Substrate and Product Inhibition in the Xanthine Oxidase Catalyzed Oxidation of Acetaldehyde†

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ABSTRACT: The xanthine oxidase catalyzed oxidation of acetaldehyde was studied in phosphate buffers at 25.0°. The reactions were carried out in the presence of ferricytochrome *c* which, upon reduction, results in an increase in optical density at 550 nm. It was observed that the enzymatic oxidation of acetaldehyde is sensitive to some form of substrate inhibition. Since acetaldehyde in aqueous solution exists in equilibrium with its hydrate, experiments were carried out to determine the nature of the actual inhibitory species, *viz.*, the aldehyde or its conjugate hydrate. Substrate inhibition was studied at various stages of the aldehyde–hydrate equilibration. It was observed that *both* the aldehyde and hydrate inhibit the enzymatic reaction, the latter being the more potent inhibitor (K_i = 1.6 × 10^{-3} M). Inhibition of the enzymatic process by acetic acid, a common contaminant of acetaldehyde and indeed the product of enzymatic oxidation, was also investigated. The acetate ion appears to function as a noncompetitive inhibitor (K_i = 7.6 × 10^{-2} M).

Xanthine oxidase catalyzes the oxidation of many purine and pteridine derivatives as well as aliphatic, aromatic, and heteroaromatic aldehydes (Booth, 1938; Bergmann and Dikstein, 1956; Bergmann *et al.*, 1959, 1960a,b; Bray, 1963; Muraoka *et al.*, 1967; Fridovich, 1970; Bray and Swann, 1972; Gregory *et al.*, 1972). The enzyme appears to be equally nonselective with respect to the many inhibitors with which it interacts (Coombs, 1927; Doisy *et al.*, 1955; Wyngaarden, 1957; Bergmann *et al.*, 1960a,b; Gilbert, 1964; Webb, 1966; Massey *et al.*, 1970). Aqueous solutions of many of the substrates and inhibitors of xanthine oxidase exist as equilibrated mixtures containing several different chemical species. Bergmann *et al.* (1960a) considered the participation of hydrated as well as various tautomeric forms of purine derivatives as the preferential substrates of xanthine oxidase. Aldehydes in aqueous solution exist in equilibrium with their hydrates, $RCHO + H_2O \rightleftharpoons RCH(OH)_2$ (Bell, 1966; Pocker *et al.*, 1967; Pocker and Dickerson, 1969), and the enzymatic oxidation of acetaldehyde by xanthine oxidase has been shown to proceed *via* the aldehyde *per se* rather than its conjugate hydrate (Fridovich, 1966; Gregory *et al.*, 1972).

The disposition of inhibitors of xanthine oxidase in aqueous solution and the actual form of the active inhibitory species have not yet been considered. Xanthine oxidase is susceptible to substrate inhibition (Dixon and Thurlow, 1924; Hofstee, 1955). From his studies, Hofstee (1955) proposed that in the enzymatic oxidation of xanthine, a second enzyme-bound molecule of xanthine interrupts the electron-transport system associated with the enzyme–xanthine–oxygen complex. Substrate inhibition is particularly severe for the aldehydic substrates of xanthine oxidase (Gregory *et al.*, 1972). In aqueous solution, many of the aldehydes studied as substrates of xanthine oxidase exist predominantly in their hydrated forms (Pocker *et al.*, 1967). Thus the question arises as to whether the inhibition observed in the enzymatic oxidation is caused by the substrate (aldehyde), its hydrate, or by a combination of both.

In the present work, substrate inhibition of the xanthine oxidase catalyzed oxidation of acetaldehyde was studied at various stages of the hydration equilibration in order to determine the relative extent to which the hydrated and unhydrated species inhibit the enzymatic process.

Experimental Section

Materials. The maintenance of oxygen free conditions for the purification and storage of acetaldehyde was necessary to avoid its oxidation to acetic acid. Commercially available acetaldehyde was twice distilled under dry nitrogen gas, then stored under nitrogen until used. The aqueous solutions of the aldehydes which were used to initiate kinetic runs were prepared in deionized water which had been purged with nitrogen.

Ferricytochrome *c* was obtained as a product of Sigma Chemical Corp., Sigma type VI. Beef liver catalase was purchased from Worthington Biochemical Corp. All buffer reagents and other inorganic chemicals were obtained from commercial sources in analytical or reagent grade. The purification and assay of xanthine oxidase have been described in our earlier work (Gregory *et al.*, 1972).

Methods. The kinetic runs were monitored on a Beckman Acta III recording spectrophotometer. The temperature of the cell chamber was maintained at 25.0° by means of a Beckman Thermocirculator accessory. A Beckman Century SS pH meter with an expanded scale was used to measure pH values of all buffered reaction mixtures to within 0.01 pH unit. Unless otherwise stated, all kinetic runs reported in this paper were carried out at pH 7.25 in 0.005 M phosphate buffers. The ionic strength of all reaction mixtures was adjusted to 0.1 M by the incorporation of the appropriate quantities of sodium chloride.

The hydration of acetaldehyde

$$CH_3CHO + H_2O \rightleftharpoons CH_3CH(OH)_2 \qquad (1)$$

is a reversible, pseudo-first-order process for which the integrated rate expression may be written

$$k_{obsd} = \frac{2.3}{t} \log \frac{[CH_3CHO]_t - [CH_3CHO]_\infty}{[CH_3CHO]_0 - [CH_3CHO]_\infty} \qquad (2)$$

The reaction was followed spectrophotometrically by recording the diminution of the carbonyl band at 278 nm. Plots were con-

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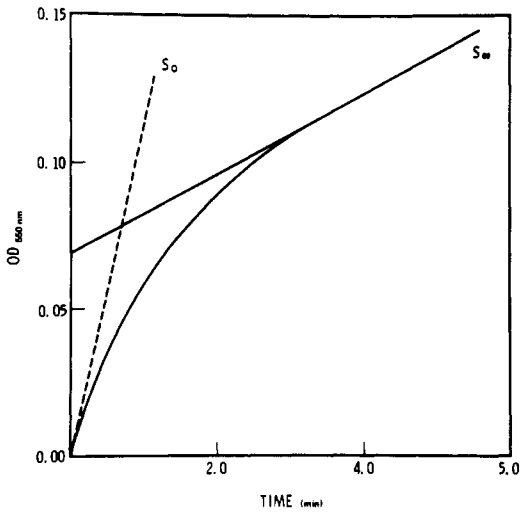

FIGURE 1: Variation in the rate of ferricytochrome *c* reduction with the hydration of acetaldehyde. S_0 and S_∞ represent initial and final slopes, respectively. Run was initiated by injecting neat acetaldehyde into a buffered solution (0.01 M phosphate, pH 7.25) containing 3.0×10^{-5} M ferricytochrome *c* and 0.25 unit/ml of xanthine oxidase.

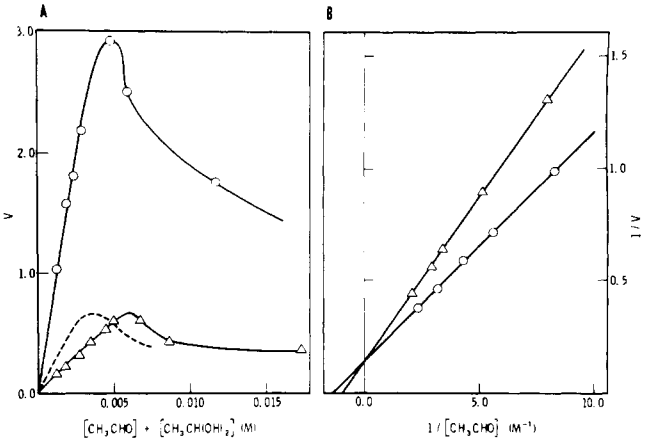

FIGURE 2: (A) Initial rate of ferricytochrome *c* reduction as a function of total aldehyde concentration. Upper solid curve: runs initiated by injection of neat acetaldehyde. Lower solid curve: runs initiated by injection of preequilibrated aqueous solutions containing both the aldehyde and the hydrate. Broken curve: data from lower solid curve which were corrected to represent the concentration of *free* aldehyde = $(1 - \chi)$[total aldehyde]. Reaction solutions contained 3.0×10^{-5} M ferricytochrome *c* and 0.045 unit/ml of xanthine oxidase. (B) Lineweaver–Burk plots of the xanthine oxidase catalyzed oxidation of acetaldehyde: (○) uninhibited process; (△) process inhibited by 5.5×10^{-4} M $CH_3CH(OH)_2$, $K_i = 1.6 \times 10^{-3}$ M.

structed of log $(A_t - A_\infty)$ *vs.* time, where A_t is the absorbancy recorded as the function of time and A_∞, the absorbancy after equilibration was established between the hydrated and unhydrated forms. The observed rate constants, k_{obsd}, were calculated from the slopes of the resultant straight lines: $k_{obsd} = -2.3 \times$ slope. The fraction of hydration, χ, for the equilibrated system was determined as in earlier work (Pocker *et al.*, 1967)

$$\chi = \frac{[CH_3CH(OH)_2]}{[CH_3CHO] + [CH_3CH(OH)_2]} = \frac{A_0 - A_\infty}{A_0} \quad (3)$$

where A_0 is the absorbancy of acetaldehyde obtained from extrapolation to the kinetic zero.

The enzymatically catalyzed *oxidation* of acetaldehyde was carried out in the presence of ferricytochrome *c*. The reduction of ferricytochrome *c* which accompanies the aldehydic oxidation[1] causes an increase in absorbancy at 550 nm. The difference in extinction coefficients between ferricytochrome *c* and ferrocytochrome *c* at this wavelength was taken to be 1.96×10^{-2} l. μmol^{-1} cm^{-1} (Horecker and Heppel, 1955). All reaction velocities are reported in terms of micromoles of ferricytochrome *c* per liter reduced per minute. Catalase (10 μg/ml) was incorporated into all reaction mixtures to destroy any hydrogen peroxide formed during the enzymatic reaction. It was observed that the presence of ferricytochrome *c*, xanthine oxidase, or catalase had no effect on the rate of hydration of acetaldehyde.

Results and Discussion

Before meaningful mechanistic conclusions can be drawn regarding the xanthine oxidase catalyzed oxidation of aldehydes, the *nature* and the *extent* of involvement by the aldehyde and its conjugate hydrate must be fully understood. In an earlier communication (Gregory *et al.*, 1972), it was shown that when anhydrous acetaldehyde is injected into a buffered solution containing ferricytochrome *c* and xanthine oxidase, two reactions commence at the kinetic zero: (i) the hydration of acetaldehyde, and (ii) the enzymatically catalyzed oxidation of the aldehyde–hydrate system. Figure 1 illustrates the variation in the rate of ferricytochrome *c* reduction as the hydration reaction progresses. It has been shown for such experiments (Gregory *et al.*, 1972) that the variation in the slope of the curve directly corresponds to the hydration process, a constant slope, S_∞, being reached upon equilibration of the hydrated and unhydrated species. Since the rate of reduction of ferricytochrome *c* decreases as the hydration of acetaldehyde proceeds, it was concluded that acetaldehyde (rather than its hydrate) is the preferential substrate of xanthine oxidase. These experiments were carried out at low enough aldehyde concentration such that the enzymatic oxidation was first order in aldehyde (Figure 2). Thus, if the variation in the rate of ferricytochrome *c* reduction illustrated in Figure 1 were due solely to the change in the concentration of free aldehyde, the initial and final rates should respectively be proportional to the initial aldehyde concentration and that after the *hydration* equilibrium had been established. Accordingly, one would anticipate that the initial and final slopes, S_0 and S_∞, of the curve in Figure 1 would allow the calculation of the fraction of hydration for the equilibrated acetaldehyde–hydrate system: $\chi_{app} = (S_0 - S_\infty)S_0$. This is not, however, the case. Instead we consistently observe from this type of analysis that $\chi_{app} = 0.9$ is much larger than the actual fraction of hydration, $\chi = 0.55$, directly determined as described in the Experimental Section.

The large value of χ_{app} results from an unexpectedly slow rate of enzymatic catalysis in the presence of hydrate which may imply that the hydrated aldehyde is an inhibitor of XO. This indeed would appear to be the case as illustrated in Figure 2A in which initial velocities of the reduction of ferricytochrome *c* are plotted against aldehyde concentration. The upper solid curve represents data for the unhydrated system in which neat aldehyde was injected last into the reaction mixture. The lower solid curve was obtained from corresponding runs in which an aqueous buffered solution of the hydrate in equilibrium with the aldehyde was added as the last component. When the points on the lower solid curve are corrected to represent the concentration of *free* aldehyde, the broken curve

[1] Oxygen functions as the primary electron acceptor and is converted into the superoxide ion. Ferricytochrome *c* is the secondary electron acceptor and nonenzymatically oxidizes the superoxide ion to molecular oxygen (Horecker and Heppel, 1955). The concentration of dissolved oxygen at atmospheric pressure in aqueous solution is *ca.* 0.001 M.

TABLE I: Calculation of Velocities of Ferricytochrome *c* Reduction as a Function of Acetaldehyde Concentration at Constant Hydrate Concentration.

$[Ald]_0 \times 10^3$ M	$[Hyd]_t \times 10^4$ M	$[Ald]_t \times 10^3$ M	t (min)	V_t[a]	$1/V_t$	$1/[Ald]_t \times 10^{-2}$ M^{-1}
1.8	5.5	1.3	0.49	0.77	1.3	7.7
2.4	5.5	1.9	0.32	1.2	0.83	5.3
3.4	5.5	2.9	0.23	1.5	0.67	3.5
3.6	5.5	3.1	0.19	1.8	0.56	3.2
4.8	5.5	4.3	0.14	2.2	0.45	2.4

[a] In μmol of ferricytochrome *c* reduced per l. min^{-1}.

results. It is immediately obvious that for a given concentration of *free* aldehyde, the presence of the hydrate causes extensive inhibition. It should be noted from the upper curve that even in the absence of the hydrated species, substrate inhibition in the usual sense is still observed. However, substrate inhibition *per se* only becomes detectable at relatively high concentrations of acetaldehyde (>0.005 M) in comparison to the much lower concentrations of hydrate which cause as much as 65% inhibition.

Other direct interpretations from Figure 2A should be made with caution since in these experiments, the concentrations of the aldehyde and the hydrate both vary. Before one may deduce either the type of inhibition by the hydrate or the inhibition constant, it is necessary to deduce rates of reduction of ferricytochrome *c* at various stages of hydration such that the velocities obtained as a function of aldehyde concentration will correspond to a constant concentration of the hydrate. Thus for each aldehyde concentration, one may choose a fixed hydrate concentration, substitute that value into a form of the integrated rate expression for the hydration reaction[2] and solve for the time, t, at which that concentration of hydrate would be present in the reaction mixture. One may then calculate the instantaneous rate of ferricytochrome *c* reduction, V, at time t from traces such as that illustrated in Figure 1. When this analysis is carried out for several aldehyde concentrations at the same fixed concentration of hydrate, a plot of the reciprocal of these instantaneous velocities *vs.* the reciprocal of aldehyde concentrations may be constructed. The approach is exemplified by the data in Table I in which the concentration of hydrate is 5.5×10^{-4} M. The Lineweaver-Burk plots of the inhibited and uninhibited exzymatic reactions are shown in Figure 2B. The hydrate appears to act as a competitive inhibitor, $K_i = 1.6 \times 10^{-3}$ M. Our assignment as to the type of inhibition, however, must be regarded as tentative since the enzymatic aldehydic oxidation, even in the absence of hydrate, does not obey normal Michaelis-Menten kinetics due to substrate inhibition by acetaldehyde. If indeed the aldehyde and its hydrate bind at a common site on the enzyme matrix, it is obvious that only the former is able to undergo the subsequent enzymatic hydroxylation (Gregory *et al.*, 1972).

In addition to hydrated acetaldehyde, small quantities of acetate ion are generally present in the reaction mixture when initial velocities of the xanthine oxidase oxidation of acetaldehyde are determined. Acetaldehyde rapidly undergoes air oxidation to acetic acid. Thus, acetic acid (converted into the acetate ion upon contact with phosphate buffers) will accompany the introduction of the substrate into the reaction mixtures. Because of the reversibility of the *enzymatic* oxidation of acetaldehyde to acetic acid, interaction of acetate with the active site on the enzyme surface would be expected. For this reason, inhibition studies using sodium acetate were carried out. It will be noted from Figure 3 that the noncompetitive inhibition caused by acetate[3] is not very potent, $K_i = 7.6 \times 10^{-2}$ M.

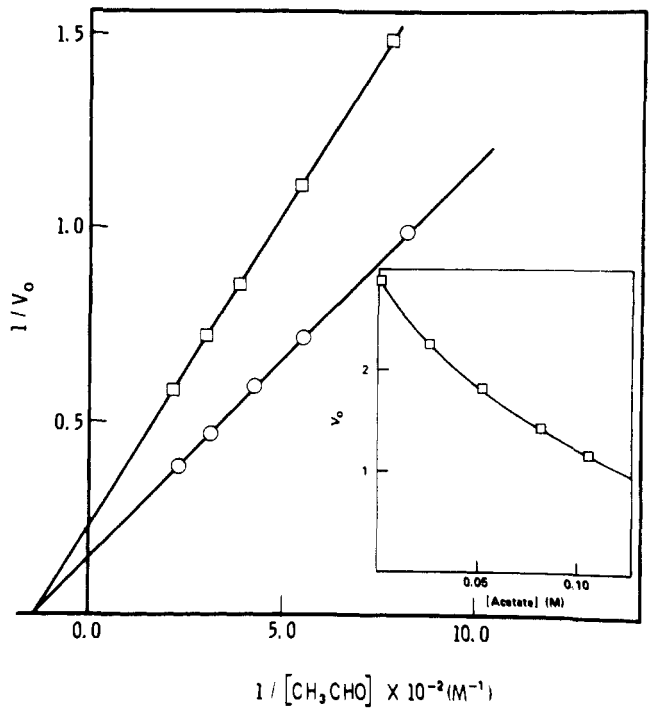

FIGURE 3: Inhibition of the xanthine oxidase catalyzed oxidation of acetaldehyde by acetate. Lineweaver-Burk plot: (○) uninhibited process; (□) process inhibited by 0.05 M sodium acetate. Inset: initial velocities as a function of sodium acetate concentration.

In summary the xanthine oxidase catalyzed oxidation of acetaldehyde, the hydrate (a species formed in a nonenzymatic side reaction), acetate (the product of the enzymatic oxidation), and acetaldehyde itself (the substrate), are all inhibitors. The hydrate is the most potent inhibitor and comprises about 55% of the overall concentration of the aldehyde-hydrate system in an equilibrated aqueous solution at 25°. Thus, it is evident that the xanthine oxidase catalyzed oxidation of aldehydes is a very complex process and even more intimately dependent upon the aldehyde-hydrate equilibrium than previously assumed. Apparent values of Michaelis-Menten parameters, after correction has been made to compensate for the fraction of aldehyde hydration, reflect the interaction between the enzyme and the hydrate as well as the aldehyde. Accordingly, in order for kinetic results associated with other xanthine oxidase catalyzed aldehyde oxidations to be meaningful, such investigations must be carried out under experimental conditions in which rates of the enzymatic oxidation are deduced prior to the formation of appreciable quantities of the hydrate. On the other hand, product inhibition by acetate is relatively mild such that when carefully purified samples of acetaldehyde are used in xanthine oxidase studies, acetate inhibition should be of little concern since the concentration of acetate formed during

[2] Equation 2 may be rewritten as

$$k_{obsd} = \frac{2.3}{t} \log \frac{\chi[CH_3CHO]_0 - [CH_3CH(OH)_2]_t}{\chi[CH_3CHO]_0}$$

[3] Parallel runs made in the presence of the corresponding concentrations of sodium chloride showed that the effects illustrated in Figure 4 are not due merely to changes in ionic strength.

the course of ferricytochrome *c* reduction is generally very small.[4]

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[4] It will be noted that in the present investigation, the concentration of ferricytochrome *c*, 3×10^{-5} M, was always much smaller than that of acetaldehyde.

Kinetics of Transamidating Enzymes. Production of Thiol in the Reactions of Thiol Esters with Fibrinoligase†

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ABSTRACT: Fibrinoligase is a transamidating enzyme, formed from the "fibrin-stabilizing factor" or "factor XIII" zymogen in two distinct steps of activation, first by thrombin and then by calcium ions. The enzyme catalyzes both the hydrolysis and the aminolysis of specific thiol ester substrates, exemplified by β-phenylpropionylthiocholine. The steady-state velocity of formation of the thiol (*e.g.*, thiocholine) product, measured by a continuous direct reaction with 5,5′-dithiobis(2-nitrobenzoic acid), follows Michaelis–Menten kinetics yielding, for example, for the hydrolysis of β-phenylpropionylthiocholine at pH 7.5 and 25° an apparent Michaelis constant of about 0.3 mM and a turnover number of 0.2 sec^{-1}. The kinetic analysis indicates that the pathway of catalysis includes an acyl-enzyme intermediate which can undergo deacylation by hydrolysis and also by aminolysis with an added amine substrate. The increased rate of thiol production in the presence of amines can be used as a quantitative index of specificity of the enzyme for various amine substrates. When kinetic experiments are carried out by mixing the thrombin-activated zymogen, calcium ions, and the ester substrate, without any prior incubation of the first two components, there is a marked delay in the onset of the steady-state formation of the thiol product. The lag phase corresponds to the calcium-dependent dissociation of the subunit structure of the thrombin-modified zymogen and it is linked to the unmasking of the active center of the transamidase. Calcium ions shorten the lag period and they also increase the steady-state rate of the enzymatic reaction. At low calcium concentration (5 mM), increase in general ionic strength accentuates these effects.

Following the demonstration that fibrinoligase (fibrin-stabilizing factor or blood coagulation factor XIII, activated by thrombin and by calcium ions) could catalyze the hydrolysis and aminolysis of certain thiol esters (Lorand *et al.*, 1972a), it became possible for the first time to analyze the kinetics of this transamidating enzyme by reactions involving exclusively synthetic substrates. Hitherto, such studies could be performed only in conjunction with protein substrates (see, *e.g.*, Lorand *et al.*, 1968, 1972b; Chung and Folk, 1972). The enzyme displays a great deal of specificity both toward the acyl portion and the thiol-containing residue in the esters, with β-phenylpropionyl- and *trans*-cinnamoylthiocholine among the best substrates examined so far giving an apparent Michaelis constant of the order of 10^{-4} M for hydrolysis. These compounds are also relatively stable in the absence of the enzyme, with a nonenzymatic rate constant for hydrolysis of about 10^{-6} sec^{-1} at pH 7.5 and 25°.

Continuous recording of the disappearance of substrates and

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