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KINETICS OF INHIBITION OF HORSE LIVER ALCOHOL DEHYDROGENASE BY *p*-METHYLBENZYL HYDROPEROXIDE

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The complex kinetic behaviour of *p*-methylbenzyl hydroperoxide in its inhibitory action on horse liver alcohol dehydrogenase was examined. The kinetic patterns are markedly different at very low ($<10^{-8}$ M) and high ($>10^{-7}$ M) hydroperoxide concentrations. In both cases very low inhibition constants (4nM and 14nM, respectively) were found. A possible mechanistic model based on these results is discussed.

KEY WORDS: Horse liver alcohol dehydrogenase, p-methylbenzyl hydroperoxide

INTRODUCTION

Horse liver alcohol dehydrogenase (HLAD, EC 1.1.1.1.) is the most thoroughly investigated representative of the wide and branched family of NAD-dependent dehydrogenases[1]. Effects (mostly inhibition) of many compounds on this enzyme have been studied[2] and in many instances inhibitors of no apparent physiological relevance have helped to reveal important features of the catalytic mechanism. Recently, *p*-methylbenzyl hydroperoxide (XyHP) was found to be a very potent reversible inhibitor[3] of ethanol/acetaldehyde interconversion, which also acts as a substrate for the enzyme[4].

This phenomenon of XyHP degradation is coupled with a NAD^+-consuming (partially enzymic) reaction, closely related to the HLAD-catalyzed conversion of NAD^+ to the unusual derivative (designated[5] NADX) at very high hydrogen peroxide concentrations.

Binding of XyHP to the enzyme-NAD^+ complex was documented[4] as the initial step in the mechanism of inhibition but no value for the inhibition constant of XyHP has been estimated yet because of the atypical kinetic behaviour of the reaction system. Here we describe an attempt to partially elucidate the interaction of XyHP with HLAD.

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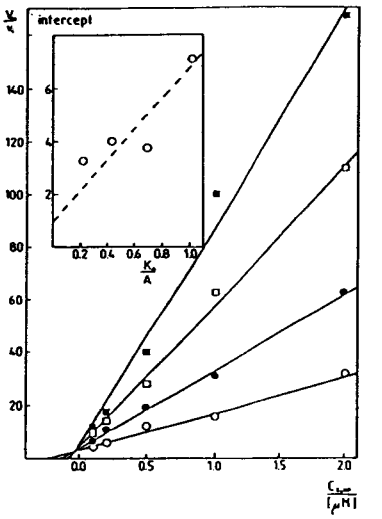

FIGURE 1 Dixon plot of the HLAD reaction inhibited by high XyHP concentrations. Velocities were measured in the standard reaction mixture (see text) containing 9nM HLAD at $K_A/A = 0.21$ (○), 0.42 (●), 0.70 (□), and 1.05 (■). The inset shows a dependence of V_m/v_i-intercepts on K_A/A values (plotted for the mechanism described in the Discussion).

MATERIAL AND METHODS

HLAD from Sigma and our own preparation of EE-isoenzyme[6] were used and gave the same results. Activity was measured spectrophotometrically according to Dalziel[4,7] and expressed as active site molar concentrations.

XyHP was prepared by a modified[3] method of Hock and Lang[8]. NAD^+ was purchased from Imuna (Czechoslovakia), common laboratory chemicals from Lachema (Czechoslovakia). Millipore^R water was used thorough the study.

Spectrophotometric measurements were performed on Shimadzu UV-3000 and Cary 118 C (Varian) spectrophotometers.

RESULTS

Kinetic measurements of the inhibition of HLAD activity were performed in a mixture consisting of 0.75mM NAD^+ in 0.05M sodium carbonate buffer, pH 10 at 30°C. The enzyme activities were measured at various concentrations of ethanol and XyHP. Series of results obtained in the absence of XyHP were used for a determination of an actual K_A values (data not shown).

Reactions in the presence of XyHP were initiated by additions of enzyme and typical progress curves with marked transient phases[4] were obtained. The decrease in the

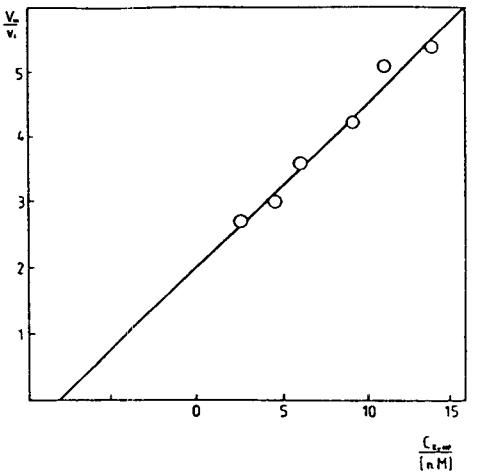

FIGURE 2 Dixon plot of the HLAD reaction inhibited by low XyHP concentrations Velocities were measured in the standard reaction mixture (see text) containing 1.5nM HLAD at $K_A/A = 1$.

reaction rate caused by introduction of the inhibitor was not abrupt and attaining a stationary value after a short time (tens of seconds).

Ratios of maximal velocity over steady-state velocities of the inhibited reaction (V_m/v_i) were plotted against XyHP concentrations (Dixon plot). At higher XyHP concentrations a set of linear plots typical of inhibition was obtained but the V_m/v_i-intercepts were slightly higher than the assumed values[c] $1 + K_A/A$ (Figure 1). Formally, this situation can by described by equation (1),

$$V_m/v_i = a + K_A/A.(b + X/K_{i,2}) \; . \tag{1}$$

An apparent inhibition constant $K_{i,2} = 14$ nM was calculated from the slopes (Figure 1). Estimated values of a and b (see inset of Figure 1) are 1–2 and 4–6, respectively. The coefficient a and b could not be evaluated exactly because the intercepts are too small in comparison with V_m/v_i-values at the XyHP concentrations used (which must be sufficiently high to obtain linear plots).

At lower concentrations of XyHP the respective plots are curved and at very low XyHP concentrations the obtained ratios fit a common Dixon plot for a competitive inhibition (Figure 2) in accord with equation (2),

$$V_m/v_i = 1 + K_A/A.(1 + X/K_{i,1}) \; . \tag{2}$$

In this case the inhibition constant $K_{i,1}$ is 4 nM.

[c] Abbreviations used: A – ethanol concentration, X – XyHP concentration, and K_A – Michaelis constant for ethanol

TABLE I
Stoichiometry of XyHP : NAD^+ consumption in the HLAD-catalyzed reaction measured as a decrease in the NAD^+ concentration (converted to NADH by addition of ethanol for determination) in the 0.1M phosphate buffer, pH7, containing 70 μM NAD^+.

Initial XyHP concentration [μM]	Decrease in NAD^+ concentration [μM]	HLAD concentration μM
27	11	0.2
30	14.5	1.0
24	16	1.0

DISCUSSION

The inhibition of the HLAD-catalyzed oxidation of ethanol by NAD^+ exhibits an unusual kinetic pattern. A curve dependence of V_m/v_i-ratios on XyHP concentration become linear only at concentrations of XyHP greater than 10^{-7} M.

A kinetic study of XyHP-NAD^+-HLAD interaction, based on the formation of a derivative absorbing[4] at 285 nm, was not possible at pH 10 because of an interference by increasing absorbance at 260 nm and 340 nm (*p*-methylbenzaldehyde + NADH or some NAD-derivatives) during the reaction at this pH (data not shown). Results obtained at pH 7 lead to a non-linear Lineweaver-Burk plot[4]; its description[4] based on the Hill equation is only formal and the system (involving also non-enzymic step(s)) is not fully saturated even at milimolar XyHP concentration (L. Žídek, unpublished results). Also, the stoichiometric ratio of consumed XyHP : NAD^+ measured by the decrease in NAD^+ concentration after addition of XyHP (20–30 μM concentration) is about 2 : 1 (Table I) in contrast to the value of 1 : 1 obtained[4,9] by "titration" of NAD^+ by small amounts of XyHP (where concentration did not exceed 2 μM).

The inhibition constants found here define XyHP as a very potent HLAD inhibitor but mere kinetic experiments cannot give an unequivocal mechanistic explanation of the phenomena observed. If we suppose the kinetical independence of HLAD subunits[10] then the hypothesis that two molecules of XyHP are bound per an inhibition cycle (Figure 3A) seems to be in a good agreement with the results obtained. Solving this mechanism graphically (Figure 3B) according to King and Altman[11], we obtained a general description of the dependence of V_m/v_i on XyHP concentration in this case (equation 3)

$$V_m/v_i = 1 + \frac{K_A}{A}\left(1 + \frac{k_1 X}{k_{-1} + k_2}\left(1 + \frac{k_2}{k_5}\,\frac{1 + ((k_{-3} + k_5)/k_3 X)}{1 + ((k_4/k_5)\,(k_{-3} + k_5)/k_3 X}\right)\right) . \quad (3)$$

If k_4 is not much greater than k_5, equation (3) can involve both equation (1) (for when $X \ll k_4/k_5 . (k_{-3} + k_5)/k_3$) and equation (2) (for when $X \gg k_4/k_5 . (k_{-3}+$

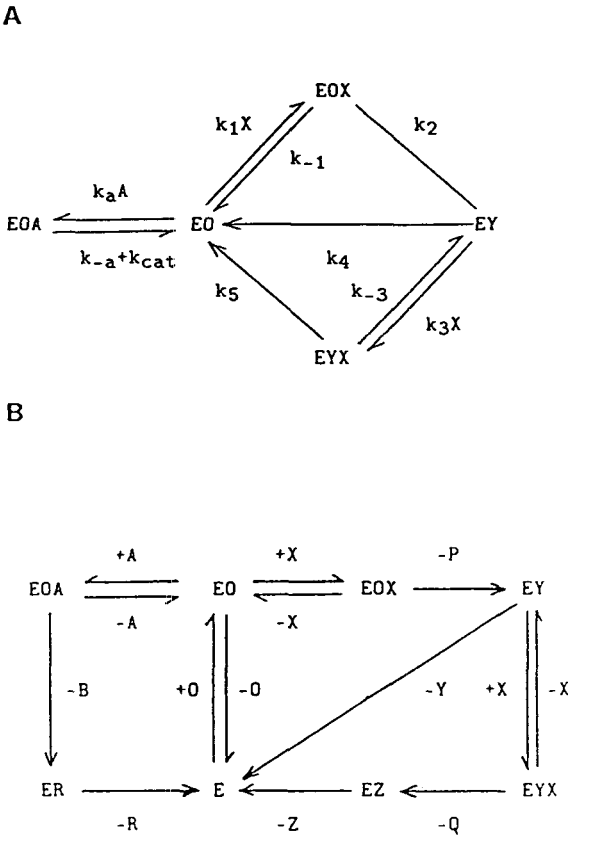

FIGURE 3 Scheme of the proposed mechanism (A) and its graphical expression (B) for zero initial concentrations of the products. E = free enzyme, A = ethanol, B = acetaldehyde, O = NAD^+, R = NADH, Y,Z = NAD-derivatives, X = XyHP, P,Q = xylene derivatives (product of XyHP conversion).

$k_5)/k_3$) with the inhibition constants expressed as $K_{i,1} = k_4/k_1 . (k_2 + k_{-1})/(k_2 + k_4)$ and $K_{i,2} = k_5/k_1 . (k_2 + k_{-1})/(k_2 + k_5)$, respectively. The coefficients a and b in the equation (1) are 1 and $1 + k_1k_2(k_{-3} + k_5)/k_3k_5(k_{-1} + k_2)$, respectively. The existence of two different binding sites for XyHP in the enzyme molecule seems also to be compatible with our experimental data but further experimental data would be required to confirm this speculation.

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