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Proton Release during the Pre-Steady-State Oxidation of Aldehydes by Aldehyde Dehydrogenase. Evidence for a Rate-Limiting Conformational Change†

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ABSTRACT: A transient release of protons with an amplitude corresponding to one proton per active site has been observed for the oxidation of propionaldehyde, acetaldehyde, and benzaldehyde by sheep liver cytoplasmic aldehyde dehydrogenase at pH 7.6 with phenol red as indicator. At saturating substrate levels, the rate constants for the proton burst are in each case the same, and for acetaldehyde and propionaldehyde show the same dependence on the concentrations of the substrates, as the rate constants for the transient production of NADH reported previously [MacGibbon, A. K. H., Blackwell, L. F., & Buckley, P. D. (1977) *Biochem. J. 167*, 469–477]. Although, with propionaldehyde as a substrate, a full proton burst is also observed at pH 6.0, no proton burst is observed at pH 9.0. For 4-nitrobenzaldehyde, there is no burst in NADH production, but a burst in proton release is observed, showing that proton release precedes hydride transfer. No protons were released during the binding of the substrate analogues acetone and chloral hydrate nor on reaction of the enzyme with the inhibitor tetraethylthiuram disulfide (disulfiram). A model is proposed in which the rate-limiting step in the pre-steady-state phase of the reaction is a conformational change which occurs after the binding of aldehydes to the enzyme. As a result of the conformational change, the environment of a functional group on the enzyme, which initially has a pK_a of about 8.5, is perturbed to give a final pK_a value for the group of less than 5. Computer simulations were used to show that the model accurately reproduces all of the experimental data. The lack of observation of a second transient proton release, as required by the overall stoichiometry, argues that its release occurs in a slow step prior to NADH dissociation.

Extensive transient kinetic studies have been carried out on the oxidation of aldehydes by the cytoplasmic aldehyde dehydrogenase from sheep liver (MacGibbon et al., 1977a,b) and have led to the following kinetic scheme (Scheme I). In Scheme I, release of NADH contributes significantly to the rate-limiting step in the steady state, and the observation of a burst in the production of NADH (in both nucleotide fluorescence and absorbance) is consistent with the first appearance of enzyme intermediates containing NADH occurring before the rate-determining step in the enzyme-catalyzed reaction. It is assumed that the ternary complexes E·NAD$^+$·Ald and E·NADH·acid are rapidly interconverted and that the concentration of E·NADH·acid is low so that no transient results from this intermediate. Computer simulations

Scheme I

$$E \underset{k_{-1}}{\overset{k_1,\ NAD^+}{\rightleftharpoons}} E{\cdot}NAD^+ \underset{k_{-2}}{\overset{k_2,\ Ald}{\rightleftharpoons}} E{\cdot}NAD^+{\cdot}Ald \xrightarrow{k_3} E{\cdot}NADH \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + NADH$$

based on Scheme I, using rate constants determined from pre-steady-state and steady-state studies (MacGibbon et al., 1977a,c), give a good approximation to the experimental data although it has been noted (MacGibbon et al., 1977a) that additional steps are required to explain some of the experimental data.

When saturating concentrations of NAD$^+$ are premixed with enzyme before reaction with propionaldehyde, Scheme I may be treated as a system of two consecutive first-order reactions (MacGibbon et al., 1977a), and an equation relating the observed burst rate constant to the kinetic constants and the concentration of aldehyde has been derived (eq 1). The

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$$1/k_{obsd} = 1/k_3 + (k_3 + k_{-2})/(k_2k_3[Ald]) \quad (1)$$

rate constant for the process controlling the burst (k_3 in Scheme I) may be obtained from the intercept of the resulting double-reciprocal plot at infinite aldehyde concentration. In this way, values of 11 and 23 s^{-1} have been obtained for k_3 (for propionaldehyde and acetaldehyde, respectively) from nucleotide fluorescence measurements (MacGibbon et al., 1977a), and kinetic isotope experiments with saturating concentrations of the corresponding deuterated aldehyde showed no kinetic isotope effect on k_3 in either case. The process which is limiting in the pre-steady-state phase of the reaction therefore clearly precedes the hydride transfer step (which is included in k_3) but has not so far been identified.

The overall stoichiometry of the reaction requires that two protons are produced per molecule of NAD^+ reduced, and thus potentially another mechanistic probe is available from a study of the transient release of protons during the oxidation of aldehydes. We have investigated the pre-steady-state release of protons by using phenol red as an indicator at pH 7.6 in the hope of obtaining further insight into the detailed sequence of events taking place during the pre-steady-state phase of the enzyme-catalyzed reaction. It will be shown that proton release precedes hydride transfer at pH 7.6 and that both steps are controlled by the same process, which is most likely a conformation change initiated by binding of the aldehyde.

Experimental Procedures

Materials

Cytoplasmic aldehyde dehydrogenase was isolated from sheep livers essentially as described by MacGibbon et al. (1979), and [1-^{2}H]propionaldehyde was prepared from 1-nitro[1,1-$^2H_2$]propane as described by MacGibbon et al. (1977a). NAD^+ was obtained from Sigma as the highest purity grade. All reagents used in these studies were obtained commercially.

Methods

Concentration of Active Sites. Aldehyde dehydrogenase activity was determined by following the increase in absorbance at 340 nm due to the production of NADH in 25 mM pH 7.6 phosphate buffer at 25 °C. With saturating conditions of propionaldehyde (20 mM) and NAD^+ (1 mM), the active site concentration could be calculated from the assay by using a value of 0.25 s^{-1} for k_{cat} (MacGibbon et al., 1977c) and an extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} (Horecker & Kornberg, 1948) for NADH. The active site concentration was also determined from an NADH titration as described previously (MacGibbon et al., 1979). The protein concentration was determined by A_{280} measurements using a value for $E^{1\%}_{1cm}$ of 11.3 (Dickinson et al., 1981).

Preparation of Solutions. Solutions were prepared for use in the stopped-flow apparatus by using 0.5 mM pH 7.6 phosphate buffer which had been degassed on a water pump for 30 min prior to use in order to remove air bubbles which if present in the observation chamber can result in erratic data. The enzyme solution was dialyzed for 3 h in 2 × 3 L of 0.5 mM pH 7.6 phosphate buffer containing 0.1% (v/v) β-mercaptoethanol. All solutions contained 10–20 μM phenol red, 0.1 M Na_2SO_4, and 0.1 M KNO_3 and were adjusted to pH 7.6 with 0.1 M HCl or 0.1 M NaOH immediately prior to use.

The enzyme was usually mixed with NAD^+ and propionaldehyde in the stopped-flow apparatus, although in some experiments it was convenient to have one of the substrates premixed with the enzyme. Unless otherwise stated, all experiments were carried out at pH 7.6.

Calibration of Activity at 340 and 560 nm. The stoichiometry of the enzyme-catalyzed oxidation of aldehydes to acids requires that at the pHs used in the study, two protons are released per NADH molecule produced:

$$H_2O + R{-}C(=O)H + NAD^+ \xrightarrow{\text{enzyme}} R{-}C(=O)O^- + 2H^+ + NADH$$

Consequently, the change in concentration per unit time determined by monitoring the reaction at 340 nm with a spectrophotometer should be half that resulting from following the reaction at 560 nm using a molar extinction coefficient of 54×10^3 M^{-1} cm^{-1} (Gutfreund, 1972). However, due to the presence of the 0.5 mM phosphate buffer in the solutions, the change in concentration per unit time at 560 nm was considerably less than that at 340 nm; thus, the same solution was assayed at both 340 and 560 nm, and the change in absorbance at 560 nm was related to that at 340 nm by a calibration factor. Usually, this was carried out prior to each stopped-flow experiment by using solutions which were to be mixed in the stopped-flow apparatus.

Stopped-Flow Measurements. Stopped-flow experiments were carried out with a Durrum-Gibson D110 stopped-flow spectrophotometer (Durrum Instruments Corp., Palo Alto, CA) in either the absorbance or fluorescence modes. Signals from the photomultiplier were passed through a log buffer amplifier to a Data Laboratories DL905 or DL901 transient recorder. The data were displayed on a Hewlett Packard 141B oscilloscope and, if a satisfactory trace was obtained, then transferred from the transient recorder as 1024 8 bit data points by means of a machine language program similar to that described by Boland & Hardman (1973). Statistical and kinetic calculations could then be carried out on the data by using a number of machine language subroutines. In each case, a permanent record of the reaction trace was printed by using a Bryans 29000 A4 XY recorder. pH burst experiments which resulted in a single first-order exponential were treated by a method described by Laidler & Bunting (1973). A least-squares analysis was used by the computer program to fit the straight line through the log difference vs. time plot, and after calculating the rate constant and amplitude of the exponential process, the computer reconstructed the burst curve by using the calculated rate constant and amplitude. The closeness of the computer-drawn curve to the original reaction trace was taken as a measure of the accuracy of the calculated values. When traces with a low signal to noise ratio were obtained, each point was computer averaged over eight runs, and kinetic calculations were performed on the averaged data.

Computer Simulations. Computer simulations were carried out by using a Burroughs continuous simulation modeling package on a Burroughs B6700 computer. The program accepted as input data the rate equations for the mechanism to be simulated, the rate constants for the individual steps in the mechanism, and the initial concentrations of all reactants, intermediates, and products involved in the proposed mechanism. The computer integrated the rate equations at time intervals specified by the program and printed the concentration of products, reactants, and intermediates at each integration interval. These data were then treated to obtain the rate constant for the simulated burst.

Results

NADH Titrations. NADH titrations with enzyme and NADH only, in 25 mM pH 7.6 phosphate buffer, yielded enzyme binding site concentrations in good agreement with those calculated by assay at pH 7.6, and a dissociation constant

of 1.0 μM was obtained, compared with the value of 1.2 μM reported by MacGibbon et al. (1979). The addition of phenol red (10 μM) to the fluorometer cell resulted in a slight decrease in the fluorescence of both the NADH and the E·NADH solutions, probably as a result of the absorption of the fluoresced radiation by the acidic form of phenol red which has a moderate absorption at 435 nm. However, both the NADH binding site concentration and the K_D value were unchanged in the presence of phenol red. Titrations carried out with chlorophenol red and thymol blue added also resulted in K_D values and NADH binding site concentrations identical within experimental error with those in the absence of any indicator. The addition of phenol red (10 μM) to assays in 25 mM phosphate buffer had no effect on V_{max}; thus, these results show that the pH indicators obviously did not bind to either the coenzyme or the substrate binding sites and hence are suitable probes for measuring the changes in proton concentration.

In order to observe changes in the proton concentration during the enzyme-catalyzed reaction, it was necessary to dialyze the enzyme against 0.5 mM pH 7.6 phosphate buffer. (In some cases, a stock enzyme solution was diluted with distilled water to give a final buffer concentration of ca. 1 mM.) Titrations carried out at 100 mM Na_2SO_4 gave the same concentration of NADH binding sites as that obtained by titration in the presence of 25 mM pH 7.6 phosphate buffer, and a value of 2.0 μM for K_D. The k_{cat} value was also unchanged at 100 mM Na_2SO_4 in 0.5 mM pH 7.6 phosphate buffer, and thus the kintic behavior of the enzyme is unchanged in this solution. Hence, the proton release data may be directly compared with the data already obtained in 25 mM pH 7.6 phosphate buffer (MacGibbon et al., 1977a–c).

Calibration of Activity between 560 and 340 nm. The recorder traces obtained by monitoring the reaction at 560 nm were generally linear for 30–60 s while at 340 nm they were linear for 2–3 min. The calibration factor was obtained by dividing the change in concentration of NADH per unit time (calculated from the absorbance data at 340 nm) by the apparent change in concentration of protons (calculated from the absorbance data at 560 nm) and multiplying by 2.

Proton Burst at pH 7.6. When a solution containing enzyme (20 μM), NAD^+ (2 mM), phenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M) was mixed with a solution containing propionaldehyde (120 μM), phenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M) in the stopped-flow apparatus, a burst in the rate of production of protons was observed followed by a slow release of protons corresponding to the steady-state rate (Figure 1). The transient phase was first order with a rate constant of 8.1 s^{-1}, and the amplitude calculated by using a calibration factor of 50 was equal to the enzyme active site concentration within experimental error, indicating that a single proton is released. A lag phrase was observable at the start of the proton burst transient, presumably caused by the buildup of the E·NAD^+·aldehyde intermediate (MacGibbon et al., 1977a).

When the propionaldehyde concentration was varied, keeping all other concentrations the same as for the burst shown in Figure 1, the observed burst rate constant (k_{obsd}) exhibited the expected hyperbolic relationship, and a plot of $1/k_{obsd}$ against 1/[propionaldehyde] was linear (Figure 1, inset) with a slope of 3×10^{-6} mol L^{-1} s and a half-saturating concentration of 36 μM. The rate constant at infinite propionaldehyde concentration (>20 mM) was 12 s^{-1}, and when the same solutions were monitored at 340-nm absorbance, a burst in the rate of production of NADH was observed with a rate constant of 13 s^{-1}. When [1-^{2}H]propionaldehyde was used as a substrate, a moderate kinetic isotope effect (2.0) was observed on the slope of the double-reciprocal plot, but there was no kinetic isotope effect on the intercept. Thus, within experimental error, the same data were obtained for propionaldehyde from measurements of the transient release of protons as were previously obtained (MacGibbon et al., 1977a) by studying the transient production of NADH.

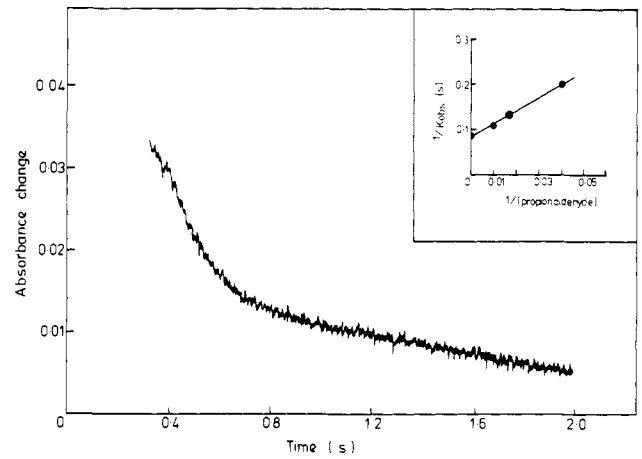


FIGURE 1: Proton burst at pH 7.6 when enzyme (20 μM) premixed with NAD^+ (2 mM) in one syringe is mixed with propionaldehyde (120 μM) from the other. Both syringes contained 0.5 mM phosphate, pH 7.6, buffer, KNO_3 (0.1 M), Na_2SO_4 (0.1 M), and phenol red (15 μM), and the reaction was followed at 560 nm in absorbance at 25 °C. Note the slight lag phase at the beginning of the trace. The burst rate constant (k_{obsd}) calculated from the data was 8.1 s^{-1}, and the burst amplitude was 9.8 μM. Insert: Plot of $1/k_{obsd}$ against 1/[propionaldehyde]. One syringe contained enzyme (20 μM), NAD^+ (2 mM) in a 0.5 mM phosphate, pH 7.6, buffer, KNO_3 (0.1 M), Na_2SO_4 (0.1 M), and phenol red (15 μM) solution while the other contained a range of propionaldehyde concentrations in the same buffer–salt solution. The slope of the plot was 3×10^{-6} mol L^{-1} s, and k_{obsd} at infinite propionaldehyde concentrations (>20 mM) was 12 s^{-1}.

If NAD^+ was not premixed with the enzyme, a proton burst was also observed with an amplitude equal to 90–100% of the enzyme active site concentration at saturating concentrations of propionaldehyde (20 mM); however, there was a slight decrease in k_{obsd} to 7 s^{-1}.

When acetaldehyde was used as a substrate, a burst in the production of protons was also observed, and the plot of $1/k_{obsd}$ against 1/[acetaldehyde] was again linear, with a slope of 8.8 $\times 10^{-3}$ mol L^{-1} s and a half-saturating concentration of acetaldehyde of 2 mM. The rate constant at infinite aldehyde concentration was 30 s^{-1}, and when [1,2,2,2-$^2H_4$]acetaldehyde was used as the substrate, a slope kinetic isotope effect of 2.1 was found at acetaldehyde concentrations less than 1 mM, though as for propionaldehyde there was no isotope effect on the intercept at higher concentrations. These results are again similar to those previously obtained by MacGibbon et al. (1977a) from measurements of nucleotide fluorescence.

No transient proton release was observed when enzyme (20 μM) was mixed with NAD^+ (2 mM), phenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M); however, a very slow proton release was observed with a rate constant of about 0.05 s^{-1}. This proton release was also observed in the absence of NAD^+ and probably resulted from the presence of dissolved CO_2 in unequal concentrations in the two syringes which can result in a proton release as shown below.

$$H_2CO_3 \rightleftarrows HCO_3^- + H^+$$

This process has a rate constant of 0.038 s^{-1} (Gutfreund, 1972).

Proton Release with Other Aldehydes. With benzaldehyde (1.0 mM) as a substrate, a proton burst was observed with

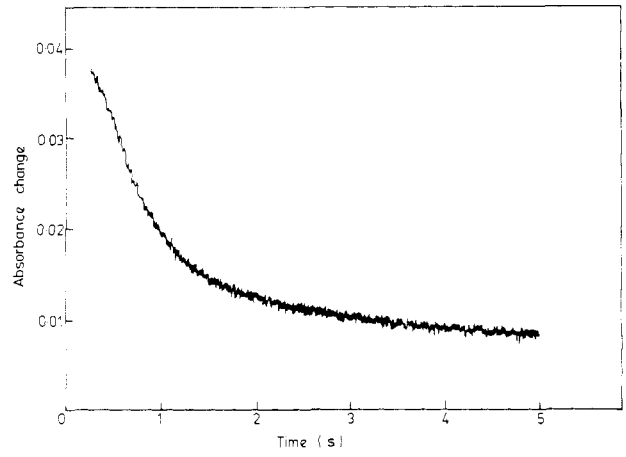


FIGURE 2: Burst in production of protons for 4-nitrobenzaldehyde. Conditions (before mixing): enzyme (27 μM), NAD^+ (2 mM), phenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M) in 0.5 mM pH 7.6 phosphate buffer (syringe 1); 4-nitrobenzaldehyde (500 μM), phenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M) in 0.5 mM pH 7.6 phosphate buffer (syringe 2) at 25 ± 0.2 °C. The burst rate constant was 3 s^{-1}, and the burst amplitude was 12.8 μM by using the calibration factor as described under Methods.

a rate constant of 2.7 s^{-1}, and the amplitude of the burst process corresponded to 90% of the enzyme active site concentration. These results were similar to those previously obtained by MacGibbon et al. (1977a) when the reaction was monitored in nucleotide fluorescence.

When 4-nitrobenzaldehyde (500 μM) was used as a substrate, a proton burst was observed at pH 7.6 with a rate constant of 3 s^{-1} and an amplitude equal to 90% of the enzyme active site concentration (Figure 2). No burst in the production of NADH was observed in either absorbance or nucleotide fluorescence in agreement with MacGibbon et al. (1977a).

Burst Experiments with Steady-State Inhibitors Present. When enzyme (38 μM) containing phenol red (14.8 μM) was mixed with disulfiram (17 μM) containing phenol red (14.8 μM), a slow release of protons (0.6 s^{-1}) was observed corresponding to 12–13% of the enzyme concentration (2.3 μM). A similar experiment was carried out with enzyme (38 μM) premixed with NAD^+ (3.2 mM) and phenol red (14.8 μM) being mixed with disulfiram (17 μM) and phenol red (14.8 μM). Again, a slow release of protons was observed (0.3 s^{-1}) which corresponded to 15% of the enzyme active site concentration. For comparison, a standard proton burst carried out by using the same enzyme and NAD^+ concentrations but mixed in the stopped-flow apparatus against propionaldehyde (240 μM) and phenol red gave a burst (7 s^{-1}) with an amplitude equal to the active site concentration.

Acetone, which is similar to acetaldehyde in structure and size, has a relatively small inhibitory effect on the steady-state reaction (MacGibbon et al., 1978). However, when it was mixed with enzyme and NAD^+ in the stopped-flow apparatus, no transient or steady-state proton release was observed. No transient release of protons was observed when chloral hydrate, a competitive inhibitor with respect to aldehyde (MacGibbon et al., 1977c), was mixed with enzyme and NAD^+.

Proton Release during NADH Displacement. When enzyme (12 μM) premixed with NADH (100 μM) in 0.5 mM phosphate, pH 7.6, buffer containing KNO_3 (0.1 M), Na_2SO_4 (0.1 M) and phenol red (15 μM) was mixed with NAD^+ (3 mM) in the same buffer–salt–indicator solution, there was no observable transient release of protons. Only a very slow release of protons (~0.05 s^{-1}) was observed which is probably due to the presence of dissolved carbon dioxide (Gutfreund, 1972).

Effect of NAD^+ Concentration on the Burst at pH 7.6. All solutions were prepared in 0.5 mM phosphate, pH 7.6, buffer containing KNO_3 (0.1 M), Na_2SO_4 (0.1 M), and phenol red (15 μM). Conditions before mixing were enzyme (30 μM) in the first syringe and propionaldehyde (40 mM) with varying concentrations of NAD^+ in the second syringe. The burst rate constant for the proton burst was found to vary with NAD^+ concentration below saturating levels. A plot of the observed rate constant vs. NAD^+ concentration resulted in a straight line with a slope of 1.14×10^5 L mol^{-1} s^{-1}.

Effect of pH on the Burst. When a solution of enzyme (20 μM), chlorophenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M) was mixed with NAD^+ (2 mM), propionaldehyde (40 mM), chlorophenol red (15 μM), KNO_3 (0.1 M), and Na_2SO_4 (0.1 M) in the stopped-flow apparatus at pH 6.0, a burst in the production of protons was observed at 580 nm. The transient was a single first-order process with a rate constant of 5 ± 1 s^{-1}, and the amplitude of the burst was equal to 90% of the enzyme active site concentration.

When the experiment was repeated at pH 9.0 with thymol blue as an indicator, there was no observable transient release of protons.

Discussion

Evidence That Proton Release Precedes Hydride Transfer at pH 7.6. The observation of a transient release (or burst) of protons during the pre-steady-state phase of the oxidation of aldehydes by cytoplasmic sheep liver aldehyde dehydrogenase indicates that at pH 7.6 a proton is released along with hydride transfer. The close similarity between the proton burst data presented in this paper and the NADH burst data of MacGibbon et al. (1977a) at saturating levels of acetaldehyde, benzaldehyde, and propionaldehyde and the similar dependence on NAD^+, acetaldehyde, and propionaldehyde concentrations suggest that the rate of proton release in the pre-steady-state phase of the reaction is limited by the same process which controls hydride transfer and NADH production. On the basis of these data alone, the proton could be released during the actual hydride transfer step. However, for 4-nitrobenzaldehyde, only a steady-state rate of production of NADH was observed[1] with a k_{cat} value of 0.013 s^{-1}, but when the same solutions were monitored for the production of protons, a full proton burst (3 s^{-1}) was still seen.[2] If the proton had been released during or after the hydride transfer step, then the rate constant for the proton release could not be greater than 0.013 s^{-1}; thus, clearly for this substrate, at pH 7.6, proton release precedes the hydride transfer step. Assuming, as seems reasonable, that the same mechanism applies to all the aldehydes (the only differences being in the relative magnitudes of the rate constants for the individual steps), it can then be concluded that proton release also precedes hydride transfer in the pre-steady-state phase of the reaction for acetaldehyde, benzaldehyde, and propionaldehyde as well. This implies that at pH 7.6 the proton release and

[1] The lack of observation of a burst in the production of NADH is in agreement with the data of MacGibbon et al. (1977a), who monitored the reaction in nucleotide fluorescence and absorbance at 340 nm. This result was interpreted as a shift to hydride transfer as the rate-limiting step in the steady-state resulting from the powerful rate-retarding effect of the 4-nitro substituent.

[2] The value of 3 s^{-1} is less than the saturating burst rate constants for acetaldehyde and propionaldehyde but is similar to the burst rate constant of 2–3 s^{-1} obtained for other aromatic aldehydes from measurements of either proton release or NADH production.

the pre-steady-state rate-limiting processes are closely connected.

Nature of the Rate-Limiting Step in the Pre Steady State. The nature of the rate-limiting step in the pre-steady-state phase of the reaction has not previously been identified, but it obviously must occur after the first step in Scheme I since no proton is released on the binding of NAD^+. Also, the second-order rate constant for the binding of propionaldehyde is of the order of 10^6 L mol^{-1} s^{-1}; thus, at the saturating levels of propionaldehyde used in the burst studies (20 mM after mixing), the rate of aldehyde binding is about 20×10^3 s^{-1}, which is 3 orders of magnitude greater than the burst rate constant of 12 s^{-1}. Thus, the rate-limiting process and accompanying proton release must occur after aldehyde binding, but before hydride transfer. The most likely possibilities for the rate-limiting step are (1) the covalent attachment of aldehyde to the enzyme after binding to form a tetrahedral intermediate with concomitant proton release and (2) a conformational change resulting from aldehyde binding.

The first explanation appears unlikely, as a rate-limiting step involving covalent attachment of the aldehyde would be expected to show faster rates for aldehydes with electron-withdrawing substituents. The Hammett value (ρ) for nucleophilic attack on a carbonyl center is usually about +2, and from the published σ^* and σ_I values for the CH_3, CH_3CH_2, C_6H_5, and $NO_2C_6H_4$ substituents (Kosower, 1968), the order of the rate constants for the proton burst should be acetaldehyde $\simeq$ propionaldehyde < benzaldehyde < 4-nitrobenzaldehyde. Experimentally however, this order was not observed since the rate constant for 4-nitrobenzaldehyde was in fact about 10 times slower than that for acetaldehyde, and the rate constant for propionaldehyde was less than half that for acetaldehyde. Also inconsistent with the first explanation is the lack of any kinetic isotope effect on the maximum burst rate constant (k_3) when the reaction is monitored by the release of protons at pH 7.6, or by the production of nucleotide fluorescence,[3] since a secondary isotope effect would be expected if the covalent attachment of the aldehyde to the enzyme were rate limiting. Since it is also clear that breaking of the C–H (or C–D) bond does not contribute significantly to the rate-limiting step of the burst, it is apparent that the chemistry of the aldehyde oxidation does not limit the rate of the transient phase.

The second explanation, that a conformational change is rate limiting in the pre steady state, is more probable. In this explanation, the pre-steady-state proton release obtained with 4-nitrobenzaldehyde and other aldehydes would result from a conformational change which follows aldehyde binding and thus would be expected to show some dependence on aldehyde structure. Such a structure dependence would explain the variation of the maximum burst rate constant obtained for different aldehydes (MacGibbon et al., 1977a), with, for example, the bulkier aromatic aldehydes causing a slower conformational change. Consistent with this view is the absence of any transient proton release when an E·NAD^+ mixture was pushed against a number of steady-state inhibitors, such as acetone and chloral hydrate, which indicates that the inhibitors, which presumably bind at the substrate binding site (chloral hydrate is a competitive inhibitor with respect to propionaldehyde at concentrations <100 μM), nevertheless do not initiate the sequence of events which leads to proton release.

Origin of the Proton Which Is Released during the Transient Phase at pH 7.6. Since proton release occurs before NADH formation, the proton cannot originate from the hydrolysis of any supposed E·NADH·acyl intermediate (resulting from the nucleophilic attack of water on this intermediate) or from the dissociation of the carboxylic acid formed as a reaction product, since both of these events take place after hydride transfer. The proton, therefore, is probably released through the ionization of some functional group at, or close to, the enzyme active site.

In order to account for the fact that the amplitude of the proton burst at both pH 7.6 and 6.0 is equal to 90–100% of the enzyme active site concentration, it is necessary to postulate an initial pK_a for the group involved of 8.5 or greater. The pK_a, however, cannot be much greater than 8.5 since within the limits of detection no burst in the production of protons was observed at pH 9.0, presumably because at this pH the functional group was already completely ionized before aldehyde binding took place. (It should, however, be noted that a small proton burst of 10–20% of the active site concentration would have been difficult to detect on fast time settings, especially since the molar extinction coefficient of thymol blue is only half that for phenol red.)

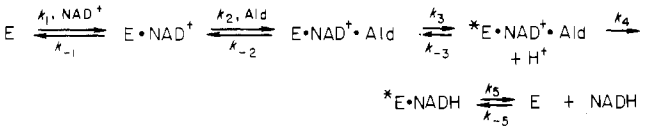
The loss of the proton from this functional group during the transient phase at pH 7.6 could be caused by a shift in the pK_a value from 8.5 to 5.0 or less after the environment of the group had been perturbed by the rate-limiting aldehyde-induced conformational change, and hence the ionizable group need not be of functional importance. Nevertheless, its presence on the enzyme has an importance in that it has provided a useful probe with which to study the transient phase of the enzymic reaction.

An alternative explanation for the loss of the proton from the functional group is possible if it is an essential group for catalytic activity. Although the chemistry of the oxidation is not rate limiting in the transient phase, if the ionizable group must be deprotonated to allow the formation of a hemiacetal intermediate, either before or after the conformational change, then the irreversible nature of the overall reaction would result in a stoichiometric release of protons at pH 7.6 at a rate which is determined by the rate of the conformational change. Currently the chemistry of the oxidation process is unknown, but several reactions between the aldehyde, functional groups on the enzyme, and the coenzyme must presumably occur in order to convert the aldehyde into a carboxylic acid. This chemistry has commonly been assumed (Eckfeldt & Yonetani, 1976; Duncan & Tipton, 1971) to resemble that proposed for glyceraldehyde-3-phosphate dehydrogenase, in which an active site thiol group attacks the carbonyl atom of the aldehyde to give a thiohemiacetal derivative which is then oxidized to give an E·NADH·acyl intermediate. If the postulated thiol group had a pK_a of 8.5, a full proton burst would be observed at pH 7.6 as discussed above.

The idea that a thiol group is involved in the reaction mechanism receives some support from the inactivation data obtained with disulfiram (Kitson, 1975, 1978) and with 5,5′-dithiobis(2-nitrobenzoate) and iodoacetamide (Hart & Dickinson, 1977). These thiol-modifying reagents cause appreciable loss of steady-state activity, suggesting the existence of a thiol group at, or near, the active site. Also, Dickinson et al. (1981) and Kitson (1982) have recently shown that disulfiram reduces the amplitude (but not the rate) of the burst in NADH production at pH 7.4 by the same extent that it

[3] There is a significant isotope effect (ca. 2.0) on the slope of the double-reciprocal plot (eq 1) for both acetaldehyde and propionaldehyde, and similar results are obtained from both the proton burst and NADH burst data. As pointed out by a referee, this result suggests that only the free aldehyde is a substrate and that the isotope effects on the slope are caused (at least in part) by the decreased level of free aldehyde present for the deuterated aldehydes.

Scheme II

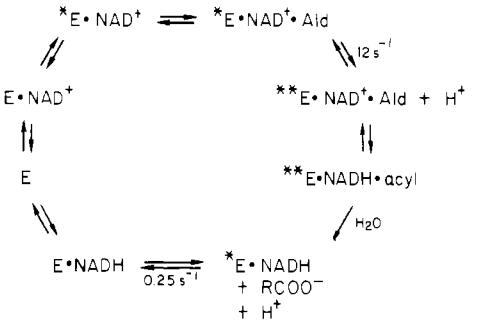


reduces the steady-state rate and that the effect occurs within the mixing time of the stopped-flow instrument. The reduction in the amplitude of the burst process can be explained if disulfiram is modifying an active site thiol group as suggested by Kitson (1978). However, this disulfiram-modifiable thiol group cannot be the source of the proton which was released during the pre steady state, irrespective of whether it is in the active site or not, since when disulfiram was mixed with either enzyme alone or E·NAD$^+$, in the stopped-flow apparatus at pH 7.6, there was no stoichiometric release of protons either on the time scale of the burst processes or over a 50-s time span. Thus, it seems apparent that the disulfiram-sensitive thiol group must be already ionized at pH 7.6, possibly as part of an ion pair similar to the E.S.$^-$–ImH$^+$ system[4] proposed for papain (Lewis et al., 1976) or as a result of some other as yet unknown unusual environmental feature. Hence, a conformational perturbation of the environment of some nonessential ionizable group [possibly still a thiol since there are 36 per tetramer (MacGibbon et al., 1976)] seems to be the most likely explanation for the origin of the proton during the transient phase.

Kinetic Scheme for Aldehyde Dehydrogenase. Modification of Scheme I by including the rate-limiting conformational change and associated proton release yields a kinetic scheme which is consistent with the experimental data (Scheme II). The model was simulated by using a continuous system modeling package as a check on self-consistency. The values used in the simulation for k_1, k_{-1}, k_2, k_{-2}, k_5, and k_{-5} were 2×10^5 L mol^{-1} s^{-1}, 1.6 s^{-1}, 10^6 L mol^{-1} s^{-1}, 50 s^{-1}, 0.25 s^{-1}, and 5×10^5 L mol^{-1} s^{-1}, respectively, as reported by MacGibbon et al. (1977a). Both the deprotonation and protonation steps were considered to be fast, and for simplicity, the proton release step was included in k_3 which was assigned a value of 12 s^{-1}. A value of 50 s^{-1} for k_4 was arrived at by comparing the rate of hydride transfer for 4-nitrobenzaldehyde (ca. 0.02 s^{-1}) with that expected for propionaldehyde by using Taft σ^* values. With k_4 equal to 50 s^{-1}, ρ is about +4, but any increase in k_4 above this value requires a ρ value greater than +4 which is highly improbable. However, a value for k_4 of much less than 50 s^{-1} would not reproduce the kinetic behavior observed when propionaldehyde is replaced by [1-^{2}H]propionaldehyde as substrate. With the assumption of an isotope effect of about 5 for the hydride transfer step, then with the deuterated substrate k_4 would be reduced to 10 s^{-1} or less. For a value of 10 s^{-1} for k_4, simulations show a pronounced lag phase at saturating [1-^{2}H]propionaldehyde concentrations (which was not observed experimentally) while at values of less than 10 s^{-1} for k_4 a kinetic isotope effect on the NADH burst should be observed (again contrary to experiment).

The simulated data obtained for the change in absorbance as a function of time for different mixing conditions and with

Scheme III



different initial concentrations of substrates closely reproduced the experimental data for the corresponding experiments. Both the proton and NADH bursts simulated by using Scheme II were found to vary with aldehyde concentration, and a double-reciprocal plot of k_{obsd} vs. propionaldehyde concentration resulted in a straight line with a slope of 5×10^{-6} mol L^{-1} s, close to the values of 3×10^{-6} and 4×10^{-6} mol L^{-1} s for the proton and NADH bursts, respectively, as found experimentally.

Mechanism for Oxidation of Aldehydes by Aldehyde Dehydrogenase. Although Scheme II gives a good fit to the experimental data, it is still a simplification. For example, the single step, k_5, for dissociation of NADH from the enzyme should be written as a two-step process, with a conformational change of the E·NADH complex preceding the dissociation of NADH (MacGibbon et al., 1977b). The conformational change of the E·NADH complex cannot however just be the microscopic reverse of the aldehyde-induced conformational change, since if this were so an uptake of protons would be expected to occur during NADH displacement experiments. In fact, no uptake or release of protons was observed during displacement of NADH from the binary E·NADH complex by NAD$^+$, and no changes in the hydrogen ion concentration were observed during the binding of NAD$^+$ or NADH. Thus, two different conformational changes must occur during the reaction.

As suggested by MacGibbon et al. (1977a), it seems probable that the first is a fast conformational change following NAD$^+$ binding, a conformational change which must on the basis of our results take place without accompanying proton loss or gain. The reverse of this conformational change would take place prior to dissociation of NADH from the E·NADH complex and constitutes the rate-limiting step in the steady-state phase of the reaction at very high concentrations of for example acetaldehyde and propionaldehyde.[5] The second conformational change (which is rate limiting in the pre-steady-state phase of the reaction) occurs on binding of the substrate with concomitant proton release. Since only a single transient with an amplitude equal to the formal enzyme concentration is observed when proton release is monitored at pH 7.6 in the stopped-flow spectrometer, this conformation change must be reversed during the steady-state phase of the reaction. It must therefore occur during a slow hydrolysis of the proposed acyl-enzyme intermediate and dissociation of the acid product with the uptake of a proton by the previously perturbed functional group when it returns to its normal pK_a value. Overall, however, as a result of these combined pro-

[4] For papain, acylation or protonation of the thiol group of the E·S$^-$ ImH$^+$ ion pair leads to a drop in the pK_a of the imidazole group of histidine from 8.7 to around 4.0. Thus, if such an ion pair were involved in aldehyde dehydrogenase catalysis, a proton release would be anticipated on reaction of the ion-pair thiolate ion with either aldehydes or disulfiram. The postulation of more basic residues such as lysine for the other partner of the ion pair does not appear to be helpful since it is difficult to imagine a pK_a shift from 11 or 12 to below 5.0.

[5] The omission of either the proposed conformational change which occurs on NAD$^+$ binding or the dissociation of NADH from the E·NADH binary complex does not affect the validity of the excellent fit obtained for the pre-steady-state data to Scheme II.

cesses, one proton is released in the steady-state phase of the reaction to give a net release of two protons for the whole reaction pathway. The rate constant for the second slow step in the steady state must be of the same order of magnitude as the slow conformational change which occurs during NADH dissociation (0.25 s^{-1}) at saturating levels of propionaldehyde (20 mM). If this step were faster, a second transient release of protons would be observed (contrary to experiment), and if it were slower, k_{cat} would be less than the observed value of 0.25 s^{-1}.

The overall mechanistic scheme for the reaction is shown in Scheme III.

Conformational changes have been observed for a number of dehydrogenases. Wonacott & Biesecker (1977) have shown by crystallographic studies that a conformational change is induced in glyceraldehyde-3-phosphate dehydrogenase from *Bacillus stearothermophilus* by NAD^+ binding, and Eklund et al. (1974) have shown that a conformational change occurs after NAD^+ binds to horse liver alcohol dehydrogenase. Parker & Holbrook (1977) have suggested that conformational changes in dehydrogenases arise because of a requirement for a nonpolar environment around the active site in order to facilitate hydride transfer from the substrate to carbon 4 of the nicotinamide ring of NAD^+, thus reducing the opportunity for water molecules to intercept the highly reactive hydride ions. Thus, for the mechanism of oxidation of aldehydes by aldehyde dehydrogenase, we believe that the conformational change which occurs on binding of the coenzyme exposes or forms the aldehyde binding site, thus accounting for the ordered mechanism (MacGibbon et al., 1977c), and the second conformational change which occurs on binding of the aldehyde is necessary in order to form a suitable nonpolar environment for the essential organic chemistry of the oxidation reaction.

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