Biochemistry

Volume 32, Number 3 January 26, 1993

Accelerated Publications

Horse Liver Alcohol Dehydrogenase-Catalyzed Oxidation of Aldehydes: Dismutation Precedes Net Production of Reduced Nicotinamide Adenine Dinucleotide†

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Received November 4, 1992; Revised Manuscript Received November 23, 1992

ABSTRACT: The oxidation of aldehydes by horse liver alcohol dehydrogenase (HL-ADH) is more complex than previously recognized. At low enzyme concentrations and/or high aldehyde concentrations, a pronounced lag in the assay progress curve is observed when the reaction is monitored for NADH production at 340 nm. When the progress of the reaction is followed by ^{1}H NMR spectroscopy, rapid dismutation of the aldehyde substrate into the corresponding acid and alcohol is observed during the lag phase. Steady-state production of NADH commences only after aldehyde concentrations drop below 5% of their initial value; thereafter, NADH production occurs with continuous adjustment of the equilibrium between aldehyde, alcohol, NADH, and NAD^+. The steady-state NADH production exhibits normal Michaelis–Menten kinetics and is in accord with earlier studies using much higher enzyme concentrations where no lag phase was reported. These results establish that the ability of HL-ADH to oxidize aldehydes is much greater than previously thought. The relationship between aldehyde dismutase and aldehyde dehydrogenase activities of HL-ADH is discussed.

Horse liver alcohol dehydrogenase (HL-ADH) catalyzes the reversible interconversion of a wide variety of aldehyde/alcohol substrate pairs (eq 1) and catalyzes the dismutation and oxidation of aldehydes (Abeles & Lee, 1960; Dalziel & Dickinson, 1965; Hinson & Neal, 1975).

$$H^+ + NADH + \text{aldehyde} \rightleftharpoons NAD^+ + \text{alcohol} \quad (1)$$

The oxidation of aldehydes is not unique to HL-ADH; many other alcohol dehydrogenases can also oxidize aldehyde substrates (Moxon et al., 1985; Eisses, 1989; Hara et al., 1991) and can catalyze sequential dehydrogenation reactions (Handlon & Oppenheimer, 1992). When NAD^+ concentrations are approximately stoichiometric with enzyme, HL-ADH catalyzes the dismutation of aldehydes to equimolar quantities of the corresponding acid and alcohol without net synthesis of NADH, which makes this reaction spectrophotometrically silent (Dalziel & Dickinson, 1965). The V_{max} for dismutation is reportedly higher than that for oxidation of the corresponding alcohol; however, the K_m values for aldehydes are high; e.g., the K_m for acetaldehyde = 100 mM.

At high concentrations of NAD^+, HL-ADH catalyzes oxidation of aldehydes with net NADH production but with a V_{max} much less than that for alcohol oxidation (Hinson & Neal, 1975). K_m values for aldehydes are in the micromolar range and are comparable to those for the corresponding alcohol substrates. Clearly, there are discrepancies regarding the kinetic values for the oxidation of aldehydes by HL-ADH, and these discrepancies are significant. It was the reported unfavorable kinetic values for aldehyde oxidation compared

† This research was supported in part by National Institutes of Health Grants AR-17323 (G.L.K.) and GM-22982 (N.J.O.). The UCSF Magnetic Resonance Laboratory is in part funded by grants from the National Science Foundation (DMB 8406826) and the National Institutes of Health (RR-01668).

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0006-2960/93/0432-735$04.00/0

to alcohol oxidation that lead to the dismissal of alcohol dehydrogenases as having any physiological function in aldehyde oxidation (Dalziel & Dickinson, 1965). As part of an investigation of the mechanistic implications of the ability of dehydrogenases to conduct alternative chemistries, we have reexamined the oxidation of aldehydes to the corresponding carboxylic acids by HL-ADH and find it to be much better than previously considered. Moreover, the kinetic values derived in earlier studies of the HL-ADH-catalyzed aldehyde oxidation are fundamentally in error because the impact of aldehyde dismutation has been overlooked.

EXPERIMENTAL PROCEDURES

Materials. HL-ADH and NAD^+ were obtained from the Sigma Chemical Co. and were used without further purification. Other chemicals, buffers, and substrates were purchased from Aldrich. Butanal was distilled under a stream of oxygen-free nitrogen and stored at 4 °C until used. NAD^+ concentrations were determined spectrophotometrically.

Enzyme Assays. Spectrophotometric assays were performed at 37 °C in 1-mL cuvettes on a Hewlett-Packard 8542A diode array spectrophotometer. Reactions were conducted in 0.1 M sodium phosphate buffer, pH 7.5, and NADH formation was monitored by measuring the time-dependent increase in A_{340}. Enzyme stock solutions were also prepared in 0.1 M sodium phosphate buffer, pH 7.5. The specific activity of HL-ADH was determined by assay in Tris buffer, pH 10, in the presence of 2.0 mM ethanol and 0.5 mM NAD^+. The duration of lag phases in progress curves was determined by extrapolation of the linear portion of the curve to its intersection with the horizontal (time) axis (Dalziel et al., 1978).

NMR Spectroscopy. Proton nuclear magnetic resonance spectra were acquired at 500 MHz on a General Electric GN-500 instrument. The probe temperature was maintained at 37 °C, and 5-mm NMR tubes were used. The total NMR sample volume was 0.5 mL. For kinetic runs, spectra were acquired every 5.1 min and consisted of 64 scans with a spectral width of ±2700 Hz, using 16K data points. The pulse width was set to correspond to a 45° tip angle, and a 2.0-s postacquisition delay was used to allow for full relaxation of the resonances. Concentrations of assay components were measured relative to the peak areas of NAD^+ (determined spectrophotometrically). The concentration of butanal was determined from the sum of the areas of the C1 proton resonances of free and hydrated aldehyde and independently calculated from the area of the α-methylene of the free aldehyde and the observed ratio of 1.6:1 for the two components. The α-methylene protons for the hydrate (δ = 1.57 ppm) overlap extensively with the β-methylene resonances of the free aldehyde (δ = 1.61 ppm) and product resonances and, hence, cannot be conveniently used for these measurements.

Buffer and coenzyme were lyophilized twice from 99.8% D_2O and then dissolved in 100% D_2O. Freshly distilled aldehyde was added to the buffer solution in 100% D_2O. Enzyme was dissolved in the buffer solution (100% D_2O) and exchanged twice with more buffer (100% D_2O) by diafiltration using a Centricon centrifugal microconcentrator, 30 000 molecular weight cutoff (Oppenheimer, 1989). To compare directly the results for the ^1H NMR and spectrophotometric experiments, and to avoid interference from solvent deuterium isotope effects, the same deuterated solutions were used for both sets of experiments and were proportioned to a stoppered 1-cm quartz cuvette and an NMR tube. The final concentrations of substrates were NAD^+, 10.8 mM, and butanal, 5.5 mM. Assays were initiated by the addition of 0.135 unit of HL-ADH for the ^1H NMR experiment (0.5-mL total volume) and 0.27 unit of enzyme for the spectrophotometric assay (1-mL total volume). These conditions produced a lag of 35 min.

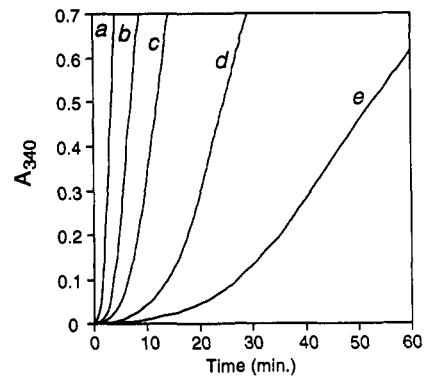

FIGURE 1: A series of assay progress curves for the oxidation of butanal by different amounts of HL-ADH: (a) 1.39 units; (b) 0.69 unit; (c) 0.35 unit; (d) 0.17 unit; (e) 0.09 unit. Final concentrations of substrates: NAD^+, 8.5 mM; butanal, 6.4 mM. Assays were conducted at 37 °C in 0.1 M sodium phosphate buffer, pH 7.5. Reactions were initiated by addition of enzyme.

RESULTS AND DISCUSSION

The underlying assumption for previous kinetic studies of alcohol dehydrogenase-catalyzed oxidation of aldehydes is that the increase in A_{340} accompanying NADH formation is a direct measure of aldehyde oxidation (eq 2). The apparent

$$R\text{–}CHO + NAD^+ \rightarrow R\text{–}COOH + NADH + H^+ \quad (2)$$

validity of this assumption was further supported with the observation that the generation of NADH by HL-ADH-catalyzed oxidation of aldehydes followed Michaelis–Menten kinetics (Hinson & Neal, 1975). When we attempted to repeat their results, we found that the reaction was more complicated. As shown in Figure 1, we can reproduce the progress curves for the oxidation of butanal using assay conditions similar to those described by Hinson and Neal (1975). As the concentration of enzyme is decreased, however, a previously unreported lag in the progress curve is observed. The lag phase is followed by a linear steady-state production of NADH. Importantly, the linear steady-state rate achieved following the lag is proportional to the enzyme concentration, exhibits Michaelis–Menten kinetics, and yields kinetic values consistent with those previously reported for higher enzyme concentrations where no lag is observed (Hinson & Neal, 1975). The lag phase is independent of NAD^+ concentration, directly proportional to the aldehyde concentration (Figure 2a), and inversely proportional to the enzyme concentration (Figure 2b). These properties can be manipulated through variation of enzyme and aldehyde concentrations to generate lag phases of any desired duration. Acetaldehyde, propanal, and octanal also exhibit lag phases similar to those observed for butanal (data not shown). The duration of these lags also appears to be a function of their Michaelis constants (work in progress). The possibility that contamination with an inhibitor might explain the lag phenomenon has been excluded. Repeated distillations of the aldehyde substrates, the use of highly purified cofactor preparations, exhaustive dialysis of the enzyme solution, and changes of buffer salts all fail to affect the duration of the lag phase. Note that lags of this nature have not been previously reported for HL-ADH.

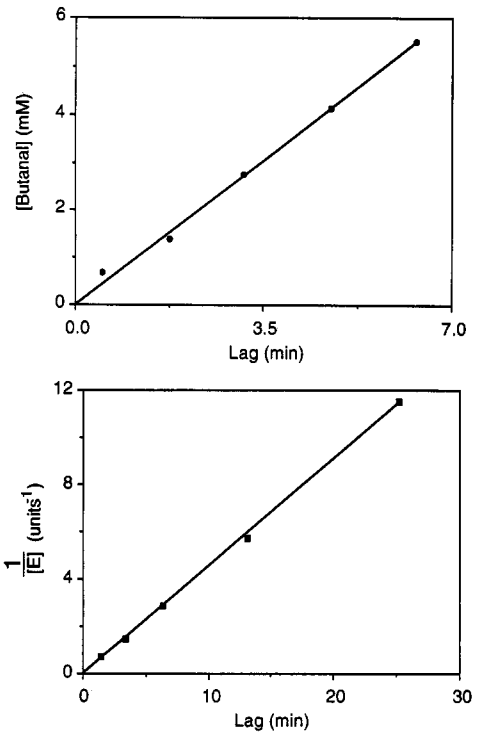

FIGURE 2: Variation of the lag phase for assay progress curves as a function of (a, top) aldehyde concentration and (b, bottom) enzyme concentration. Final concentrations of assay components: (a) NAD^+, 8.5 mM; HL-ADH, 0.35 unit; (b) NAD^+, 8.5 mM; butanal, 6.4 mM. Reactions were initiated by addition of enzyme.

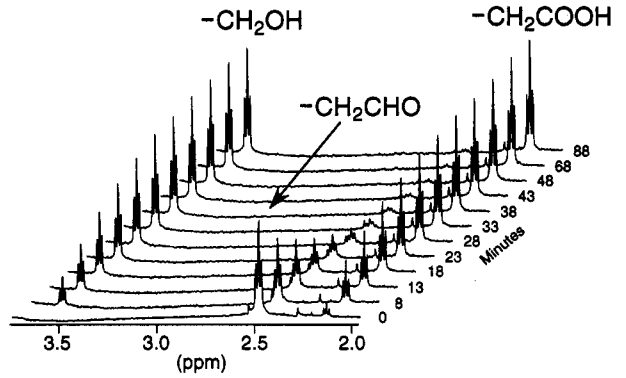

FIGURE 3: Stacked plot of a portion of the ^{1}H NMR spectra showing the time-dependent changes in an assay of butanal oxidation by HL-ADH. The assay was carried out in 0.1 M sodium phosphate buffer, pD 7.5, at 37 °C in a final volume of 0.5 mL. Initial concentrations of assay components: NAD^+, 10.8 mM; butanal, 5.5 mM. The reaction was initiated by the addition of 0.135 unit of HL-ADH.

To establish unequivocally the time course of changes occurring in the assay components during the lag phase, we investigated the oxidation of butanal using ^{1}H NMR. The methylene protons of butanol, butanal (free aldehyde form), and butyric acid give rise to readily identifiable and clearly separated resonances at δ 3.60, 2.49, and 2.13 ppm, respectively (Figure 3), and at the aldehyde concentration used the resonances are readily observable by ^{1}H NMR. As shown in Figure 3, the concentration of aldehyde rapidly decreases with a concomitant appearance of acid and alcohol in a 1:1 ratio. By 40 min butanal is unmeasurable, acid and alcohol concentrations plateau, and their combined concentrations equal that of the initial concentration of aldehyde (see Figure 4).

Plots of the time-dependent changes in concentration of butanal, butanol, and butyric acid, derived by ^{1}H NMR, and of NADH, determined spectrophotometrically, are shown in

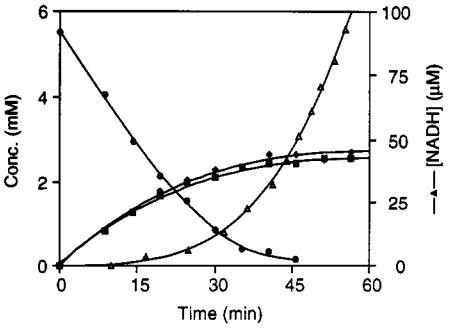

FIGURE 4: Plot of the change in concentration of reaction components with time for the oxidation of butanal by HL-ADH. The concentration of total butanal (●), butanol (■), and butyric acid (◆) was measured by ^{1}H NMR as described under Experimental Procedures. NADH (▲) was determined spectrophotometrically by measurement of A_{340}. The assays were as described for Figure 3.

Scheme I

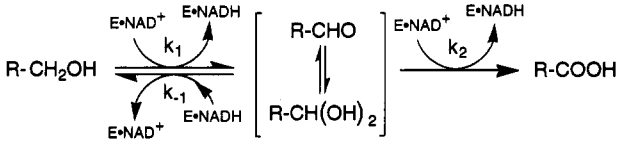

Figure 4. These data establish that during the lag HL-ADH catalyzes the dismutation of the aldehyde to a mixture of the corresponding alcohol and acid. Most importantly, the steady-state production of NADH commences only after the aldehyde concentration has fallen to <5% of its initial concentration. On the basis of the plots shown in Figure 4, aldehyde decreases linearly at an initial rate of 160 nmol/min. This is 43 times faster than the subsequent steady-state rate of NADH production at 3.7 nmol/min. The rapid rate of substrate turnover during the dismutation phase is consistent with previous observations (Dalziel & Dickinson, 1965).

The above properties of HL-ADH-catalyzed oxidation of aldehydes can be explained by the mechanism shown in Scheme I. In the early stages of the reaction the enzyme catalyzes primarily dismutation. E·NAD^+ binds aldehyde to generate E·NADH and acid, governed by the rate constant k_2. E·NADH can either dissociate, a slow process, or bind another molecule of aldehyde to regenerate E·NAD^+ and alcohol, governed by the rate constant k_{-1}. Note that aliphatic aldehydes are present in solution as interconverting aldehyde and hydrated forms. Under the conditions used in these experiments, the rate of equilibration was fast relative to depletion of total aldehyde. The free aldehyde form is the presumed substrate for reduction (Naylor & Fridovich, 1968; Eisses, 1989) whereas the hydrated *gem*-diol is the substrate for oxidation (Abeles & Lee, 1960). As the alcohol concentration increases, it begins to compete with the diminishing concentration of aldehyde for E·NAD^+ and is reoxidized back to aldehyde; thus the effect of k_1 becomes increasingly significant. Eventually the alcohol/aldehyde concentrations establish a dynamic equilibrium between alcohol oxidation and aldehyde reduction defined by their equilibrium redox potential (eq 3). Aldehyde continues to be oxidized to acid,

$$k_1[NAD^+][\text{alcohol}] = k_{-1}[NADH][\text{aldehyde}] \quad (3)$$

but the net production of NADH occurs with the continual readjustment of the concentrations of all the species involved; that is, alcohol is consumed to maintain the low, steady-state concentration of aldehyde to replace that which is being oxidized to acid. With an apparent K_{eq} at pH 7.0 of 1.94×10^{-11} M (Cornell et al., 1979), the reaction lies in favor of

alcohol/NAD^+; thus the anticipated concentrations of aldehyde and NADH will be small, as we observe.

The model explains the variation of the lag phase with substrate and enzyme concentration (Figure 2). Increasing the aldehyde concentration lengthens the lag by increasing the time taken to reach equilibrium. Increasing the enzyme concentration in the assay mixture shortens the lag by decreasing the time needed to reach equilibrium. Indeed, it is likely that the lag went unnoticed by earlier workers (Hinson & Neal, 1975) because of the high enzyme concentrations in their assays. Under their conditions the aldehyde would have fully dismutated during the mixing time of their experiment.

The proposed mechanism is fully consistent with existing knowledge of HL-ADH kinetics (Pettersson, 1987). Obviously, failure to account for substrate depletion in the lag phase and buildup of a competitive inhibitor in >40 fold molar excess will lead to a calculation of inaccurate kinetic constants. Clearly, aldehyde oxidation occurs much faster and more efficiently than indicated by the results obtained from the steady-state production of NADH. These results put HL-ADH into the class of enzymes that catalyze sequential oxidation of alcohols to carboxylic acids. This property raises the question as to the mechanism by which hydride equivalents are transferred with near equal efficiency from substrates whose external equilibrium constants differ by over 10 orders of magnitude (Handlon & Oppenheimer, 1992). The data also demonstrate that aldehyde dismutation and aldehyde oxidation are the same catalytic process viewed at different times along the assay progress curve. Aldehyde dehydrogenase activities of alcohol dehydrogenases may well have gone unnoticed with other enzymes because the standard procedure of using high "saturating" concentrations of aldehyde and coenzyme to look for such activity would generate a long lag phase while the enzyme silently catalyzed dismutation prior to commencement of net NADH production. The competence of alcohol dehydrogenases to catalyze oxidation of aldehydes raises questions regarding what, if any, evolutionary relationships might exist between the alcohol and aldehyde dehydrogenases (Handlon & Oppenheimer, 1992). Finally, these results have far-ranging implications for the physiological function of alcohol dehydrogenases. Clearly, the previous dismissal of the liver ADH in oxidative aldehyde metabolism is unfounded and must be reinvestigated. A full exploration of the ability of alcohol dehydrogenases to conduct oxidation of aldehydes is underway and will be reported in subsequent publications.

ACKNOWLEDGMENT

We thank Dr. G. L. Kenyon for helpful discussions.

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