Hutchins, C. J., Rathjen, P. D., Forster, A. C., & Symons, R. H. (1986) *Nucleic Acids Res. 14*, 3627-3640.
Johnston, P. D., & Redfield, A. G. (1978a) *Nucleic Acids Res. 4*, 3599-3615.
Johnston, P. D., & Redfield, A. G. (1978b) *Nucleic Acids Res. 5*, 3913-3927.
Johnston, P. D., & Redfield, A. G. (1979) *Transfer RNA: Structure, Properties, and Recognition*, pp 191-206, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Johnston, P. D., & Redfield, A. G. (1981) *Biochemistry 20*, 1147-1156.
Kearns, D. R. (1976) *Prog. Nucleic Acid Res. Mol Biol. 18*, 91-149.
Kearns, D. R. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 477-523.
Kearns, D. R., & Shulman, R. G. (1974) *Acc. Chem. Res 7*, 33-39.
Keese, P., & Symons, R. H. (1987) in *Viroids and Viroid-like Pathogens* (Semancik, J. S., Ed.) pp 1-47, CRC Press, Boca Raton, FL.
Kneale, G., Brown, T., Kennard, O., & Rabinovich, D. (1985) *J. Mol. Biol. 186*, 805-814.
Leroy, J.-L., Broseta, D., & Guéron, M. (1985) *J. Mol. Biol. 184*, 165-178.
Lowary, P., Sampson, J., Milligan, J., Groebe, D., & Uhlenbeck, O. C. (1987) *NATO ASI Ser. 110*, 69-76.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Prody, B. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., & Breuning, G. (1986) *Science 231*, 1577-1580.
Reid, B. R. (1981) *Annu. Rev. Biochem. 50*, 969-996.
Reid, B. R. & Hurd, R. E. (1977) *Acc. Chem. Res. 10*, 396-402.
Reid, B. R., & Hurd, R. E. (1979) *Biochemistry 18*, 3996.
Reid, B. R., Ribeiro, N. S., Gould, G., Robillard, G., Hilbers, C. W., & Shulman, R. G. (1975) *Proc. Natl. Acad. U.S.A. 72*, 2049-2055.
Reid, B. R., Ribeiro, N. S., McCollum, L., Abatte, J., & Hurd, R. E. (1977) *Biochemistry 16*, 2086-2094.
Roy, S., & Redfield, A. G. (1981) *Nucleic Acids Res. 9*, 7073-7083.
Sanchez, V., Redfield, A. G., Johnston, P. D., & Tropp, J. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5659-5662.
Schimmel, P. R., & Redfield, A. G. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 181-221.

# Kinetic Isotope Effect Studies on Milk Xanthine Oxidase and on Chicken Liver Xanthine Dehydrogenase†

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ABSTRACT: The effect of isotopic substitution of the 8-H of xanthine (with $^2H$ and $^3H$) on the rate of oxidation by bovine xanthine oxidase and by chicken xanthine dehydrogenase has been measured. $V/K$ isotope effects were determined from competition experiments. No difference in $^{H/T}(V/K)$ values was observed between xanthine oxidase (3.59 ± 0.1) and xanthine dehydrogenase (3.60 ± 0.09). Xanthine dehydrogenase exhibited a larger $^{T/D}(V/K)$ value (0.616 ± 0.028) than that observed for xanthine oxidase (0.551 ± 0.016). Observed $^{H/T}(V/K)$ values for either enzyme are less than those $^{H/T}(V/K)$ values calculated with $^{D/T}(V/K)$ data. These discrepancies are suggested to arise from the presence of a rate-limiting step(s) prior to the irreversible C-H bond cleavage step in the mechanistic pathways of both enzymes. These kinetic complexities preclude examination of whether tunneling contributes to the reaction coordinate for the H-transfer step in each enzyme. No observable exchange of tritium with solvent is observed during the anaerobic incubation of [8-$^3H$]xanthine with either enzyme, which suggests the reverse commitment to catalysis ($C_r$) is essentially zero. With the assumption of adherence to reduced mass relationships, the intrinsic deuterium isotope effect ($^Dk$) for xanthine oxidation is calculated to be 7.4 ± 0.7 for xanthine oxidase and 4.2 ± 0.2 for xanthine dehydrogenase. By use of these values and steady-state kinetic data, the *minimal* rate for the hydrogen-transfer step is calculated to be ~75-fold faster than $k_{cat}$ for xanthine oxidase and ~10-fold faster than $k_{cat}$ for xanthine dehydrogenase. This calculated rate is consistent with data obtained by rapid-quench experiments with XO. A stoichiometry of 1.0 ± 0.3 mol of uric acid/mol of functional enzyme is formed within the mixing time of the instrument (5-10 ms). The kinetic isotope effect data also permitted the calculation of the $K_d$ values [Klinman, J. P., & Matthews, R. G. (1985) *J. Am. Chem. Soc. 107*, 1058-1060] for substrate dissociation, including all reversible steps prior to C-H bond cleavage. Values calculated for each enzyme ($K_d$ = 120 μM) were found to be identical within experimental uncertainty.

In spite of a considerable number of pre-steady-state kinetic studies (Olson et al., 1974a,b; Brugeura et al., 1988; Coughlan & Rajagopalan, 1980; Rajagopalan & Handler, 1967; Edmondson et al., 1973) there are still a large number of questions unanswered regarding the initial reductive step in the catalytic mechanism of xanthine oxidase (XO)[1] or of xanthine dehydrogenase (XDH). The involvement of the molybdenum center as the initial site of entry of reducing

† This work was supported by grants from the National Science Foundation (DMB-8616952) and from the National Institutes of Health (GM-29433).

[1] Abbreviations: XO, xanthine oxidase; XDH, xanthine dehydrogenase; $NAD^+$, nicotinamide adenine dinucleotide, oxidized; HPLC, high-pressure liquid chromatography; ESR, electron spin resonance.

0006-2960/90/0429-9046$02.50/0 

equivalents from the substrate is now well established for both enzymes. The reduced Mo(IV) [and/or MO(V)] center subsequently transfers electrons to the two $Fe_2/S_2$ centers and/or to the FAD center with the latter serving as the site for $O_2$ reduction to either $H_2O_2$ or $O_2^{\bullet-}$ in the case of the oxidase (Hille & Massey, 1981; Porras et al., 1981) or as the site for $NAD^+$ reduction in the case of XDH. Both XO and XDH exhibit many of the same properties (including cofactor content), are found to be interconvertible in some tissues (Waud & Rajagopalan, 1976), and differ mainly in their respective reactivities with oxidizing substrates. The common view is that their respective Mo centers are quite similar and that the mechanisms of xanthine oxidation are also thought to be similar, if not identical, for both enzymes.

Current information in the literature on XO permits the following descriptions of the mechanism of xanthine oxidation to uric acid. Two electrons are transfered from substrate to Mo(VI) concomitant with C–H bond cleavage at the 8-position of the purine ring to form Mo(IV). Whether this electron transfer occurs in single- or two-electron steps is currently unknown. The 8-H of the substrate is transferred to a ligand of the Mo [as detected from alterations in the hyperfine splitting of the "rapid" Mo(V) ESR signal when deuterium is substituted for hydrogen (Edmondson et al., 1973; Gutteridge & Bray, 1978)]. This proton subsequently exchanges from the Mo(V) center with solvent with a rate constant of 85 $s^{-1}$ (Gutteridge & Bray, 1978). Whether the rate of proton exchange with solvent is similar for the Mo(IV) species is unknown. Oxygen incorporated into the 8-position of the purine ring to form uric acid is derived from solvent, and convincing evidence has been published in support of an unknown group on the enzyme serving as the oxygen donor to the substrate intermediate (Hille & Specher, 1987). Release of product (uric acid) from the active site is considered to be the rate-limiting step in catalytic turnover from the results of a number of studies [cf. Davis et al. (1984)].

Absorption stopped-flow kinetic studies on these enzymes mainly follow changes in the redox level of the Fe/S and FAD centers. Since 3 mol of substrate is required for complete reduction of all of the redox centers (Olson et al., 1974a), observed rates of reduction of the visible absorbance will be limited by the rate of product dissociation. Using violopterin as a substrate for XO, Davis and Palmer (1984) have found a transient intermediate [attributed to a product–Mo(IV) complex] which decays at a rate similar to catalytic turnover. No such transient intermediate attributable to the product–Mo(IV) has been observed for the reaction of xanthine with XO, although Nishino et al. (1987) have observed a rapid increase in absorption at 360 nm on mixing xanthine with XDH ($k$ = 36 $s^{-1}$ at 4 °C), which they suggest to be due to reduction of the molybdenum center. Current knowledge regarding the rate of Mo(VI) reduction by the substrate has been mainly limited to observations of the formation and decay of Mo(V) with the rapid freeze–quench ESR technique. Neither Mo(VI) nor Mo(IV) can be monitored in these experiments, so the total level of Mo reduction is unknown. Thus, uncertainties exist whether the Mo(V) monitored in these experiments is due to the one-electron *reduction* of Mo(VI) by substrate or due to the one-electron *oxidation* of Mo(IV) by the Fe/S and FAD centers.

As an alternative approach to complement existing data on the mechanism of XO and XDH, we report here the results of kinetic isotope studies in which the 8-H of the substrate has been replaced by either deuterium or tritium and the effects of such replacement on the kinetic properties exhibited upon interaction of these labeled substrate analogues with XO and XDH. Competition experiments using these labeled substrates permit calculation of the intrinsic kinetic isotope effect for the C–H bond cleavage step (Northrop, 1975; Klinman, 1978), which allows estimation of microscopic rate constants (Miller & Klinman, 1982). The determination of $^{H/D}V$ and $^{H/D}(V/K)$ values for an enzyme system also permits the calculation of a $K_d$ value for substrate binding (Klinman & Matthews, 1985). Previous studies have shown XO to exhibit a small isotope effect on $V_{max}$ (Edmondson et al., 1973), and as shown in this paper, XDH also exhibits similar properties. The results presented here show that, due to kinetic complexity, no tunneling contribution to C–H bond cleavage (Klinman, 1989) can be observed for either XO or XDH catalysis and that the *minimal* rate estimated for the C–H bond cleavage step is faster for bovine XO than for chicken XDH. The rate of substrate reduction of Mo(VI) for each enzyme is estimated to be much faster than catalytic turnover. Both XO and XDH exhibit similar calculated $K_d$ values for xanthine binding. These conclusions will be discussed in terms of previous kinetic studies on these enzymes.

## Materials and Methods

*Enzymes and Labeled Substrates.* Xanthine oxidase was purified from fresh bovine cream (obtained from the University of Georgia Dairy, Athens, GA) according to the method of Massey et al. (1969). Xanthine dehydrogenase was isolated from fresh chicken livers purchased at a local market according to the procedure (with only minor modifications) described by Rajagopalan and Handler (1967). Both enzyme preparations were of 50–60% functionality.

[8-$^3$H]Xanthine (5.0 Ci/mmol) and [8-$^{14}$C]xanthine (54.7 mCi/mmol) were purchased from NEN Du Pont. [8-$^2$H,8-$^{14}$C]Xanthine was prepared by refluxing a 1% (w/v) solution of xanthine containing 5 μCi of [8-$^{14}$C]xanthine in 0.3 N NaOD in 99.8% $D_2O$. Proton–deuterium exchange at the 8-position was monitored by NMR (360 MHz) and the exchange reaction continued until the proton resonance at 7.3 ppm disappeared. Exchange was complete after 6 h of refluxing. The solution was then diluted to 30 mM with water and neutralized with HCl. Purity of the labeled xanthine thus prepared was monitored by HPLC ($C_{18}$ column, isocratic conditions, 4 mM sodium phosphate, pH 6.0). After further dilution into the enzyme assay, the final $D_2O$ concentration was 0.5% (v/v). It should be noted that introduction of $^{14}$C in the 8-position of xanthine may lead to a small underestimation of $^{H/T}(V/K)$ and $^{D/T}(V/K)$ values depending on the value of the $^{12}C/^{14}C$ isotope effect on the rate of C–H or C–D bond cleavage. Hermes et al. (1982) have shown deuteration to increase the size of the $^{13}$C isotope effect if the two isotopically sensitive steps are the same as is the case here. Carbon isotope effects are found to be small [cf. $^{13}(V/K)$ = 1.0165–1.0316 for glucose-6-dehydrogenase] (Hermes et al., 1982) and, when converted to $^{14}$C isotope effects, would be in the range of $^{14}(V/K)$ = 1.031–1.059. Alterations of this magnitude would be in the range of uncertainty of the measurements presented here.

*Enzyme Assay Methods.* Xanthine oxidase activity was measured spectrophotometrically at 295 nm and at 25 °C in 0.1 M sodium pyrophosphate, pH 8.5. Xanthine dehydrogenase activity was also measured spectrally at 340 nm and at 25 °C with 0.5 mM $NAD^+$ as electron acceptor in 50 mM potassium phosphate–0.1 mM EDTA, pH 7.9. The fractional conversion ($f$) of xanthine to uric acid was measured spectrally at 295 nm with a delta molar extinction coefficient of 9600 $M^{-1}$ $cm^{-1}$ (Avis et al., 1956b). A molar extinction

of 6200 $M^{-1}$ $cm^{-1}$ (Horecker et al., 1948) was used to calculate the level of NADH formation in XDH experiments. Fractional conversions were also determined from the amount of [$^{14}$C]uric acid produced after separation from labeled xanthine by HPLC ($C_{18}$ column, 4 mM sodium phosphate, pH 6.0, isocratic conditions, flow rate 1 mL/min). Under these conditions, $^3H_2O$ eluted at 3 min, uric acid at 9 min, and xanthine at 13 min. HPLC fractions were collected and counted in a Beckman LS 5801 scintillation counter programmed to correct for quenching and for overlap of the $^3$H channel and the $^{14}$C channel. $^3$H and $^{14}$C standards supplied by Beckman were used to convert cpm to dpm.

*Competition Experiments.* $^{H/T}(V/K)$ and $^{D/T}(V/K)$ values for XO and for XDH were determined in the following manner. Catalytic amounts of either enzyme (typically 5.0 nM functional XO active sites or 2.0 nM functional active sites of XDH) were incubated at 25 °C with a xanthine concentration of $2K_m$ to $4K_m$. In the case of XO, air-saturated solutions (~240 μM) were used, which are ~5-fold greater than the $K_m[O_2]$ of 50 μM (Massey et al., 1969). In the XDH reaction, 0.5 mM $NAD^+$ was present as electron acceptor, which is 28-fold greater than the $K_m[NAD^+]$ of 18 μM (Rajagopalan & Handler, 1967). Levels of radiolabeled xanthine used in each experiment were 0.16 μCi/mL [8-$^3$H]xanthine and 0.09 μCi/mL [8-$^{14}$C]xanthine. For the XO reaction, ~1100 international units of catalase and ~50 international units of superoxide dismutase were included to prevent any reaction of hydrogen peroxide, superoxide, or hydroxyl radical (generated as products of the reaction) with either xanthine or uric acid (Kuppusamy & Zweir, 1989). At various levels of fractional conversion, aliquots of the reaction mixture were quenched by the addition of 10% (w/v) trichloroacetic acid, and the products were separated from substrates by HPLC under the conditions given above. Competition experiments to determine $^{T/D}(V/K)$ were performed as described above except that [8-$^2$H,8-$^{14}$C]xanthine was used in place of [8-$^{14}$C]xanthine.

Competitive ($V/K$) isotope effects were calculated from quantitation of radioactivity in the $H_2O$ and in the uric acid fractions separated from each reaction mixture by use of (Simon & Palm, 1966; Cleland, 1982)

$$^{D/T}(V/K) \text{ or } ^{H/T}(V/K) = \frac{\ln (1 - f)}{\ln [1 - f(R_p t/R_0)]}$$

where $f$ is the fractional conversion of substrate to products, $R_p t$ is the $^3H/^{14}C$ ratio of products at time $t$, and $R_0$ is the $^3H/^{14}C$ ratio of the substrate at $t = 0$. Ratios of $^3H/^{14}C$ at 100% conversion of reactants to products were the same as the ratio determined for the substrate at 0% conversion correction for $^3$H exchange with $H_2O$. These data serve as a check for the absence of any radiochemical impurities in the labeled xanthine preparations.

## Results

*Measurement of V/K Isotope Effects.* The $^{H/T}(V/K)$ isotope effect for XO was measured by the competitive method as outlined under Materials and Methods. Preliminary experiments demonstrated the results were more reproducible when superoxide dismutase and catalase were included in the assay mixture to eliminate the breakdown of labeled substrates or products. This breakdown presumably occurs by reaction with $O_2^{\bullet-}$, $H_2O_2$, or $^{\bullet}OH$ generated during catalytic turnover. The conversion of [8-$^3$H]xanthine to uric acid is monitored by determination of $^3H_2O$ formed during the time course of the reaction while the conversion of [8-$^{14}$C]xanthine was monitored by the formation of [8-$^{14}$C]uric acid after separation by HPLC. Table I shows the results of a number of representative experiments. Recovery of counts ($^3$H and $^{14}$C) from HPLC separations were always 95% or greater. The data in Table I for $^{H/T}(V/K)$ values were calculated at fractional conversions ranging from 5.4% to 75.2% and were found to be independent of the level of fractional conversion. Deviations from the mean value (3.59) of $^{H/T}(V/K)$ reflect counting uncertainties and other systematic errors (2.8%). Control samples of labeled xanthine were incubated under identical conditions without enzyme and analyzed in the same manner to correct for any $^3$H exchange with solvent. The amount of background T–H exchange from [8-$^3$H]xanthine with solvent was found to be less than 4.5% over the time course of the experiments. When similar competition experiments were performed with chicken liver XDH (Table II), the $^{H/T}(V/K)$ value determined, 3.60, was found to be identical (within experimental error) with that determined for XO. Data were collected at fractional conversions of less than 20% to maximize the observed discrimination and to reduce errors expected at large fractional conversions (Duggleby & Northrop, 1989). The experimental uncertainty in $^{H/T}(V/K)$ determined for XDH is 2.4%.

Table I: $^{H/T}(V/K)$ and $^{T/D}(V/K)$ Isotope Effect Measurements on Xanthine Oxidase[a]

| $^{H/T}(V/K)$ | $^{T/D}(V/K)$ | $^{H/T}(V/K)$ | $^{T/D}(V/K)$ |
|---|---|---|---|
| 3.41 | 0.579 | 3.60 | 0.549 |
| 3.80 | 0.537 | 3.65 | 0.537 |
| 3.62 | 0.565 | 3.49 | 0.561 |
| 3.43 | 0.548 | 3.39 | 0.574 |
| 3.75 | 0.534 | av: 3.60 ± 0.086 | av: 0.551 ± 0.015 |
| 3.78 | 0.533 | | |

[a] Enzyme catalysis was measured in 0.1 M sodium pyrophosphate, pH 8.5, 25 °C. Counting error for $^{14}$C ranged from 1.5 to 3.3%. Counting error for $^3$H ranged from 1.1 to 3.5%.

Table II: $^{H/T}(V/K)$ and $^{T/D}(V/K)$ Isotope Effect Measurements on Xanthine Dehydrogenase[a]

| $^{H/T}(V/K)$ | $^{T/D}(V/K)$ | $^{H/T}(V/K)$ | $^{T/D}(V/K)$ |
|---|---|---|---|
| 3.47 | 0.570 | 3.60 | 0.619 |
| 3.54 | 0.652 | 3.71 | 0.609 |
| 3.69 | 0.658 | av: 3.60 ± 0.086 | av: 0.616 ± 0.028 |
| 3.64 | 0.590 | | |

[a] Enzyme catalysis was measured in 0.05 M $K_2HPO_4$–0.1 mM EDTA, pH 7.9, with 0.5 mM $NAD^+$, 25 °C. Counting error for $^{14}$C ranged from 1.5 to 3.3%. Counting error for $^3$H ranged from 1.1 to 3.5%.

Previous steady-state kinetic data (Edmondson et al., 1973) showed a $^{H/D}(V/K)$ value of 1.90 for XO which was measured under conditions identical with those used in this study. Steady-state studies on chicken liver XDH showed a value for $^{H/D}(V/K)$ of 1.97. To provide additional confirmation of these values, $^{T/D}(V/K)$ values were measured for both enzymes. In principle, $^{H/D}(V/K)$ isotope effects could also be measured by competition experiments; however, restrictions of the radiolabeling of the xanthine ring to only $^{14}$C made $^{T/D}(V/K)$ competition experiments the most reasonable approach due to the ease of preparation of the required labeled substrate analogues. Conversion of [8-$^2$H,8-$^{14}$C]xanthine could be monitored by the formation of [8-$^{14}$C]uric acid after separation from xanthine by HPLC. The data shown in Table I demonstate a mean $^{T/D}(V/K)$ value for XO of 0.551 (2.9% error), and in Table II, a mean value of 0.616 (4.6% error) for XDH is shown.

It is of interest to compare calculated values of $^{H/D}(V/K)$ values from the above measurements with $^{H/D}(V/K)$ values

Table III: Steady-State and Calculated Values of $^{D}(V/K)$ from $^{T}(V/K)$ and $^{T/D}(V/K)$ for Xanthine Oxidase and for Xanthine Dehydrogenase

| | competition experiment $^{D}(V/K)^{a}$ | steady-state $^{D}(V/K)^{b}$ |
|---|---|---|
| xanthine oxidase | 1.98 (11.6% error) | 1.90 (9.7% error) |
| xanthine dehydrogenase | 2.21 (11.0% error) | 1.97 (9.8% error) |

[a] $^{D}(V/K)$ was determined by multiplication of the average of $^{T}(V/K)$ and $^{T/D}(V/K)$ experimental values for both enzymes. Experimental error was propogated by the method described by Bevington (1969). [b] Error in these measurements was determined by unweighted least-squares regression analysis of the slopes from Lineweaver–Burk plots.

determined directly from steady-state measurements since $^{H/T}(V/K)^{T/D}(V/K) = {}^{H/D}(V/K)$. Table III shows the calculated values from the competition experiments to be in good agreement with the values determined from steady-state experiments. Although this internal consistency between the two approaches is satisfying, the errors (either from experimental uncertainty or from propogation of errors) in $^{H/D}(V/K)$ values for either enzyme are too large to permit their use in calculation of the respective intrinsic kinetic isotope effects (see below).

The demonstration of tunneling in hydrogen-transfer reactions catalyzed by alcohol dehydrogenase (Cha et al., 1989) and by bovine plasma amine oxidase (Grant & Klinman, 1989) prompted the examination of the data in Tables I and II to determine if any contribution of tunneling occurs in the enzyme-catalyzed hydrogen transfer in xanthine oxidation. Klinman (1989) has pointed out that the semiclassical relation between D/T and H/T kinetic isotope effects should break down if significant tunneling were to occur (Saunders, 1985). In the absence of tunneling

$$^{D/T}(V/K)^{3.26} = {}^{H/T}(V/K)_{calc} = {}^{H/T}(V/K)_{obs}$$

whereas in the presence of a tunnel contribution (Klinman, 1989)

$$^{D/T}(V/K)^{3.26} = {}^{H/T}(V/K)_{calc} < {}^{H/T}(V/K)_{obs}$$

From the experimental values determined for both XO and XDH (Tables I and II), the following relationships are observed:

for XO

$$^{D/T}(V/K)^{3.26} = {}^{H/T}(V/K)_{calc} = 6.92 \pm 0.08 > {}^{H/T}(V/K)_{obs} = 3.59 \pm 0.10$$

for XDH

$$^{D/T}(V/K)^{3.26} = {}^{H/T}(V/K)_{calc} = 4.82 \pm 0.14 > {}^{H/T}(V/K)_{obs} = 3.60 \pm 0.09$$

For both enzyme systems, the calculated values of $^{H/T}(V/K)$ are greater than those observed experimentally although the difference observed for XO is greater than that observed for XDH. If any significant tunnel contribution were occurring in either enzyme system, then it is expected that $^{H/T}(V/K)_{calc} < {}^{H/T}(V/K)_{obs}$ (Cha et al., 1989). The finding that the calculated value is larger than that determined experimentally for both XO and XDH arises from isotope-insensitive rate-limiting steps in both catalytic mechanisms which occur prior to C–H bond cleavage since, as will be shown below, the C–H bond cleavage step is irreversible. These results are likely due to a partial rate-determining release of substrate from the E–S complex which has a greater contribution in the XO reaction than in the XDH reaction.[2] For the situation observed here, Cha et al. (1989) have pointed out that the difference in commitment factors ($C_H$ and $C_D$; see Table IV) in the relationship describing the isotope effect on $V/K$ and the corresponding intrinsic isotope effect can account for the inequality of observed and calculated $^{H/T}(V/K)$ values. Since $C_H$ values differ from $C_D$ values by the magnitude of the intrinsic isotope effect (Cha et al., 1989), the greater difference in calculated and observed $^{H/T}(V/K)$ values for XO suggests the magnitude of the intrinsic kinetic isotope effect is greater for bovine XO than for chicken XDH.

*Experimental Evaluation of the Reverse Commitment Factor.* The standard relation for the estimation of intrinsic kinetic isotope effects for enzyme-catalyzed reactions (Northrop, 1975) is

$$^{D}(V/K) - 1 = \frac{^{D}k - 1 + C_r(^{D}K_{eq} - 1)}{1 + C_f + C_r} \quad (1)$$

where $C_r$ and $C_f$ are the reverse and forward commitments to catalysis and $^{D}K_{eq}$ is the isotope effect on the equilibrium constant for hydrogen transfer from the substrate to an acceptor group. The above equation is simplified if either $C_r = 0$ or $^{D}K_{eq} = 1$. To test whether $C_r = 0$ or has a finite value, aliquots of XO and XDH were incubated in separate experiments with [8-$^3$H]xanthine under anaerobic conditions to prevent turnover. If $C_r$ were to have a finite value for either enzyme tested, when the 8-$^3$H should extensively exchange with the solvent on prolonged incubation. On the other hand, if $C_r = 0$, the tritium should remain on the substrate. Incubation of [8-$^3$H]xanthine with XO or with XDH under anaerobic conditions for 1 h at 25 °C (followed by anaerobic acid quenching of the reaction and HPLC separation of the reaction products) showed no $^3$H exchange from the substrate to the solvent over that observed with a control sample in which no enzyme was present. Thus, for the general mechanism (Northrop, 1975)

$$E_1 \underset{k_2}{\overset{k_1[X]}{\rightleftharpoons}} E_2 \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E_3 \underset{k_6^*}{\overset{k_5^*}{\rightleftharpoons}} E_4 \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E_5 \underset{k_{10}[UA]}{\overset{k_9}{\rightleftharpoons}} E_6 \overset{k_{11}}{\rightharpoonup} E_7 \quad (2)$$

$C_r = (k_6/k_7)(1 + k_8/k_9)$ and $k^*$ denotes the isotopically sensitive steps in the catalytic sequence. For the situation where $C_r = 0$, $k_6 <<< k_7$.

From the kinetic data of Olson et al. (1974b) and Davis et al. (1984) on xanthine oxidase, the minimal kinetic mechanistic pathway for XO and for XDH catalysis can be viewed as follows:

$$E_{ox} + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}X \underset{k_{-2}^*}{\overset{k_2^*}{\rightleftharpoons}} E_rX' \underset{+H_2O}{\overset{k_3}{\rightharpoonup}} E_r + UA \quad (3)$$

$$E_r + O_2 \text{ (or } NAD^+) \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E_rO_2 \overset{k_5}{\rightharpoonup} E_{ox} + H_2O_2 \text{ or } O_2^{\cdot-} \text{ (or NADH)}$$

Northrop's general mechanism (eq 2) may be viewed as an expansion of the above minimal mechanism. For the XO mechanism, $k_2^*$ indicates the isotopically sensitive step, $C_r = k_3/k_2 = 0$, and $k_3$ is assumed to be essentially irreversible due to the high molar ratio of water present during the reaction.

*Estimation of the Intrinsic Kinetic Isotope Effect for XO and for XDH.* The relation between the $V/K$ isotope effect and the intrinsic kinetic isotope effect under the condition where $C_r = 0$ is

$$^{H/D}(V/K) - 1 = \frac{^{D}k - 1}{1 + C_f} \quad (4)$$

and

[2] We thank one of the reviewers for pointing out this possibility.

$$^{H/T}(V/K) - 1 = \frac{^{D}k^{1.44} - 1}{1 + C_f} \quad (5)$$

The ratio of these two equations results in the cancellation of the $C_f$ term, and the resulting equation allows calculation of the intrinsic kinetic isotope effect (Northrop, 1975):

$$\frac{^{H/D}(V/K) - 1}{^{H/T}(V/K) - 1} = \frac{^{D}k - 1}{^{D}k^{1.44} - 1} \quad (6)$$

Although the $^{H/T}(V/K)$ values in Tables I and II are of sufficient accuracy to use in this equation, the $^{H/D}(V/K)$ values (Table III) are not. As an alternative approach, the competition $V/K$ data are expressed as $^{D/T}(V/K)$ and $^{H/T}(V/K)$. This substitution changes the exponential to 3.26 from the reduced mass consideration (Saunders, 1985):

$$(k_D/k_T)^{3.26} = k_H/k_T \quad (7)$$

so that after substitution and rearrangement of eqs 4 and 5 (D. B. Northrop, personal communication), eq 6 becomes

$$\frac{^{T/D}(V/K)}{^{T/H}(V/K) - 1} = \frac{^{T/D}k - 1}{^{T/D}k^{3.26} - 1} = \frac{^{H}k^{-0.442} - 1}{^{H}k^{-1.442} - 1} \quad (8)$$

This rearrangement of eqs 4 and 5 to give eq 8 results in a common commitment factor $C_T$ in both $V/K$ expressions which can be factored out. Implicit in these calculations is the application of standard reduced mass relations which, in the absence of tunneling [cf. Klinman (1989)], can be used to estimate the intrinsic isotope effect for XO and for XDH. Although no evidence for tunneling in these two reactions has been found (see below), we cannot unequivocally conclude that it does not occur. With these limitations and with the *assumption* that reduced mass relations are applicable to XO and XDH kinetic isotope effect data, the intrinsic deuterium isotope effect value for XO is calculated to be 7.4 ± 0.7, and the value for XDH is calculated to be 4.2 ± 0.2. (These calculations were carried out with a computer program kindly supplied by Prof. D. W. Northrop, University of Wisconsin.) Uncertainties in these values were estimated by standard expressions of error propogation (Bevington, 1969). As suggested from considerations of $C_H$ and $C_D$ commitment factors (see above), the intrinsic kinetic isotope effect for the C–H step is larger for bovine XO than for chicken XDH. This is not a unique situation in comparison of similar enzymes from different sources as, for example, alcohol dehydrogenases isolated from yeast (Klinman, 1972) and from horse liver (Shore & Gutfreund, 1970) have been shown to also exhibit different values of $^{D}k$. The lower value of $^{D}k$ for XDH suggests the structure of the transition state for the C–H bond cleavage step exhibits isotope-sensitive vibrations to a greater degree than in the case of XO. This situation could arise from differences in geometries as well as the degree of hydrogen transfer in the respective transition states for the two enzymes.

*Use of Kinetic Isotope Effect Data in Calculation of Microscopic Rate Constants and Substrate Binding Affinities for XO and for XDH.* Palcic and Klinman (1983) have shown that estimates of the microscopic rate constant for the C–H bond cleavage step in the plasma amine oxidase reaction can be made once values for the intrinsic isotope effect, $^{D}k$, and $k_{cat}$ are determined. For the general mechanism given in eq 2 (Northrop, 1975), the following equation has been derived (Palcic & Klinman, 1983):

$$\frac{k_{C-H}}{1 + E_f} = \frac{k_{cat}(^{D}k - 1)}{^{H/D}k_{cat} - 1} \quad (9)$$

All of the terms of eq 9 are of standard definition with the exception of $E_f$, which is comprised of a collection of equilibrium terms occurring prior to C–H bond cleavage. Using the mechanism given in eq 2 (Northrop, 1975; Palcic & Klinman, 1983)

$$E_f = (1 + k_4/k_3) \quad (10)$$

With the small values observed from calculated steady-state values for $^{H/D}k_{cat}$, XO (1.1) and XDH (1.2), relative to their respective values for their intrinsic kinetic isotope effects, the rate of C–H bond cleavage estimated for either enzyme will be *minimally* much faster than catalytic turnover. On the basis of the assumption that $E_f \ll 1$ [cf. Palcic and Klinman (1983)], the rate of C–H bond cleavage [and therefore Mo(VI) reduction] is ~75-fold faster than $k_{cat}$ for XO and ~10-fold faster than $k_{cat}$ for XDH. If the value of $E_f$ were to approach unity, then the corresponding values for $k_{C-H}$ would double. From estimates of the $K_d$ values of substrate dissociation from all preisotopic complexes (see below), the values of $E_f$ for XO and for XDH are suggested to be similar. Given these considerations and the similar values of $k_{cat}$ determined from steady-state experiments for XO (18 $s^{-1}$) and for XDH (19 $s^{-1}$), the rate of C–H bond cleavage ($k_2$) and therefore Mo(VI) reduction is calculated to be 7-fold faster for XO than for XDH (see Table IV).

Table IV: Calculated Kinetic Parameters for Xanthine Oxidase and Xanthine Dehydrogenase

| | xanthine oxidase | xanthine dehydrogenase |
|---|---|---|
| $^{D}k$ (intrinsic kinetic isotope effect on $k_2$) | 7.4 ± 0.7 | 4.20 ± 0.2 |
| $C_{f(H)}$[a] | 5.6 | 1.6 |
| $C_{f(D)}$[a] | 0.75 | 0.37 |
| $C_r$[b] | 0 | 0 |
| $K_D$ (μM)[c] | 120 | 120 |
| $k_2$ ($s^{-1}$) | 1400 | 200 |

[a] Forward commitment to catalysis ($k_2/k_{-1}$) (see eq 3). [b] Reverse commitment to catalysis. [c] Minimal rate assuming $E_f \ll 1$ (see text).

Klinman and Matthews (1985) have described a simple but elegant approach to determine $K_d$ values for substrate binding to the catalytic sites of enzymes from measurements of isotope effects on $k_{cat}$ and $V/K$. The equation relating $K_m$ and $K_d$ is

$$\frac{^{H/D}k_{cat} - 1}{^{H/D}(V/K) - 1} = \frac{K_m}{K_d} \quad (11)$$

$K_d$ represents the dissociation constant from *all* complexes formed prior to the isotopically sensitive step (Klinman & Matthews, 1985). For both XO and XDH, $K_d$ is calculated to be greater than $K_m$ and is similar for each enzyme (120 μM).

To provide supporting evidence that the rate of C–H bond cleavage in XO catalysis occurs at a minimal rate of at least 1400 $s^{-1}$, rapid chemical quench studies were performed. Although rapid-mixing techniques do not have the time resolution sufficient to determine a rate constant of each magnitude, a "burst" formation of 1 mol of uric acid/mol of functional active center of XO should be observed in the shortest possible time (5–10 ms) if the rate of C–H bond cleavage were much greater than $k_{cat}$. [8-$^{14}$C]Xanthine (1 mM final concentration) was mixed with 30 μM XO in an Update Instruments Ram System and the reaction quenched with 10% (w/v) trichloroacetic acid (final concentration after mixing) introduced through a second mixing chamber. Uric acid formation was determined by scintillation counting after separation from xanthine by HPLC. A stoichiometry of 1.0 ± 0.3 mol of uric acid/mol of functional enzyme (average from

four determinations) was observed to be formed within a 10-ms reaction time. By use of

$$E + X \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EX \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E_rUA$$

since $k_{-2} = 0$ (see above), under conditions of the experiment, a half-time for the formation of uric acid of 0.8 ms is estimated given a $K_d$ value of 120 $\mu$M and a value of $k_2$ of 1400 $s^{-1}$. Since this half-time is much faster than that possible to measure with rapid-mixing devices, this experiment provides independent support for the notion that the rate of C–H bond cleavage is a very fast process relative to $k_{cat}$.

## Discussion

The results of this study provide some new insights in the catalytic mechanisms of XO and XDH as well as complementing existing views of their respective mechanistic pathways. The kinetic isotope methodologies used in this study are standard approaches developed principally in the laboratories of Klinman and Northrop. This study, to our knowledge, represents the first systematic application of these approaches to xanthine-oxidizing molybdoenzymes.

The finding of a small kinetic isotope effect on $k_{cat}$ and the formation of a stoichiometric "burst" of uric acid within the smallest mixing time of a rapid-quench experiment provides direct support for the view that product release constitutes the major contribution to the rate limitation of catalytic turnover for XO (Davis et al., 1984). This view may also be applicable to chicken XDH since $^{H/D}k_{cat}$ measured in this study is of the same magnitude as that measured with XO. However, with regard to the rates of enzyme reoxidation, the reaction of reduced XDH with $NAD^+$ is not a simple one (Nishino et al., 1987). We have measured the rate of NADH formation on reaction of $NAD^+$ with XDH reduced with 3 mol of dithionite/mol of enzyme and found a limiting rate of 56 $s^{-1}$ at 25 °C. This value is approximately twice that reported by Nishino et al. (1987) for experiments performed at 4 °C.

A significant aspect of these studies that has not been appreciated is the magnitude of the rate of substrate C–H bond cleavage [and Mo(VI) reduction] relative to catalytic turnover. Calculations from the kinetic isotope effect data (supported by rapid acid quench experiments on XO) show a *minimal* rate for xanthine oxidation by Mo(VI) to be at least 1400 $s^{-1}$ for XO and at least 200 $s^{-1}$ for XDH (it should again be stressed that these values are minimal estimates, and in fact, they could be larger). A large number of rapid freeze–quench ESR kinetic studies have been done on bovine milk XO to measure the formation and decay of the "very rapid" and "rapid" Mo(V) ESR signals during turnover (Palmer et al., 1964; Bray & George, 1985; Edmondson et al., 1973). The formation of the "very rapid" Mo(V) signal of XO occurs with a $t_{1/2}$ of ~5 ms (Edmondson et al., 1973). While an absolute rate constant for this formation cannot be unequivocally calculated due to the time resolution of the measurement, this half-time would be consistent with a more rapid formation of Mo(IV) as found in this study. The estimates presented here for the rates of Mo reduction by substrate suggest that the formation and decay of the Mo(V) species observed in kinetic ESR experiments are a result of *oxidation* of Mo(IV) by the Fe/S and FAD centers in the enzyme. This view has also been proposed without evidence in previous work (Olson et al., 1974a; Bray, 1980). In a recent report, Bray and George (1985) have described the room temperature Mo(V) ESR kinetic properties of XO with a slow substrate (2-oxo-6-methylpurine) ($k_{cat}$ = 0.3 $s^{-1}$). These workers calculate the rate of production of Mo(IV) to be ~1 $s^{-1}$ in order to fit their ESR kinetic data. If this value could be verified experimentally, it would have important mechanistic consequences since the rate is $\sim 10^3$ slower than that reported here for XO with xanthine as a substrate. Kinetic isotope effect approaches such as outlined here would be valuable as an auxillary approach with other rapid-mixing kinetic techniques to probe mechanistic details involved in the oxidation of this slow substrate.

It remains for future work to determine the mechanism of hydrogen transfer from the 8-position of the substrate to a ligand on the Mo. None of the possibilities (proton abstraction, hydrogen atom abstraction, or hydride transfer) can be ruled out at this time. Even though no evidence for any tunneling contribution to the hydrogen-transfer step was found in this study, our knowledge regarding this phenomenon in enzyme catalysis is still at an early stage (Klinman, 1989). Therefore, our view is that to totally dismiss it as a factor in molybdoenzyme catalysis is at this stage premature.

## Acknowledgments

We thank Dr. D. B. Northrop, University of Wisconsin, for the computer program used in calculation of intrinsic isotope effects and Dr. J. P. Klinman, University California, Berkeley, for helpful discussions.

## References

Avis, P. G., Bergel, F., & Bray, R. C. (1956) *J. Chem. Soc.*, 1219.

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, p 60, McGraw-Hill, New York.

Bray, R. C., & George, G. N. (1985) *Biochem. Soc. Trans. 13*, 560.

Bruguera, P., Lopez-Cabrera, A., & Canela, E. I. (1988) *Biochem. J. 249*, 171.

Cha, Y., Murray, C. J., & Klinman, J. P. (1989) *Science, March 10*, 1325.

Cleland, W. W. (1982) *Crit. Rev. Biochem. 13*, 385.

Coughlan, M. P., & Rajagopalan, K. V. (1980) *Eur. J. Biochem. 105*, 81.

Davis, M. D., Olson, J. S., & Palmer, G. (1984) *J. Biol. Chem. 259*, 3526.

Duggelby, R. G., & Northrup, D. B. (1989) *Bioorg. Chem. 17*, 177.

Edmondson, D. E., Ballou, D., van Heuvelen, A., Palmer, G., & Massey, V. (1973) *J. Biol. Chem. 248*, 6135.

Grant, K. H., & Klinman, J. P. (1989) *Biochemistry 28*, 6597.

Gutteridge, S., & Bray, R. C. (1978). *Biochem. J. 175*, 887.

Hermes, J. D., Roeske, C. A., Oleary, M. H., & Cleland, W. W. (1982) *Biochemistry 21*, 5106.

Hille, R., & Massey, V. (1981) *J. Biol. Chem. 256*, 9090.

Hille, R., & Sprecher, H. (1987) *J. Biol. Chem. 262*, 10914.

Horecher, B. L., & Doreberg, A. (1948) *J. Biol. Chem. 175*, 385.

Klinman, J. P. (1972) *J. Biol. Chem. 247*, 7977.

Klinman, J. P. (1989) *Trends Biochem. Sci. 14*, 368.

Klinman, J. P., & Matthews, R. G. (1985) *J. Am. Chem. Soc. 107*, 1058.

Kuppusamy, P., & Zweir, J. L. (1989) *J. Biol. Chem. 264*, 9880.

Massey, V., Brumby, P. E., Komai, H., & Palmer, G. (1969) *J. Biol. Chem. 244*, 1682.

Miller, S. M., & Klinman, J. P. (1982) *Methods Enzymol. 87*, 711.

Nishino, T., Tsushima, K., Schopter, L., & Massey, V. (1987) in *Flavins and Flavoproteins* (Edmondson, D. E., & McCormick, D. B., Eds.) pp 409–412, W. deGruyter, Berlin.

Northrop, D. B. (1975) *Biochemistry 14*, 2644.

Northrop, D. B. (1977) in *Isotope Effects in Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) University Press, Baltimore, MD.
Northrop, D. B. (1982) *Methods Enzymol. 87*, 607.
Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974a) *J. Biol. Chem. 249*, 4350.
Olson, J. S., Ballou, D. P., Palmer, G., & Massey, V. (1974b) *J. Biol. Chem. 249*, 4363.
Palmer, G., Bray, R. C., & Beinert, H. (1964) *J. Biol. Chem. 239*, 2657.
Palcic, M. M., & Klinman, J. P. (1983) *Biochemistry 22*, 5957.
Porras, A. G., Olson, J. S., & Palmer, G. (1981) *J. Biol. Chem. 256*, 9096.
Rajagopalan, K. V., & Handler, P. (1967) *J. Biol. Chem. 242*, 4097.
Saunders, W. H., Jr. (1985) *J. Am. Chem. Soc. 107*, 164.
Schopfer, L. M., Massey, V., & Nishino, T. (1988) *J. Biol. Chem. 263*, 13528.
Shore, J. D., & Gutfreund, H. (1970) *Biochemistry 9*, 4655.
Simon, H., & Palm, D. (1966) *Angew. Chem., Int. Ed. Engl. 5*, 920.
Waud & Rajagopalan, K. V. (1976) *Arch. Biochem. Biophys. 172*, 365.

# Phosphorescence and Optically Detected Magnetic Resonance Studies of Echinomycin–DNA Complexes†

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*Received March 15, 1990; Revised Manuscript Received June 22, 1990*

ABSTRACT: Echinomycin complexes with polymeric DNAs and model duplex oligonucleotides have been studied by low-temperature phosphorescence and optical detection of triplet-state magnetic resonance (ODMR) spectroscopy, with the quinoxaline chromophores of the drug used as intrinsic probes. Although not optically resolved, plots of ODMR transition frequencies versus monitored wavelength revealed heterogeneity in the phosphorescence emission of echinomycin, which was ascribed to the presence of two distinct quinoxaline triplet-state environments (referred to as the blue and red triplet states of echinomycin in this report). We think that a likely origin of the two triplet states of echinomycin is the occurrence of two or more distinct conformations of the drug in aqueous solutions. Spectroscopically observed perturbations of the triplet-state properties of echinomycin such as the phosphorescence emission spectrum, phosphorescence lifetime, ODMR spectrum, and zero-field splitting (zfs) energies were investigated upon drug binding to the double-stranded alternating copolymers poly(dG-dC)·poly(dG-dC) [abbreviated as poly[d(G-C)$_2$]] and poly(dA-dT)·poly(dA-dT) [abbreviated as poly[d(A-T)$_2$]], the homopolymer duplexes poly(dG)·poly(dC) [abbreviated as poly(dG·dC)] and poly(dA)·poly(dT) [abbreviated as poly(dA·dT)], and the natural DNAs from *Escherichia coli*, *Micrococcus lysodeikticus*, and calf thymus. Echinomycin bisintercalation complexes with the self-complementary oligonucleotides d(ACGT), d(CGTACG), and d(ACGTACGT), which are thought to model drug binding sites, were also investigated. Phosphorescence and ODMR spectroscopic results indicate that the quinoxaline chromophores of the drug are involved in aromatic stacking interactions in complexes with the natural DNAs as evidenced by red shifts in the phosphorescence 0,0 band of the drug, a small but significant reduction in the phosphorescence lifetime of the red triplet state, and reduction of the zfs *D*-value of both the blue and red triplet states upon drug complexation. These changes in the triplet-state properties of echinomycin are consistent with stacking interactions that increase the polarizability of the quinoxaline environment. The extent of the reduction of the *D* parameter for the red triplet state upon complexation with the polymeric DNAs was found to correlate with the binding affinities measured for these targets [Wakelin, L. P. G., & Waring, M. J. (1976) *Biochem. J. 157*, 721–740], with the single exception of the drug–poly[d(G-C)$_2$] complex, for which an increase in the *D*-value was noted. In addition, upon drug binding to the natural DNAs, there is a reversal of signal polarity in the ODMR spectra of the red triplet state. Among the synthetic DNA polymers investigated, a reversal of ODMR signal polarity was found only with the echinomycin–poly[d(A-T)$_2$] complex. The polarity reversals apparently result from changes in the triplet sublevel decay constants upon binding to DNA. Echinomycin binding to the duplex oligonucleotides employed in this work gave results similar to those obtained with the natural DNAs. In relation to the other DNA complexes, the echinomycin–d(CGTACG) complex exhibited significantly enhanced ODMR signals for a blue-shifted triplet state, which were ascribed to solvent-exposed quinoxaline rings at either end of the oligonucleotide lattice as deduced from X-ray diffraction studies [Ughetto, G., et al. (1985) *Nucleic Acids Res. 13*, 2305–2323].

Quinoxaline antitumor antibiotics, the natural products of several species of *Streptomycetes*, are composed of two planar quinoxaline-2-carboxamide moieties linked by a cross-bridged, cyclic octadepsipeptide. Two families of quinoxaline antibiotics are known, the triostins and the quinomycins, that differ in their respective cross-bridge structure. Echinomycin (Figure 1) is the most prominent member of the quinomycins, which contain a thioacetal cross-linkage. Its biological activity as

† This research was partially supported by NIH Grant ES-02662 and by a grant from the University of California, Davis, Committee on Research.

0006-2960/90/0429-9052$02.50/0 