Rate-limiting Steps for the Esterase and Dehydrogenase Reaction Catalyzed by Horse Liver Aldehyde Dehydrogenase*

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Horse liver aldehyde dehydrogenase, like other aldehyde dehydrogenases, is capable of hydrolyzing esters such as nitrophenyl acetate. Pre-steady state and burst kinetics were performed using substrate levels of enzyme to determine whether the rate-limiting step occurred prior to or after the formation of an acyl enzyme intermediate. A burst was found by both techniques for the dehydrogenase reaction, but no burst was found for the esterase reaction. These data show that the rate-limiting step for the two reactions catalyzed by the enzyme differs. For dehydrogenase it occurs after the formation of the acyl intermediate, but for esterase it occurs prior to its formation.

Mammalian liver aldehyde dehydrogenase, a nonspecific NAD-dependent oxidoreductase enzyme, has been shown to possess esterolytic activity toward p-nitrophenyl acetate in addition to its dehydrogenase activity. This dual activity was first demonstrated with a horse liver enzyme (1) and later shown to exist for the human liver enzyme (2), as well as for all the isozymes isolated from rat liver. Although the mechanisms of action for these enzymes have not been elucidated, it has been suggested (1) that the enzyme reaction is analogous to that catalyzed by glyceraldehyde-3-phosphate dehydrogenase (3). According to this proposal in the first step a nucleophile (ZV), presumably a sulfhydryl group, adds to the carbonyl carbon of the substrate to form a tetrahedral intermediate:

\[
E \quad N\quad C\quad CH_3
\]

or

\[
E\quad N\quad C\quad O\quad NO_2
\]

In the dehydrogenase reaction the hydride is then removed to form an ester \(E\quad N\quad C\quad CH_3\). In the esterolytic reaction nitrophenol is eliminated to form the same acyl enzyme intermediate.

In the initial characterization of the horse liver enzyme it was concluded that the rate-limiting step in the dehydrogenase reaction involved a nucleophilic attack (1). This could either be step \(k_2\) or \(k_4\). When studying the esterase reaction we then concluded the rate-limiting step occurred prior to formation of the acyl enzyme intermediate. Reasoning by analogy it was suggested that the rate-limiting step for the dehydrogenase reaction might then also occur prior to the formation of the proposed acyl intermediate.

A reinvestigation of the rate-limiting step for the dehydrogenase and esterase reaction of horse liver aldehyde was undertaken using pre-steady state analysis with substrate levels of enzyme. If the rate-limiting step occurred after the formation of the acyl enzyme intermediate a burst of product would be observed. If, however, the rate-limiting step occurred prior to the formation of the acyl enzyme no burst would be observed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Propionaldehyde was obtained from Eastman Organic Chemicals and p-nitrophenyl acetate from Aldrich. NAD\(^+\) and NADH were purchased from P-L Biochemicals. Deionized, distilled water was used in the buffer made from sodium phosphate salts obtained from Mallinckrodt Chemical Works. Dithiothreitol was obtained from Sigma.

**Preparation of Aldehyde Dehydrogenase**—The isolation of the pl = 5 isozyme of horse liver aldehyde dehydrogenase as well as the determination of specific activity and concentration was performed as previously reported (4). The purified enzyme was dialyzed 4 hours against 0.1 M phosphate, pH 7.5, buffer used in the stopped flow studies. The buffer used in the burst studies contained, in addition, 0.5 mM dithiothreitol.

**Stopped Flow Burst and Burst Studies**—Stopped flow burst experiments were performed in 0.1 M phosphate buffer, pH 7.5, 25°C. A Durrum-Gibson stopped flow spectrophotometer with a 2-cm light path
equipped with a pneumatic drive attachment was used. One of the drive syringes contained NAD and propionaldehyde at concentrations of approximately 650 and 300 \mu M, respectively. The other drive syringe contained 3 \mu M enzyme in the same buffer. The rate of formation of NADH was followed at 340 nm. The changes of transmittance were monitored by a Tektronix type 564 storage oscilloscope. The oscilloscope traces were photographed with a Polaroid camera. The transmittance at selected times from the photographs were converted to absorbances and plotted as a function of time.

Horse liver aldehyde dehydrogenase was assayed for an esterase and a dehydrogenase burst in a Gilford 240 spectrophotometer connected to a Houston Instrument, series 2000 Omnigraphic recorder. The reactions were initiated by the addition of either propionaldehyde or p-nitrophenyl acetate, to the enzyme solutions in 1-cm microcuvettes and the changes in absorbance were recorded as a function of time. The formation of NADH was followed at 340 nm to monitor dehydrogenase activity and the formation of p-nitrophenol was followed at 400 nm to monitor esterase activity. Reactions were performed in 0.1 M phosphate, pH 7.5, containing 0.5 mM dithiothreitol at 25°C. The esterase activity was corrected for the small spontaneous hydrolysis of p-nitrophenyl acetate in the absence of enzyme.

The concentration of NAD\(^+\) and NADH were determined spectroscopically using extinction coefficients of 18 \times 10^3 M\(^{-1}\) cm\(^{-1}\) (260 nm) and 6.22 \times 10^3 M\(^{-1}\) cm\(^{-1}\) (340 nm), respectively. The extinction coefficient used to determine the concentration of p-nitrophenol was 12.8 \times 10^3 M\(^{-1}\) cm\(^{-1}\) (400 nm) at pH 7.5.

RESULTS AND DISCUSSION

In Fig. 1 is presented a representative photograph of the output of a stopped flow experiment. This experiment was performed using substrate level concentrations of enzyme. The results definitely show that a burst does indeed occur. The magnitude of burst extrapolates to 2 mol of NADH produced/mol of enzyme. This is consonant with the stoichiometry of two NADH per mol of enzyme found by binding studies (5). The enzyme contains four apparently identical subunits and the explanation or significance of the half of the sites reactivity is under investigation. The burst in the pre-steady state kinetics indicates that the rate-limiting step for dehydrogenase occurs after the formation of the acyl intermediate.

A similar technique was used to determine the rate-limiting step for the esterase reaction. Since we were not interested in the rate of formation of intermediates, burst experiments were performed using a spectrophotometer. A representative recorder tracing presented in Fig. 2A shows that no burst was obtained for the esterase reaction. Control experiments verified that a burst could have been detected using a spectrophotometer. The dehydrogenase reaction, when extrapolated to zero time, revealed, as presented in Fig. 2B, the formation of 2 coenzyme molecules per molecule of enzyme showing that this technique gives the same results as those obtained using the stopped flow instrument. In order to verify our ability to measure a burst with a nitrophenyl acetate reaction the experiment was repeated with chymotrypsin. The expected burst (6) was obtained. Thus we conclude that for the esterase reaction a step leading to the acyl enzyme intermediate is rate-limiting and therefore the esterase and dehydrogenase reaction have different rate-limiting steps.

![Figure 1](https://via.placeholder.com/150)

**FIG. 1.** Stopped flow burst kinetics for the dehydrogenase activity. After mixing, the reaction mixture contained 1.5 \mu M enzyme; 0.43 mM NAD\(^+\); 100 \mu M propionaldehyde. A, oscilloscope tracing. Ordinate, 10 mV/division; abscissa, 20 ms/division; B, absorbance at 340 nm as a function of time is plotted. The extrapolation from the steady state portion corresponds to a burst of 3.05 \mu mol of NADH/1.5 \mu mol of enzyme.

![Figure 2](https://via.placeholder.com/150)

**FIG. 2.** Burst kinetics for the esterase and dehydrogenase reaction. A, esterase reaction: enzyme 2.68 \mu M; substrate, p-nitrophenyl acetate, 1 mM. The theoretical intercept for a burst under these experimental conditions is 0.034 A per active site; thus the expected intercept if a burst had occurred would have been either 0.069 or 0.138. B, dehydrogenase reaction: enzyme, 5 \mu M; 0.5 mM NAD; substrate propionaldehyde, 70 \mu M. The extrapolated intercept corresponds to a burst of 9.7 \mu mol of NADH/5 \mu mol of enzyme.
Additional experiments performed in the presence of coenzyme also show that no burst occurs for the esterase reaction, although the reaction velocity is increased. This finding supports our earlier report (1) that the velocity of the esterase reaction is increased by coenzyme. The increase is not due to a change in rate-limiting step, but more likely is due to the increased reactivity of a nucleophilic amino acid residue in the presence of the coenzyme.

Although no direct proof for the existence of the tetrahedral intermediates shown in Equations 1 or 2 has been presented, it is well documented that an esterase forms a tetrahedral intermediate with its substrate (7). The fact that esterase reaction exhibits saturation kinetics shows that some intermediate complex forms prior to the formation of acyl enzyme.

For the dehydrogenase reaction the rate-limiting step would be $k_r$. The rate-limiting step for esterase cannot be $k_r'$, since the reaction exhibits classical Michaelis-Menten kinetics. To determine whether $k_r'$ or $k_r''$ was the rate-limiting step a second substrate, p-nitrophenyl benzoate was employed. It is known that for the base catalyzed hydrolysis of esters, which is analogous to the scheme presented in Equation 2 that the breakdown of the tetrahedral intermediate ($k_r'$) is much less sensitive to the nature of the acyl group than is the formation of the intermediate ($k_r''$) (8). Thus with the enzyme, it can be assumed that $k_r''$ would change less than would $k_r'$ when acetates are compared to benzoates. It was found that p-nitrophenyl acetate and p-nitrophenyl benzoate substrates having different $K_m$ values, 30 and 1200 $\mu$M, respectively, are hydrolyzed by the enzyme at the same rate (9). The observation of the same maximum velocity with these two esters is more consistent with $k_r''$, being rate-limiting for ester hydrolysis.

The data presented here clearly substantiate that the rate-limiting step for the esterase reaction is prior to the formation of the acyl enzyme intermediate (1). However, we now prove that the rate-limiting step for the dehydrogenase occurs after acyl enzyme formation.

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