bromoxanthine perturbs the environment of MoV in the active site of xanthine oxidase in much the same way as do xanthine and urate.

Room Temperature Potentiometric Titrations of Xanthine Oxidase in the Presence of 8-Bromoxanthine—From thermodynamic considerations, if 8-bromoxanthine binds more tightly to the reduced molybdenum center of xanthine oxidase than to the oxidized form, it must raise the oxidation-reduction midpoint potential of that site. This is known to be the case with urate, which raises the midpoint potential of the molybdenum center by approximately 45 mV (27). These results were obtained at liquid nitrogen temperatures, however, and it has been shown that the various oxidation-reduction potentials of xanthine oxidase are extremely sensitive to experimental conditions (temperature, pH, and buffer, Refs. 13 and 30). In order to determine the effect of 8-bromoxanthine as well as urate on the oxidation-reduction potentials of the molybdenum center at room temperature under the present experimental conditions, potentiometric titrations of enzyme in the presence of 8-bromoxanthine or urate were performed using an apparatus similar to that described by Porras and Palmer (13), with 0.1 M pyrophosphate, pH 8.5, 0.3 mM EDTA as buffer.

Fig. 7 shows the reduction profiles for (Rapid) MoV in the course of potentiometric titrations performed in the absence and presence of uric acid and 8-bromoxanthine. In the absence of either purine (open circle) the profile for the functional molybdenum center is best fit using potentials for the MeV'/MoV and MoV/MorV couples of −415 and −430 mV, respectively. These potentials are appreciably lower than those obtained by Spence et al. using microcoulometry and 0.05 M pyrophosphate, pH 8.2, 1.0 mM EDTA, but not outside the...
The equilibrium constants thus obtained are 1.5 mM and 0.9 mM for 8-bromoxanthine binding to MoVI and MoV, respectively. Two observations are noteworthy in regard to these values. First, the results indicate that the change in the chemistry of the molybdenum center that occurs on reduction of the metal (as reflected in the increased affinity for inhibitor on reduction) is associated for the most part with the MoV/MoVI couple. Thus, at least in regard to the binding of 8-bromoxanthine (but also urate) MoV more resembles MoVI in its chemistry than it does MoV. Second, the KD calculated for binding of 8-bromoxanthine to MoVI is the same as that determined experimentally for inhibitor binding to oxidized enzyme, monitoring a spectral change that is clearly due to a perturbation of the flavin environment. It is possible that this is merely a coincidence with two separate binding events (one at the molybdenum center, the other at the FAD) having the same KD, but we cannot at present rule out the possibility that inhibitor binding to oxidized enzyme occurs exclusively at the molybdenum center (albeit in a way that cannot involve the essential sulfur of the active site as binding to oxidized desulfoenzyme also gives the spectral change) and perturbs the flavin environment by means of a conformational change.

**CONCLUSIONS**

The substrate analog 8-bromoxanthine has been found to be an inhibitor of xanthine oxidase, having a KD determined from steady state analysis of approximately 400 μM. Inhibition is uncompetitive with respect to xanthine and is found to be due to the preferential binding of inhibitor to the reduced form of the molybdenum center of enzyme (resulting in the inability of enzyme to participate in the reductive half-reaction of the catalytic cycle). This conclusion is supported by the observation of a rather tight KD for inhibitor binding to reduced enzyme (18 μM) determined from spectrophotometric titration and by the observation that inhibitor dramatically slows the anaerobic reduction of enzyme by xanthine but has only a minor effect on the reduction of oxidized enzyme by molecular oxygen. The KD for 8-bromoxanthine determined from the reductive half-reaction is 395 μM in good agreement with the steady state results, but at variance with the KD of 18 μM determined from equilibrium titration. This discrepancy is to be expected, however, if the inhibitory complex involves MoV. In the steady state and reductive half-reaction experiments the inhibitor interacts with only partially reduced enzyme species, and the fraction of molybdenum centers present as MoV is much less than 1.0. The result is that a large fraction of the enzyme population does not have molybdenum in the appropriate oxidation state for complex formation, and the observed KD will, therefore, be considerably higher than the KD for inhibitor binding to completely reduced enzyme (containing 100% MoVI) as is observed.

Given the evidence that 8-bromoxanthine inhibits xanthine oxidase by binding to the reduced form of the molybdenum center, it might seem anomalous that the inhibitor would slow the rate of reduction of oxidized enzyme by xanthine. It must be remembered, however, that three substrate molecules are required to completely reduce xanthine oxidase and that the second and third xanthine molecules interact with enzyme that is already partially reduced. It is these two steps that are affected by inhibitor. Furthermore, although the intrinsic rate constants associated with the three steps in enzyme reduction must decrease in the order k2 > k3 > k, as the molybdenum center encountered by substrate becomes increasingly likely to become reduced, a semilogarithmic plot of the kinetic data appears to accelerate (31). This phenomenon tends to mask the slowing of the latter rates of reduction by 8-bromoxan-
thine. Thus, while a rapid phase followed by a slow phase in the kinetic timecourse might be expected a \emph{a priori} in the presence of 8-bromoxanthine (as the second and third steps in reduction are appreciably slowed compared to the first step) this is not observed.\footnote{That the distribution of reducing equivalents at the microscopic level within xanthine oxidase can affect the macroscopic properties of the enzyme has been observed before in a comparative study of $V_{\text{max}}$ for xanthine oxidase containing a series of flavin analogs having the similar enzyme xanthine dehydrogenase using xanthine as a substrate. It has been shown, when the steady state level of reduced enzyme is lower (32), making it less likely for the Mo$^{\text{IV}}$-xanthine complex to form.

It is somewhat surprising that 8-bromoxanthine, urate, and xanthine would bind to reduced xanthine oxidase in the same way, as in apparently the case. The bulky bromine atom especially might be expected to exert considerable steric hindrance. It is conceivable that all three purines bind via the pyrimidine subnucleus in exerting their effect on the molybdenum center. This appears unlikely, however, on the basis of preliminary results of experiments using x-ray absorption spectroscopy\footnote{S. P. Cramer and R. Hille, unpublished results.} that places the bromine atom of 8-bromoxan-

thine approximately 4 Å from the molybdenum atom, far too close for binding to be taking place via the pyrimidine subnucleus, with the likelihood of an intervening atom to explain the long Mo-Br distance.

Given that the Mo$^{\text{IV}}$-urate complex is almost certainly a catalytic intermediate (in fact the most accessible intermediate, experimentally, after $E_{\text{m}}$ and $E_{\text{m}}^{\text{red}}$) it is worth noting that 8-bromoxanthine is a more appropriate analog of product than of substrate. In this light the optical absorbance change associated with purine binding and particularly the ability to discern bromine in the extended x-ray absorption fine structure analysis of molybdenum provide the opportunity to gain important new insight into the interaction of purines with the active site of xanthine oxidase as well as the structure of the molybdenum center.

Acknowledgments—We are indebted to Dr. James A. Fee, Biophysics Research Division and Department of Biological Chemistry, University of Michigan, in whose laboratory the EPR work was performed, and his assistance is gratefully acknowledged. We wish to thank Drs. Vincent Massey and Stephen P. Cramer for helpful discussions.

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J. Biol. Chem. 1984, 259:1570-1576.

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