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HYDROPEROXIDIC INHIBITOR OF HORSE LIVER ALCOHOL DEHYDROGENASE ACTIVITY, TIGHTLY BOUND TO THE ENZYME-NAD$^+$ COMPLEX, CHARACTERISTICALLY DEGRADES THE COENZYME

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The strong inhibition of horse liver alcohol dehydrogenase (HLAD) by *p*-methylbenzyl hydroperoxide (XyHP)[7] is only transient, XyHP behaves also as a pseudo-substrate of the enzyme and in the presence of NAD$^+$, is degraded by HLAD to (as yet unidentified) non-inhibiting products while the NAD$^+$ is converted to a derivative similar to the "NADX", originally observed in an analogous reaction of HLAD with hydrogen peroxide.[4] The apparent K_M for XyHP is approximately 10^4 times smaller than that for H_2O_2. The catalytic constant k_{cat} for HLAD degradation of XyHP is two orders of magnitude less than that for ethanol dehydrogenation. XyHP inhibits both directions of the alcohol-aldehyde interconversion with equal potency. The first step of the inhibition mechanism is a tight binding of XyHP to the binary HLAD-NAD$^+$ complex.

KEY WORDS: Horse liver alcohol dehydrogenase, *p*-methylbenzyl hydroperoxide, tight-binding inhibition.

INTRODUCTION

Horse liver alcohol dehydrogenase (HLAD) is the most thoroughly researched representative of the family of NAD-dependent enzymes[1] having numerous divergent physiological functions.[2] HLAD also exhibits several alternative catalytic activities.[2,3] Among them the "peroxidase activity"[4] is the most unusual one. It is responsible for the *in vitro* conversion, in the presence of H_2O_2, of NAD$^+$ into a derivative termed[4] NADX, with a partially degraded nicotinamide ring.[5] The K_M of HLAD for H_2O_2 as a substrate for the NADX-forming reaction being as high[6] as 100 mM can hardly support speculation on the possible physiological role of HLAD in degrading H_2O_2 and no biological function of NADX has been considered yet.

Here we report on the ability of HLAD to degrade an organic derivative of H_2O_2, *p*-methylbenzyl hydroperoxide (XyHP), by a reaction related to the "peroxidase activity"[4] of HLAD. The affinity of the HLAD/NAD$^+$ system for XyHP is so high that in the presence of minute traces of XyHP the alcohol-aldehyde interconverting

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activity of HLAD is extremely strongly inhibited.[7] The inhibition is only transient and ceases with the concomitant decomposition of XyHP catalysed by HLAD. The initial phase of this unusual inhibitory process has been closely examined in this work.

MATERIALS AND METHODS

HLAD: Three commercial batches (Sigma, Boehringer and Fluka) and our own preparation of pure "EE isoenzyme"[8] were used with purity[9] ranging from 50 to 90%. The enzyme was dialyzed against 0.1 M sodium phosphate buffer before use. The active site zinc-depleted HLAD-variety[10] was a gift from Prof. M. Zeppezauer.

NAD^+ from Imuna (Czechoslovakia) and Boehringer were interchangeable in this work, NADH was purchased from Boehringer. Common laboratory chemicals were of standard quality, mostly from Lachema (Czechoslovakia). Millipore® water was used throughout the study.

Preparation of XyHP was by a modified[7] method of Hock and Lang.[11]

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

Determination of Hydroperoxide Concentration

Assays of hydroperoxides and H_2O_2 were routinely performed using the procedure[12] based on photometry of the ferric-thiocyanate complex formed from the ferrous salt upon oxidation by the hydroperoxide. The accuracy of the method[12] was shown by comparison with the method described by Gebicki and Guille.[13]

Chromatographic Procedures

Analytical thin layer chromatography of XyHP and of related products was performed as previously[7] described.

Gas chromatography: A fused silica capillary column (0.25 mm d. × 17 m) with SE-54 stationary phase using H_2 or a Carbowax 20 M filled column (120 × 4 mm) using N_2 with FID detector were used to separate the *p*-methylbenzyl alcohol and the corresponding aldehyde.

HLAD Concentration and Activity

Direct determination of the enzyme active site concentration (two independent active sites in one 80 kD molecule) is possible by the "pyrazole titration"[14] only with rather concentrated enzyme solution. As found by Dalziel[9] the results of routine activity assays with very small amounts of HLAD can also be expressed in terms of the active site concentration using a simple formula. Standard activity measurements in this work have been performed at 25°C in 0.1 M sodium glycinate buffer, pH 10.0 with 0.5 mM NAD^+ and 10 mM ethanol concentrations in 1 ml plastic cuvettes with 1 cm light path. In the formula

$$e = \frac{7{,}470}{z \cdot v} \qquad (1)$$

"e" denotes the HLAD active sites concentration (in micromolarity[14]) in the sample from which the volume "v" (in microliters) in 1 ml reaction mixture causes the initial 340 nm absorbance to increase by 0.200 in "z" seconds.[14] The coefficient in the above formula differs slightly from that used by Dalziel[15] due to later refinement (S. Taniguchi and H. Theorel, personal communication). With the formula (1) we obtained for ethanol dehydrogenation a turnover number (molecular activity) for our enzyme preparations of $k_{cat} = 3 +/- 0.5\ s^{-1}$, which is in reasonable agreement with the published value.[2]

The acetaldehyde reducing activity of HLAD was measured at 25°C in 0.1 M sodium phosphate buffer, pH 7.

RESULTS AND DISCUSSION

XyHP as a Substrate for HLAD

Consumption of XyHP by the HLAD/NAD$^+$ system

HLAD is not oxidatively attacked[7] by XyHP but on the contrary, prolonged exposure of HLAD to high concentrations of XyHP reveals that the enzyme (in the presence of an excess of NAD^+) degrades the inhibitor with restoration of complete enzyme activity. From a typical experiment (see Figure 1) it is possible to calculate that under the given conditions the average rate of XyHP degradation is approximately one molecule of XyHP per minute per single enzyme active site. The absence of

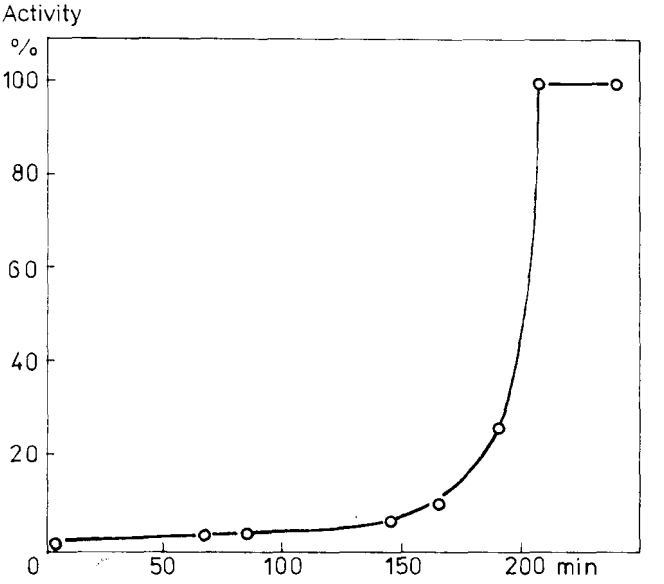

FIGURE 1 Gradual recovery of HLAD activity caused by degradation of XyHP. Addition of XyHP (0.86 mM final concentration) to a mixture of 4.1 μM HLAD and 3.9 mM NAD^+ completely abolished the enzyme activity (measured in an aliquot sample). When the enzyme activity reappeared the XyHP was not detectable in the mixture.

XyHP in the mixture was verified by a negative result using the sensitive potassium iodide oxidation test on TLC plates.[7]

In the absence of NAD^+ the reactivation of HLAD does not occur and XyHP is not decomposed within a much longer time periods (data not shown).

Formation of a NAD^+ Derivative

The ability of HLAD (in the presence of NAD^+) to degrade XyHP is an obvious analogy of the reaction with hydrogen peroxide discovered[4] and further studied[3,5] by Favilla *et al.* and this prompted us to test whether NAD^+ is chemically modified under the conditions used here. This was found to be so from repeated scans of the UV spectra of the mixture containing HLAD, NAD^+ and XyHP at pH 7.0 (the value at which the reaction with H_2O_2 was studied[3]) (see Figure 2). The final

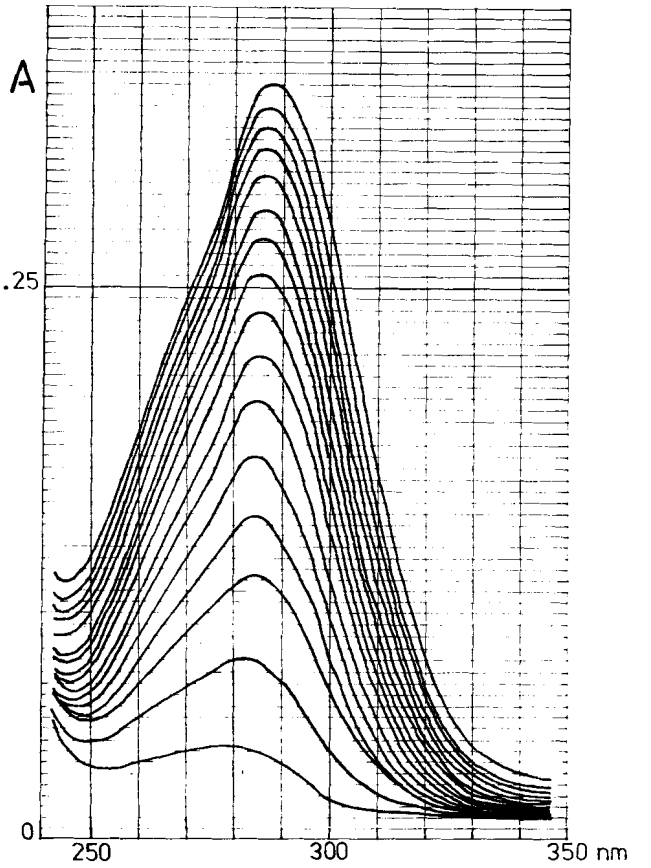

FIGURE 2 Spectral changes during the incubation of 400 nM HLAD with 20 μM NAD^+ and 3.5 μM XyHP. Represented are the difference spectra of individual stages (10 min apart) based on conditions before initiation of the reaction by addition of HLAD.

absorption maximum at 300 nm which is equal[3,4] to that of NADX indicates at least some similarity between our final product(s) and NADX. Gradual shift of the absorption maximum from 285 nm to its final value of 300 nm indicates a process involving intermediates.

Reaching the final value of the absorption maximum takes approximately ten times longer than that required for the complete degradation of XyHP. Hence it can be inferred that the ultimate steps of the final product formation do not depend on XyHP and probably do not require the enzyme either; this represents another similarity with the reaction of Favilla *et al.*[3]

Stoichiometry and Products of XyHP Degradation

In experiments on XyHP degradation by HLAD with an excess of NAD^+ we tried to enzymatically[16] determine the remaining coenzyme after the complete disappearance of XyHP and full recovery of HLAD activity (data not shown). This revealed a stoichiometric ratio between NAD^+ and XyHP consumed of approximately unity. Precise figures were difficult to obtain, possibly due to the interference of subsequent enzymic reactions between NAD^+ and XyHP degradation products.

Concerning these final products to which XyHP has been converted no clear data is available yet. Qualitatively both the corresponding aldehyde and alcohol (*p*-methylbenzaldehyde and *p*-methylbenzyl alcohol) can be detected after XyHP has disappeared. Whether either or both of them is the immediate product of the XyHP degrading process is not clear since the aldehyde might also originate from the enzymic conversion of the alcohol on account of the excess of NAD^+ present. Both benzylalcohol and benzaldehyde are good substrates[2] of HLAD and the same applies to their *p*-methyl derivatives (L. Žídek, unpublished results). Quantitative measurements were not performed since contamination of the used XyHP solution with either or both of the alcoholic or aldehydic HLAD substrates cannot be excluded. These two compounds are easily formed by a radical dismutation[17] reaction from the XyHP. That it is virtually impossible to have a contaminant-free XyHP has also been documented.[18] Exact determination of those two products in the presence of an excess of XyHP has not yet been accomplished but our findings indicate that the content of aldehyde and/or alcohol was less than 10%.

Reaction rates and kinetic characteristics

When XyHP proved to behave as a pseudo-substrate, by forming a characteristically absorbing product (see Figure 2), it seemed appropriate to determine the Michaelis constant.

In experiments with variable XyHP concentrations the rate of absorbance increase at 285 nm gave a nonlinear double reciprocal plot (not shown). To exclude the possibility that at this wavelength possible XyHP degradation products (especially *p*-methylbenzaldehyde, $\lambda_{max} = 260$ nm), could interfere, the formation of the NAD derivative was followed at 300 nm, a wavelength at which the aldehyde does not absorb.

A nonlinear double reciprocal plot under these conditions was also observed (see inset in Figure 3). Using the Hill's kinetic model (instead of the Michaelis-Menten one) and a computer program,[19] suitable values for kinetic parameters were obtained: $K_M = 1.7 +/- 0.4\ \mu M$, and Hill coefficient $0.56 +/- 0.08$. The Michaelis constant

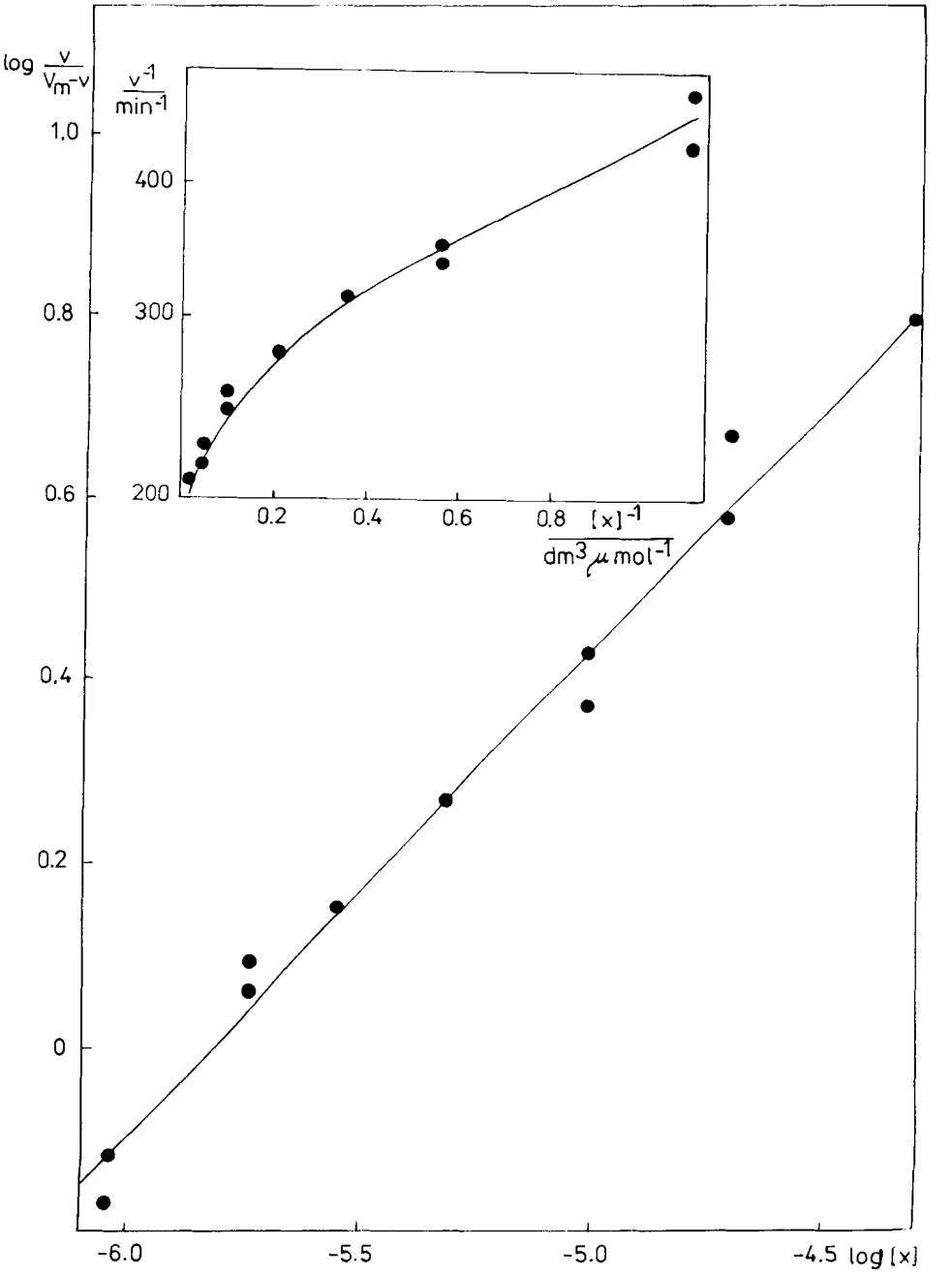

FIGURE 3 A Hill plot of rate of NAD-derivative formation (dA_{300}/dt) versus XyHP concentration (M). The data (obtained in 0.1 M sodium phosphate buffer pH 7 at 30°C and NAD^+ and HLAD (active site concentrations 0.5 mM and 0.27 μM, respectively) gave a non-linear Lineweaver-Burk plot (inset). The Hill coefficient h = 0.56 + / − 0.08; [×] denotes concentration of XyHP in μM.

for this reaction is almost 10,000 times smaller than the K_M for the reaction of HLAD for hydrogen peroxide.[6]

The maximum velocity was tentatively converted into the molecular activity and gave $k_{cat} = 0.009 +/- 0.001\ s^{-1}$. The calculated value for k_{cat} assumes that $\varepsilon_{NAD\,derivative}$ is equal to that[3] of the NADX, i.e. 15,000 $M^{-1}\,cm^{-1}$.

HLAD stripped off its "catalytic" zinc[10] does not catalyze the NAD^+ derivatization by XyHP until Zn^{2+} has been reincorporated (M. Řezáč, unpublished). This indicates that the phenomenon does not depend on non-HLAD contaminants of the enzyme preparations used.

In comparison with the value of $0.017\ s^{-1}$ calculated from the average rate of XyHP disappearance (from Figure 1) the value of $0.009\ s^{-1}$ seems to be in reasonable accord. Actually, the average rate (calculated from the data of Figure 1) should not be much different from the initial one since for most of the incubation period the enzyme was well saturated with both XyHP and NAD^+. Probably a higher pH value (e.g. pH 10) might have exhibited a positive influence on the reaction rate.

Nevertheless, in comparison with the value of $k_{cat} = 290\ min^{-1}$ reported[6] for the reaction of HLAD with H_2O_2, this value appears astonishingly low. On the other hand this value does support the concept of HLAD being "locked" by the peroxide degradation with regard to the dehydrogenase reaction. As far as the high k_{cat} value for H_2O_2 is concerned there is an element of uncertainty since it is higher than the $k_{cat} = 181\ min^{-1}$ for ethanol dehydrogenation.[2] Unfortunately missing data in the original report[6] concerning enzyme concentration does not clarify this uncertainty.

While the maximum velocity for the reaction of HLAD/NAD^+ with hydrogen peroxide[6] had to be measured at concentrations which cause irreversible inactivation this does not occur with XyHP. As shown in Figure 1 even when the enzyme is fully inactivated by the presence of almost 0.1 M XyHP, its activity is completely restored when XyHP has been removed by degradation.

XyHP as Inhibitor of HLAD

Inhibition of alcohol oxidation

Standard kinetic parameters of the inhibition of HLAD by XyHP have not yet been determined because of its atypical kinetic pattern due to the non-Michaelis character of the XyHP/HLAD-NAD^+ interactions (see Figure 3). Since the K_i value cannot be determined the potency of XyHP as an inhibitor of HLAD was characterized by the IC_{50} value. The data obtained (Table I) show that this ratio is virtually constant

TABLE I

Influence of enzyme and substrate concentrations on the IC_{50} values for XyHP on the ethanol dehydrogenating activity of HLAD

	Active site conc. [nM][a]			Ethanol concentration [mM][b]			
	290	80	26	1.0	2.1	4.2	6.3
IC_{50} XyHP [nM]	190	40	13	36	35	42	39

[a]Standard assay conditions—see Methods. [b]70 nM enzyme at standard assay conditions (except ethanol concentration).

and very small; ethanol concentration did not influence it. The tests for uncompetitive behaviour of XyHP towards ethanol could not be reliably evaluated since the reaction rate changes within the assay procedure (see below).

Decomposition of XyHP is accompanied by gradual recovery of the alcohol dehydrogenating activity of HLAD (Figure 1) and was also directly observed photometrically on the progress curve of the inhibited HLAD reaction if studied for a longer period of time. As shown in Figure 4 the rate of NADH production, strongly depressed by the presence of XyHP, commenced to increase after one or two minutes: presumably in connection with the exhaustion of XyHP. However the very beginning of the progress curve (which could be overlooked if the chart speed and scale range are not suitably chosen) is atypical. During the first pre-steady state phase the reaction rate gradually decreases and achieves its steady value within approximately 20–30 s. The steady reaction rate then gradually starts to increase again, obviously due to the exhaustion of XyHP which, as shown above, is degraded by the HLAD/NAD^+ system (Figure 4, curve A).

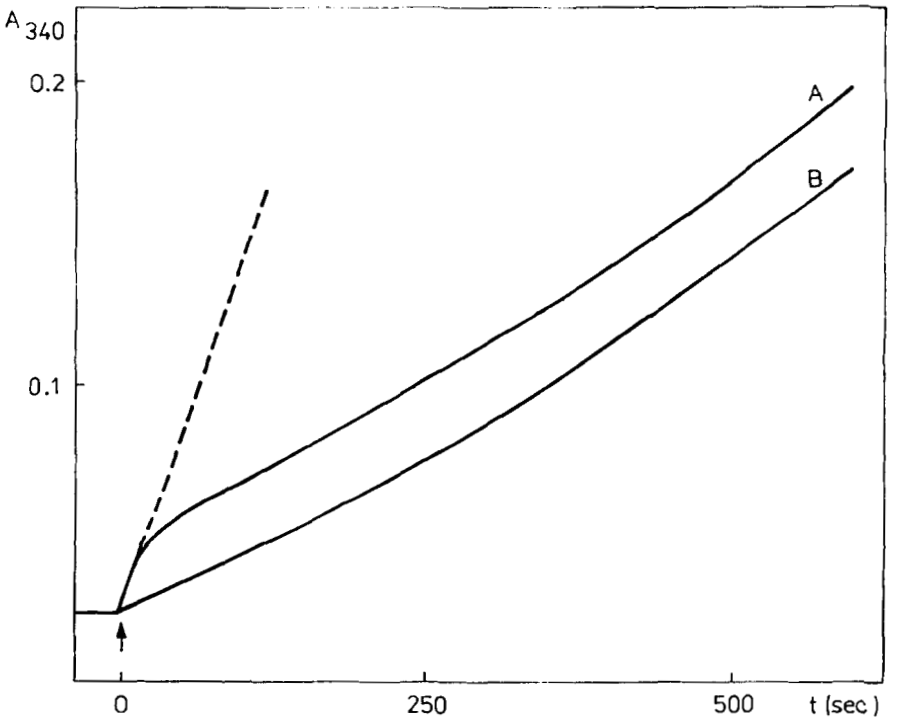

FIGURE 4 Progress curve of the uninhibited (----) and XyHP inhibited (———) ethanol dehydrogenation. The reaction mixture consisted of 10 nM HLAD, 0.5 mM NAD^+, 10 mM ethanol and 160 nM XyHP in 0.1 M sodium glycinate buffer pH 10 at 25°C. (A) An identical graph was obtained regardless of whether the reaction was started (arrow) by the addition of the enzyme or NAD^+ (after 30 s preincubation of the other components), (B) curve was obtained when ethanol was the component starting the enzymic reaction.

When this experiment was modified so that NAD^+, enzyme, and XyHP in the cuvette were preincubated for 30 s and the reaction was started by ethanol, the pre-steady state phase of the progress curve failed to appear (Figure 4, curve B). When started by the addition of NAD^+ (after a 30 s preincubation of HLAD, ethanol and XyHP) the reaction showed a progress curve identical with that of curve A (Figure 4).

There are two hypothetical mechanisms which explain the observed phenomena. (1) The conclusion drawn from the gradual onset of inhibition (Figure 4, curve A) and its absence in curve B (Figure 4) is that the inhibitory species could be enzymatically generated from two components: NAD^+ and XyHP. This transient inhibitor and its effects then should fade as noted for XyHP itself. (2) Another possible conclusion is that the observed depression of the NADH formation was due to its reoxidation by some radical species derived from XyHP and O_2. A similar process in which lactate dehydrogenase-bound NADH is oxidized to NAD^+ (via the NAD^+ radical) with the participation of oxygen was described by Chan and Bielski.[20] If this conclusion is true, the reverse reaction of HLAD, the aldehyde reduction, ought to be apparently activated by XyHP. This second alternative was disproved by the following experiments.

Inhibition of acetaldehyde reduction

XyHP also inhibits the acetaldehyde reduction. At 0.2 mM NADH and 0.25 mM acetaldehyde, XyHP concentrations of 25 nM and 43 nM were sufficient to decrease the NADH consumption rates by 7 nM and 22 nM HLAD, respectively to half their original values. This shows that 50% inhibition is achieved at total XyHP concentration not significantly different from half the enzyme concentration and that the strength of inhibition of the acetaldehyde reduction reaction is very similar to that observed for alcohol dehydrogenation (Table I). Furthermore the shape of the progress curve of the inhibited acetaldehyde reduction is analogous to that of alcohol dehydrogenation (Figure 5). The gradual onset of inhibition is understandable if the inhibitory species is being formed in the cuvette during the enzymic acetaldehyde reduction. However, in contrast to the dehydrogenation reaction (see Figure 4) the order of mixing the reaction components does not influence the shape of the progress curve. From the curves shown in Figure 5 the following can be deduced: (a) The enzyme-inhibitor complex does not arise from the incubation of XyHP, NADH, and acetaldehyde (without the enzyme) (curve A), (b) the enzyme-inhibitor complex is not formed in the reaction of HLAD, XyHP, and NADH (curve B). (c) The enzyme-inhibitor complex does arise from the incubation of NAD^+, XyHP, and HLAD, and functions in the subsequently started acetaldehyde reduction (curve D) and (d) NADH interferes with the enzyme-inhibitor complex formation from NAD^+, XyHP, and HLAD during the preincubation (curve C).

The above observations can be summarised as follows. The inhibitory species in aldehyde reduction (and obviously also alcohol dehydrogenation) is generated from NAD^+ and XyHP by the action of the enzyme. The interference (see item [d] above) of NADH with the enzyme-inhibitor complex formation is easily explained by the competition of the two coenzyme forms for the enzyme; as reported[21] the dissociation constant of the binary complex HLAD-NADH at pH 7 is 400 times less than that for the analogous complex containing NAD^+.

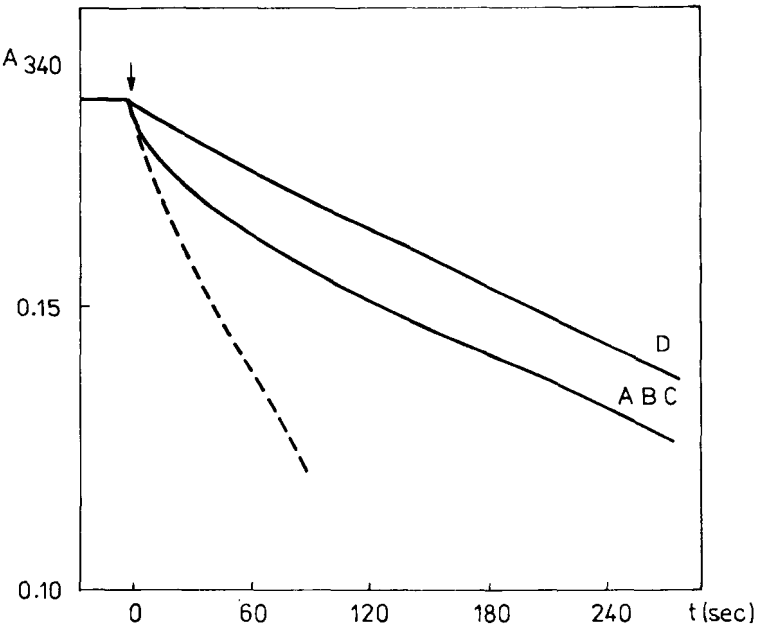

FIGURE 5 Progress curve of the uninhibited (----) and XyHP inhibited (———) acetaldehyde reduction. The reaction mixture consisted of 14 nM HLAD, 0.25 mM acetaldehyde, 0.24 M NADH, and 150 nM XyHP in 0.1 M phosphate pH 7 at 25°C. (A) and (B) The reactions were started (arrow) by enzyme and acetaldehyde addition, respectively. (C) The reaction was started by acetaldehyde addition after a 200 s preincubation of the complete reaction mixture with 15 μM NAD^+ added. (D) The reaction was started after a 30 s preincubation by simultaneous addition of NADH and acetaldehyde to a mixture containing XyHP, enzyme, and 15 μM NAD^+.

CONCLUSIONS

The finding that the derivative of hydrogen peroxide in the HLAD/NAD^+ system elicits the conversion of NAD^+ to a similar, if not identical, derivative as does H_2O_2 is not surprising. But one of the most interesting features of the behavior of HLAD towards XyHP is the powerful reversible inhibition by XyHP of the enzymic alcohol dehydrogenation activity in contrast to the irreversible inactivation[4,6] of HLAD by H_2O_2.

The very high affinity (approximately micromolar K_M) of HLAD for XyHP could be explained partly by hydrophobic interaction between the XyHP and the hydrophobic pocket which is well defined[22] in the enzyme's substrate binding site but some mechanistic factors could have some role to play.

The shape of the progress curves (see Figures 4, 5) does permit qualitative judgements on the mechanism of inhibition without rapid-mixing experiments.

In both directions of the HLAD catalyzed alcohol-aldehyde interconversion (see Figures 4, 5) the long pre-steady state period can only be eliminated by a preincubation of the apoenzyme with NAD^+. This indicates that the binding of XyHP to the binary HLAD-NAD^+ is the decisive step in the inhibition mechanism but

ternary HLAD-NAD^+-XyHP complex formation is obviously also the first step in the XyHP degradation and NAD-derivative production. The available data for the observed phenomenon of inhibition of the enzyme-catalyzed reaction does not differentiate between dependence on this HLAD-NAD^+-XyHP complex or on some of the subsequent steps of its transformation.

The turnover number for XyHP consumption is low (ca. $10^{-2}\,s^{-1}$). The initial step of the inhibitory mechanism, which is reflected by the long pre-steady state phase (theoretically treated in reference 23), is slow due to the extremely low concentration of the tightly bound XyHP. Nevertheless, the rate constant for this first step is reasonably high as argued below.

Using the data generated here and simple Michaelis-Menten analysis of the process (see equation (2)) the lower limit of the value of the k_1 can be assessed.

$$\text{XyHP} + \text{E-NAD}^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{“EI”} \xrightarrow{k_2} \text{E} + \text{product(s)} \qquad (2)$$

Since the value of $k_1 = (k_2 + k_{-1})/K_M$ should be greater than that for k_2/K_M, insertion of values of $k_2 = 10^{-2}\,s^{-1}$ and $K_M = 10^{-6}\,M$ into equation (2) gives a calculated value for $k_1 > 10^4\,s^{-1}\,M^{-1}$ which is the value published for binding ethanol to the HLAD-NAD^+ complex.[21]

Acknowledgements

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