Pre-Steady-State Kinetic Studies on Cytoplasmic Sheep Liver Aldehyde Dehydrogenase

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Stopped-flow experiments in which sheep liver cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3) was rapidly mixed with NAD⁺ and aldehyde showed a burst of NADH formation, followed by a slower steady-state turnover. The kinetic data obtained when the relative concentrations and orders of mixing of NAD⁺ and propionaldehyde with the enzyme were varied were fitted to the following mechanism:

where the release of NADH is slow. By monitoring the quenching of protein fluorescence on the binding of NAD⁺, estimates of $2 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and 2 s^{-1} were obtained for k_{+1} and k_{-1} respectively. Although k_{+3} could be determined from the dependence of the burst rate constant on the concentration of propionaldehyde to be 11 s^{-1} , k_{+2} and k_{-2} could not be determined uniquely, but could be related by the equation: $(k_{-2} + k_{+3})/k_{+2} = 50 \times 10^{-6} \text{ mol} \cdot \text{litre}^{-1}$. No significant isotope effect was observed when $[1\text{-}^2\text{H}]$ propionaldehyde was used as substrate. The burst rate constant was pH-dependent, with the greatest rate constants occurring at high pH. Similar data were obtained by using acetaldehyde, where for this substrate $(k_{-2} + k_{+3})/k_{+2} = 2.3 \times 10^{-3} \text{ mol} \cdot \text{litre}^{-1}$ and k_{+3} is 23 s^{-1} . When $[1,2,2\text{-}^2\text{H}]$ acetaldehyde was used, no isotope effect was observed on k_{+3} , but there was a significant effect on k_{+2} and k_{-2} . A burst of NADH production has also been observed with furfuraldehyde, *trans*-4-(*NN*-dimethylamino)cinnamaldehyde, formaldehyde, benzaldehyde, 4-(imidazol-2-ylazo)benzaldehyde, *p*-methoxybenzaldehyde and *p*-methylbenzaldehyde as substrates, but not with *p*-nitrobenzaldehyde.

There have been several reports of steady-state-kinetic studies on aldehyde dehydrogenases (EC 1.2.1.3) from a variety of sources (Freda & Stoppani, 1970; Duncan & Tipton, 1971; Feldman & Weiner, 1972; Sidhu & Blair, 1975; Eckfeldt & Yonetani, 1976; MacGibbon *et al.*, 1977a), which have shown that, in general, compulsory-order kinetics are obeyed, with NAD⁺ binding before the aldehyde, although some evidence has been obtained for random order of binding (Sidhu & Blair, 1975; MacGibbon *et al.*, 1977a). Only recently have the first reports of pre-steady-state studies on this enzyme appeared. Eckfeldt & Yonetani (1976) observed a burst phase when a premixed solution of NAD⁺ and the F1 isoenzyme of aldehyde dehydrogenase from horse liver was mixed with acetaldehyde in the stopped-flow spectrophotometer, and Weiner *et al.* (1976) also observed a burst when a similar experiment was carried out on the F2 isoenzyme from horse liver with propionaldehyde as substrate. Eckfeldt & Yonetani (1976) observed only a small kinetic isotope effect on the burst when $[1\text{-}^2\text{H}]$ acetaldehyde was used as substrate, a result that indicates that the

rate-limiting step for the burst lies after NAD⁺ reduction.

We report here the results of a pre-steady-state study on the cytoplasmic aldehyde dehydrogenase from sheep liver, which has been carried out to obtain more detailed information about the mechanism of the enzyme-catalysed reaction.

Methods

Stopped-flow spectrophotometric studies were carried out with a Durrum-Gibson D110 stopped-flow spectrophotometer. The nucleotide fluorescence was excited at 340 nm, with a 9 nm band width, and the fluorescence at 90° to the exciting light was observed through Wratten 47B and 2B filters, which together have a maximum transmission at 435 nm. Protein fluorescence was excited at 290 nm and the fluorescence at 90° was monitored through a Wratten 18A filter, which has a maximum transmission at 335 nm. The solutions were thermostatically maintained at 25°C and 0.025 M-sodium phosphate buffer, pH 7.6, was used unless otherwise stated. The

stopped-flow traces were collected on a Hewlett-Packard model 141B storage oscilloscope and photographed on 35mm film. The traces were projected on to graph paper, from which the data were obtained.

Computer simulations were carried out on the Burroughs B6700 computer, by using the Continuous System Modelling Program (CSMP73) designed to facilitate the digital simulation of continuous processes.

The voltage changes observed when the instrument was used in the fluorescence mode were calibrated before commencing a series of kinetic runs by measuring the difference in voltage between the buffer alone and a standard NADH solution ($5\mu\text{M}$) in the same buffer.

Materials

Cytoplasmic aldehyde dehydrogenase from sheep liver was purified essentially as described previously (Crow *et al.*, 1974). Deamino-NAD⁺, pyridine-3-carbaldehyde-adenine dinucleotide, 3-acetylpyridine-adenine dinucleotide, *trans*-4-(*NN*-dimethylamino)-cinnamaldehyde and NAD⁺ (grade III) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The NADH was obtained as preweighed vials and the concentration was checked by A_{340} , assuming a molar absorption coefficient of 6.22×10^3 litre·mol⁻¹·cm⁻¹ (Horecker & Kornberg, 1948). Deionized distilled water was used in the preparation of all reagent solutions. Acetaldehyde was obtained from BDH (Poole, Dorset, U.K.) and propionaldehyde from Koch-Light Laboratories (Colnbrook, Bucks., U.K.). Propionaldehyde and acetaldehyde were distilled before use. The concentrations of the stock solutions of these aldehydes were determined by forming the semicarbazones and measuring the A_{220} . The concentrations quoted are always total concentrations of aldehyde and do not take account of the presence of hydrated aldehyde in the *gem*-diol form. [1,2,2,2-²H]Acetaldehyde was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and [1-²H]propionaldehyde, b.p. 48–52°C, $n_D^{20} = 1.3637$, was prepared from 1-nitro[2,2-²H]propane by the Nef reaction (Noland, 1955) (nuclear-magnetic-resonance and mass spectra were consistent with the assumed structure and showed virtually complete deuteration of the aldehydic hydrogen).

4-(Imidazol-2-ylazo)benzaldehyde was prepared by the method described by Bernhard *et al.* (1970). All other aldehydes and substrates or inhibitors were commercial products of the highest purity available and were used without further purification.

Results

When aldehyde dehydrogenase was mixed with NAD⁺ and propionaldehyde in a Durrum-Gibson

Fig. 1. Reaction of aldehyde dehydrogenase with NAD⁺ and propionaldehyde monitored by changes in nucleotide fluorescence

(a) The reaction contained $3.6\mu\text{M}$ -aldehyde dehydrogenase and $825\mu\text{M}$ -NAD⁺ from one syringe and 20mM -propionaldehyde from another in 0.025M -phosphate buffer, pH 7.6 at 25°C. The vertical scale is 0.1V/division and the horizontal scale is 50ms/division. (b) Plot of $\log(F_{\infty} - F)$ (the difference between the steady-state rate of fluorescence change extrapolated back to zero time at particular times minus the corresponding experimental fluorescence value) against time. The derived first-order rate constant was 11.7s^{-1} . 0.14V = fluorescence of $5.33\mu\text{M}$ -NADH solution. Amplitude of the change corresponds to an active-site concentration of $2.4\mu\text{M}$ in the cuvette.

stopped-flow spectrophotometer operating in the fluorescence mode, there was a rapid initial increase in nucleotide fluorescence followed by a slower linear

increase that represented the steady-state production of NADH (Fig. 1). The rate constant (k_b) for the pre-steady-state phase (burst) was determined from the slope of the plot obtained by extrapolating the steady-state rate back to zero time and plotting the logarithm of the difference between this extrapolated fluorescence at specified time intervals and the corresponding experimental fluorescence value against time (Laidler & Bunting, 1973). The rate of production of the transient was first order (Fig. 1b), with a rate constant of $11 \pm 2 \text{ s}^{-1}$ and a steady-state parameter $V/[E]$ equal to 0.28 s^{-1} (compared with that obtained by steady-state measurements of 0.25 s^{-1} ; MacGibbon *et al.*, 1977a).

A similar burst experiment carried out by following the A_{340} on the stopped-flow spectrometer gave burst rate constants and steady-state rates that were identical within experimental error with those obtained by monitoring nucleotide fluorescence. All subsequent measurements were in the fluorescence mode.

Fig. 2. Dependence of the observed burst rate constant on the NAD^+ concentration

To obtain the results shown in the Figure, $5 \mu\text{M}$ -aldehyde dehydrogenase and $800 \mu\text{M}$ -propionaldehyde from one syringe were mixed with a new variable concentration of NAD^+ from the other syringe, in 0.025 M -phosphate buffer at 25°C . ● represents a plot of the observed burst rate constant against the concentration of NAD^+ , and ○ is a re-plot of the data at low NAD^+ concentrations, where the NAD^+ concentration is one-tenth of that shown on the scale. The slope of this line is $2.3 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$.

Effect of NAD^+ on the burst

When aldehyde dehydrogenase was mixed with a solution containing saturating concentrations of propionaldehyde ($415 \mu\text{M}$) and various concentrations of NAD^+ that had been purified as described by Whitaker *et al.* (1974), it was found that at low concentrations the rate constant of the pre-steady-state phase was proportional to the NAD^+ concentration, the graph having a slope of $2.3 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, and at high concentrations reached a maximum value (Fig. 2). This maximum value was identical within experimental error with that obtained when enzyme premixed with similar high concentrations of NAD^+ was mixed with saturating concentrations of propionaldehyde.

NAD^+ quenching of protein fluorescence

The protein fluorescence was quenched on the binding of NAD^+ to the enzyme. Although such quenching may exhibit a non-linear relationship to the increase in the fractional saturation of coenzyme-binding sites (Holbrook, 1972), when a range of NAD^+ concentrations was mixed with enzyme in the stopped-flow spectrophotometer the decrease in protein fluorescence obeyed first-order kinetics and a plot of the calculated rate constant against NAD^+ concentration was linear, with a slope of $1.8 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and an intercept of approx. 2 s^{-1} . The slope of such a plot represents an apparent rate constant for NAD^+ binding to the enzyme and the intercept an apparent dissociation rate constant. The rate constant determined from the slope is in good agreement with the value found from the NAD^+ -dependence of the burst rate constant at low NAD^+ concentrations (Fig. 2), confirming that the same process is being followed in each case.

When the enzyme in one syringe was mixed with NAD^+ and propionaldehyde from the other syringe in the stopped-flow spectrophotometer, a decrease in protein fluorescence was observed, with amplitude and rate constant virtually the same as that observed when enzyme and NAD^+ were mixed. However, a small increase in protein fluorescence was observed when premixed NAD^+ (1 mM) and the enzyme ($1.9 \mu\text{M}$) were mixed with propionaldehyde ($772 \mu\text{M}$), with a rate constant similar to that measured for the burst when nucleotide fluorescence was being monitored. The increase in protein fluorescence arises because NAD^+ quenches protein fluorescence more than does NADH (MacGibbon, 1976).

Effect of propionaldehyde on the burst

The effect of mixing various concentrations of propionaldehyde with a solution of aldehyde dehydrogenase which had been premixed with a

Fig. 3. Double-reciprocal dependence of the observed burst rate constant on propionaldehyde concentration

To obtain the results shown in Fig. 3, $4.8\text{ }\mu\text{M}$ -aldehyde dehydrogenase and $580\text{ }\mu\text{M}$ - NAD^+ were mixed with a variable concentration of propionaldehyde from the other syringe, in 0.025 M -phosphate buffer, $\text{pH } 7.6$ at 25°C . The slope of the line is $4.54 \times 10^{-6}\text{ s}\cdot\text{mol}\cdot\text{litre}^{-1}$, and the concentration of propionaldehyde at one-half the maximum rate constant is $50\text{ }\mu\text{M}$.

saturating concentration of NAD^+ ($240\text{ }\mu\text{M}$) on the observed first-order rate constant for the burst is shown in Fig. 3. The best-fit straight line for the double-reciprocal plot has a slope of $4.5 \times 10^{-6}\text{ s}\cdot\text{mol}\cdot\text{litre}^{-1}$, a half-saturating concentration of $50\text{ }\mu\text{M}$ -propionaldehyde and a transient rate constant at infinite substrate concentration of 11 s^{-1} .

A similar double-reciprocal plot was obtained with $[1\text{-}^2\text{H}]$ propionaldehyde as substrate, with a half-saturating concentration of $57\text{ }\mu\text{M}$ and a rate constant at infinite propionaldehyde concentration of 8.3 s^{-1} . Although there appears to be a small isotope effect ($k_b^{\text{H}}/k_b^{\text{D}} = 1.3$) on the maximum burst rate constant obtained by extrapolation of the data obtained at lower propionaldehyde concentrations, when very high concentrations of propionaldehyde ($2\text{--}20\text{ mM}$) were used no isotope effect was discernible.

Various concentrations of the enzyme ($0.47\text{--}3.8\text{ }\mu\text{M}$) when preincubated with NAD^+ ($337\text{ }\mu\text{M}$) and mixed with propionaldehyde ($410\text{ }\mu\text{M}$) gave a burst rate constant that was independent of the enzyme concentration.

A series of compounds were tested for their inhibitory effect on the transient rate constant obtained with propionaldehyde as a substrate. However, acetone (8.6 mM), methyl ethyl ketone (6.9 mM), methyl acetate (6.7 mM) and ethyl acetate (5.7 mM) had no effect on the rate constant when premixed with propionaldehyde ($90\text{ }\mu\text{M}$) and mixed with the enzyme ($1.8\text{ }\mu\text{M}$) and NAD^+ ($413\text{ }\mu\text{M}$). α -Bromophenylacetic acid (1.6 mM) slightly inhibited the transient rate constant when added with unsaturating propionaldehyde ($51\text{ }\mu\text{M}$) and mixed with enzyme ($3\text{ }\mu\text{M}$) and NAD^+ ($180\text{ }\mu\text{M}$), by decreasing the observed rate constant from 6.5 s^{-1} to 5.4 s^{-1} .

Table 1. pH-dependence of the burst rate constant with propionaldehyde as substrate

The reaction mixtures contained enzyme ($5.2\text{ }\mu\text{M}$) and NAD^+ ($350\text{ }\mu\text{M}$) from one syringe, and propionaldehyde ($280\text{ }\mu\text{M}$) in the appropriate 0.05 M -pyrophosphate buffer from another, at 25°C . The rate constants were calculated as shown in Fig. 1.

Final pH	Burst rate constant (s^{-1})
6.3	2.6
7.3	6.2
8.3	9.9
9.3	15.8
9.8	20.5

The effect of pH on the burst rate constant was determined by using saturating concentrations of both NAD^+ ($300\text{ }\mu\text{M}$) and propionaldehyde ($300\text{ }\mu\text{M}$) in a series of 0.05 M -sodium pyrophosphate buffers (Table 1). The burst rate constant increased 8-fold in the pH range $6.3\text{--}9.8$.

Burst experiments with acetaldehyde as substrate

When various concentrations of acetaldehyde were mixed with a preincubated mixture of the enzyme and NAD^+ , a hyperbolic relationship between the burst rate constant and the acetaldehyde concentration (Fig. 4) was again obtained. However, the maximum burst rate constant (23 s^{-1}) was double that observed for propionaldehyde, and the half-saturating acetaldehyde concentration (2.3 mM) was 50 times the value for propionaldehyde.

A double-reciprocal plot was also obtained when $[1,2,2\text{-}^2\text{H}]$ acetaldehyde was used (Fig. 4) with the same maximum rate constant as for acetaldehyde but a half-saturating concentration of 6.5 mM . Thus although experimentally a kinetic isotope effect ($k_b^{\text{H}}/k_b^{\text{D}}$) of about 2.6 was observed at low acetaldehyde concentrations (less than 1 mM), this was decreased to unity at saturating concentrations.

Other substrates

Some other aldehydes produced a pre-steady-state phase when the nucleotide fluorescence was monitored (Table 2). It should be noted that no burst was observed when *p*-nitrobenzaldehyde was used as the substrate, even though the usual steady-state production of NADH was observed.

For the chromophore *trans*-4-(*NN*-dimethylamino)cinnamaldehyde, the burst could also be monitored by the decrease in A_{400} after the formation of *trans*-4-(*NN*-dimethylamino)cinnamic acid. The rate constant obtained from such absorbance measurements when *trans*-4-(*NN*-dimethylamino)cinnamaldehyde ($36\text{ }\mu\text{M}$) was mixed with the enzyme

Fig. 4. Plots of the inverse of the observed burst rate constant against the inverse of the aldehyde concentration for acetaldehyde (●) and [1,2,2,2-²H]acetaldehyde (○). To obtain the results shown in Fig. 4, 3.6 μM-aldehyde dehydrogenase and 780 μM-NAD⁺ from one syringe were mixed with a variable concentration of the aldehyde from the other syringe, in 0.025 M-phosphate buffer, pH 7.6 at 25°C.

Table 2. Burst experiments with a range of aldehydes. The reaction mixtures contained enzyme (5.2 μM) and NAD⁺ (350 μM) from one syringe and the appropriate aldehyde at the concentration stated from another syringe in pH 7.6 buffer at 25°C. The rate constants are calculated as shown in Fig. 1.

Aldehyde	Concentration	Burst rate constant (s ⁻¹)
Furfuraldehyde	11.1 mM	3.0
Formaldehyde	0.39 M	3.0
	0.039 M	3.2
p-Methoxybenzaldehyde	67 μM–1 mM	1.0
p-Methylbenzaldehyde	3.7 mM	2.5
Benzaldehyde	2–5 mM	3.0
4-(Imidazol-2-ylazo)-benzaldehyde	6.5 μM	4.0
p-Nitrobenzaldehyde	50–500 μM	No observable burst

(4.5 μM) and NAD⁺ (560 μM) was 8 s⁻¹, which is comparable with the value obtained under the identical reaction conditions by monitoring the nucleotide A₃₄₀ (13.9 s⁻¹).

The effect of pH on the burst rate constant obtained with benzaldehyde was also measured (Table 3).

Although when 3-acetylpyridine-adenine dinucleotide was used in place of NAD⁺ no burst was observed, in the analogous experiment with deamino-

Table 3. pH-dependence of the burst rate constant with benzaldehyde as substrate

The reaction mixtures contained enzyme (5.8 μM) and NAD⁺ (542 μM) from one syringe and benzaldehyde (2.3 mM) in the appropriate 0.05 M-pyrophosphate buffer from another, at 25°C.

Final pH	Burst rate constant (s ⁻¹)
6.3	1.7
7.3	2.4
8.3	2.8
9.3	8.1
9.8	17.5

NAD⁺ and propionaldehyde as substrates a burst was detected. When various concentrations of propionaldehyde were mixed with a solution containing the enzyme (1 μM) and deamino-NAD⁺ (950 μM) a double-reciprocal plot of the inverse of the observed burst rate constant against the inverse of the propionaldehyde concentration could be fitted to a straight line, which gave a maximum rate constant of 26 ± 6 s⁻¹ and a half-saturating propionaldehyde concentration of 230 μM. It was found that when premixed enzyme and propionaldehyde (0.9 mM) were made to react with even 1.35 mM-deamino-NAD⁺ the observed rate constant for the burst was only 6 s⁻¹. Since this rate constant is much less than the maximum value (26 s⁻¹) obtained when the enzyme and deamino-NAD⁺ were premixed, it is clear that at this concentration the binding of deamino-NAD⁺ is still rate-limiting.

Discussion

The observation of a burst in the kinetics of aldehyde dehydrogenase indicates that the first appearance of enzyme intermediates containing NADH occurs before the rate-determining step in the enzyme-catalysed reaction. Since it has been shown for this enzyme that the release of NADH contributes significantly to the rate-limiting step in the steady state (MacGibbon *et al.*, 1977a,b) and because there was no significant isotope effect on the burst rate constant with [1-²H]propionaldehyde, then the simplest reaction scheme that can be used to describe the enzyme-catalysed reaction is (where Ald is aldehyde):

where it is assumed that E^{NAD⁺}_{Ald} and E^{NADH}_{Acid} are rapidly interconverted and that the concentration of E^{NADH}_{Acid} is low, so that no burst corresponding to this complex is seen. Although NADH can bind to the free enzyme and is a competitive inhibitor of the steady-

state reaction (MacGibbon *et al.* 1977a), such inhibition need not be considered under the conditions used to study the burst, and for simplicity the reverse of the step with rate constant k_{+4} is not shown in eqn. (1).

However, when saturating concentrations of NAD^+ are premixed with enzyme before reaction with propionaldehyde, then eqn. (1) may be simplified to:

The rate of dissociation of NADH is very slow (MacGibbon *et al.*, 1977b) and may be ignored when considering only the burst (Shore & Gutfreund, 1970). Since in all the burst experiments the concentration of aldehyde is much greater than that of E^{NAD^+} , the addition of aldehyde becomes pseudo-first-order, and the sequence of reactions shown in eqn. (2) can be treated in the way described by Frost & Pearson (1961) for a system of first-order reactions. Using the notation of Frost & Pearson (1961), the system can be described in general by two first-order decay constants λ_2 and λ_3 , which are related to the rate constants for the steps in eqn. (2) thus:

$$\lambda_2 = \frac{1}{2}(p+q) \quad (3)$$

$$\lambda_3 = \frac{1}{2}(p-q) \quad (4)$$

where

$$p = k_{+2}[\text{Ald}] + k_{-2} + k_{+3}$$

and

$$q = (p^2 - 4k_{+3}k_{+2}[\text{Ald}])^{\frac{1}{2}}$$

It can also be shown (Frost & Pearson, 1961) that if $[\text{E}^{\text{NAD}^+}]_0$ is the concentration of the enzyme- NAD^+ complex initially, then the dependence on time of $[\text{E}^{\text{NADH}}]$ is given by the expression:

$$\begin{aligned} [\text{E}^{\text{NADH}}] = & [\text{E}^{\text{NAD}^+}]_0 \left(\frac{k_{+3}k_{+2}[\text{Ald}]}{\lambda_2\lambda_3} \right. \\ & + \frac{k_{+3}k_{+2}[\text{Ald}]}{\lambda_2(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_2 t} \\ & \left. - \frac{k_{+3}k_{+2}[\text{Ald}]}{\lambda_3(\lambda_2 - \lambda_3)} \cdot e^{-\lambda_3 t} \right) \end{aligned} \quad (5)$$

Since E^{NADH} is the species that is being monitored as a function of time in the stopped-flow spectrophotometer, then eqn. (5) can be used to relate the relative amplitudes of the two phases that could be experimentally observed. It is clear from eqn. (5) that if λ_2 and λ_3 are very different in magnitude and only a single process is observed, as for the burst kinetics, then it will be the slower process with decay constant λ_3 .

Suitable rearrangement of eqns. (3) and (4) gives:

$$\frac{1}{\lambda_3} = \frac{1}{k_{+3}} + \frac{(k_{+3} + k_{-2} - \lambda_3)}{k_{+2}k_{+3}[\text{Ald}]} \quad (6)$$

Provided that the value of $(k_{+3} + k_{-2})$ is much greater than λ_3 , then eqn. (6) can be simplified to give:

$$\frac{1}{\lambda_3} = \frac{1}{k_{+3}} + \frac{(k_{+3} + k_{-2})}{k_{+2}k_{+3}[\text{Ald}]} \quad (7)$$

a result that is identical in form with the equation obtained by Shore & Gutfreund (1970), who made the steady-state assumption with respect to an intermediate in the burst which is equivalent to $\text{E}^{\text{NAD}^+}_{\text{Ald}}$ in reaction eqn. (2).

From the studies on the quenching of protein fluorescence by NAD^+ , estimates of the rate constants k_{+1} and k_{-1} of reaction eqn. (1) of 2×10^5 litre $\cdot$ mol $^{-1}$ s $^{-1}$ and 2 s $^{-1}$ respectively were obtained. The dissociation constant for NAD^+ calculated from these rate constants (approx. 10 μM) is in good agreement with the value of 8 μM obtained from the steady-state measurements (MacGibbon *et al.*, 1977a). Although the k_{+1} value obtained from the steady-state measurements ($k_{+1} = 5 \times 10^4$ litre $\cdot$ mol $^{-1}$ s $^{-1}$; MacGibbon *et al.*, 1977a) was lower than the values obtained from the stopped-flow measurements, if the $V/[E]$ value obtained at high propionaldehyde concentrations is used in determining k_{+1} , a value of 1.2×10^5 litre $\cdot$ mol $^{-1}$ s $^{-1}$ is obtained, which is in good agreement with the stopped-flow measurements.

The value estimated for the NAD^+ -binding rate from steady-state and pre-steady-state measurements is low compared with the corresponding value for other dehydrogenases (Borgmann *et al.*, 1975), but is similar to that for pig heart cytoplasmic malate dehydrogenase (Frieden & Fernandez-Sousa, 1975), as well as to that which can be estimated from the data reported by Eckfeldt & Yonetani (1976) for the horse liver cytoplasmic (F1) enzyme. Coenzyme-binding rates are often close to 10^6 – 10^7 litre $\cdot$ mol $^{-1}$ s $^{-1}$, not far below that calculated by assuming diffusion as the rate-determining step (Eigen & Hammes, 1963). The lower values found for sheep liver cytoplasmic enzyme may indicate that binding of the coenzymes is followed by some other process such as a conformational change. Evidence has been produced for a two-step process on binding of NADH (MacGibbon *et al.*, 1977b), and a conformational change on binding of NAD^+ could result in the observed quenching of protein fluorescence (Wolfe *et al.*, 1977).

As shown in Fig. 4, for the experiments where saturating concentrations of NAD^+ are premixed with the enzyme, a plot of the inverse of the burst rate constant against the inverse of the propionaldehyde concentration can be fitted by a straight line. Such an observation is consistent with eqn. (2) provided that the value for $(k_{+3} + k_{-2})$ is much greater than λ_3 (eqn. 7).

From the intercept of the double-reciprocal plot a value of 11 s $^{-1}$ can be calculated for k_{+3} . Since k_{+3} is

of the same order of magnitude as λ_3 , in order for eqn. (7) to apply k_{-2} must be a good deal greater than either k_{+3} or λ_3 . Although k_{+2} and k_{-2} cannot be separately determined from the experimental data, eqn. (7) can be rearranged to give:

$$(k_{-2} + k_{+3})/k_{+2} = (\text{slope/intercept}) \quad (8)$$

If the slope and intercept calculated from the data in Fig. 3 are substituted in eqn. (8) then the following equation is obtained:

$$(k_{-2} + k_{+3})/k_{+2} = 50 \times 10^{-6} \text{ mol} \cdot \text{litre}^{-1} \quad (9)$$

For values of k_{-2} less than 40 s^{-1} , the plot of $1/\lambda_3$ against $1/[\text{Ald}]$ will deviate significantly from linearity [the approximation used in obtaining eqn. (7) being no longer valid], therefore from eqn. (9) k_{+2} must be greater than $10^6 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$.

By using the lowest determinations of k_{-2} and k_{+2} that are consistent with the experimental data, λ_2 can be calculated from eqn. (3) (assuming a propionaldehyde concentration of $10 \mu\text{M}$) to be 59 s^{-1} . From eqn. (5) it can be shown, by substituting this value of λ_2 and the known value of λ_3 at a propionaldehyde concentration of $10 \mu\text{M}$, that the fast phase will have an amplitude only 3% of the slow phase, which under the conditions used would be difficult to observe. All other allowed values of λ_2 are larger and will have correspondingly smaller amplitude compared with the slow phase.

The best-fit parameters for the pre-steady-state phase of the oxidation of propionaldehyde according to eqn. (1) are thus:

$$k_{+1} = 2 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$$

$$k_{-1} = 2.0 \text{ s}^{-1}$$

$$k_{+3} = 11 \text{ s}^{-1}$$

$$(k_{-2} + k_{+3})/k_{+2} = 50 \times 10^{-6} \text{ mol} \cdot \text{litre}^{-1}$$

It should be noted that as the reaction is irreversible, pre-steady-state studies in the reverse direction cannot be undertaken to elucidate further steps in the mechanism.

As a further check on the self-consistency of the mathematical treatment based on eqn. (1) the model was simulated by using the continuous system-modelling package which is available on the Burroughs B6700 computer. A value of 50 s^{-1} was assigned to k_{-2} and k_{+2} was calculated from eqn. (9) as $1.2 \times 10^6 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$. From a study of the rate of NADH binding to and dissociation from the enzyme, values of 0.2 s^{-1} and $2 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ were obtained for k_{+4} and k_{-4} (the rate constant for the reverse of step 4) respectively (MacGibbon *et al.*, 1977b). Only the species E·NADH and NADH were considered to be fluorescent, with a fluorescence ratio of 5.6:1 (MacGibbon, 1976). These parameters, together with the best-fit values of k_{+1} , k_{-1} and k_{+3} listed above, were used in the simulation.

Fig. 5. Burst of NADH obtained when enzyme is not pre-incubated with NAD^+ , measured by nucleotide fluorescence (a) The reaction mixture contained aldehyde dehydrogenase ($6 \mu\text{M}$) from one syringe and propionaldehyde ($830 \mu\text{M}$) and NAD^+ ($39.8 \mu\text{M}$) from another in 0.025 M -phosphate buffer pH 7.6 at 25°C . The vertical scale is 0.5 V/division and the horizontal scale is 100 ms/division . (b) Plot of $\log(F_\infty - F)$ (the difference between the steady-state rate of fluorescence change extrapolated back to zero time at particular times minus the corresponding experimental value) against time. There is a pronounced lag phase for the first 100 ms . The derived first-order rate constant is 4.4 s^{-1} .

The simulated data obtained for the change in fluorescence as a function of time for different mixing conditions and with different initial concentrations of substrates closely reproduced the experimental data for the corresponding experiments. When the reaction at low NAD^+ concentrations and saturating propionaldehyde was simulated, it was found that (as seems intuitively reasonable) the slope of a plot of k_b^0 (the burst rate constant calculated from the simulated data) against NAD^+ was the rate constant for NAD^+

binding used in the simulation. Another noteworthy feature of the simulated plots was the observation at low NAD^+ concentrations (when the enzyme was not preincubated with NAD^+) of a lag phase at the start of the reaction, caused in the simulation by the slow build-up of E^{NAD^+} a feature which had previously been observed in the experimental data (Fig. 5).

For the same mechanism as represented in reaction eqn. (2) the K_m , which was obtained from steady-state studies with propionaldehyde as a substrate, can be related to the individual rate constants thus:

$$K_m = \frac{k_{+4}(k_{+3} + k_{-2})}{k_{+2}(k_{+3} + k_{+4})}$$

(King & Altman, 1956; Cleland, 1963). Since the pre-steady-state equations have the same form as the steady-state equations no additional rate constants can be determined; however, the consistency between the data collected by the two methods can be checked.

If the values of k_{+3} and $(k_{+3} + k_{-2})/k_{+2}$ obtained from the intercept and slope of the best straight-line fit of the data in Fig. 4 are substituted into this equation together with the measured K_m of $1.1 \mu\text{M}$ (MacGibbon, 1976), a value for k_{+4} of 0.24 s^{-1} is obtained. This value is similar to the slow NADH -displacement rate constant (MacGibbon *et al.*, 1977b) and to the steady-state parameter $V/[E]$ (MacGibbon *et al.*, 1977a), demonstrating the consistency of the steady-state and pre-steady-state kinetic data.

Studies carried out by mixing enzyme-deamino- NAD^+ complexes with various concentrations of propionaldehyde gave results which fitted a linear double-reciprocal relationship, similar to that obtained with NAD^+ as coenzyme but with a maximum burst rate constant, k_{+3} , of 26 s^{-1} . From the slope and intercept of the double-reciprocal plot, k_{+2} and k_{-2} can be related by the equation:

$$(k_{-2} + k_{+3})/k_{+2} = 230 \times 10^{-6} \text{ mol} \cdot \text{litre}^{-1}$$

Since this equation cannot be solved in terms of the same k_{+2} and k_{-2} values as those obtained with NAD^+ , then not only does the maximum burst rate constant change with the cofactor, but also the rate constants for association or dissociation of propionaldehyde from the enzyme-coenzyme complex change. From the result of the experiment where deamino- NAD^+ is not premixed with the enzyme, it appears that this NAD^+ analogue binds much more slowly than NAD^+ , which suggests that the amino group is directly involved in the binding step.

The k_b -pH profile showed that the step involved in the burst was pH-dependent, with the greatest rate constant occurring at high pH values (a trend that is shown even more dramatically by benzaldehyde). The removal of a proton is involved either directly in this step or indirectly through the ionization of an adjacent group.

A plot of the inverse of the observed burst rate constant against the inverse of the acetaldehyde concentration, for kinetic studies in which the enzyme had been premixed with a saturating concentration of NAD^+ ($390 \mu\text{M}$), is shown in Fig. 4. From the intercept a maximum burst rate constant of 23 s^{-1} is obtained, and if it is assumed that eqn. (2) adequately describes the oxidation of acetaldehyde then the relationship between k_{-2} and k_{+2} is given by:

$$(k_{-2} + k_{+3})/k_{+2} = 2.5 \times 10^{-3} \text{ litre} \cdot \text{mol}^{-1}$$

Thus the maximum burst rate constant is about double that for propionaldehyde, whereas the half-saturating concentration of acetaldehyde is 50 times the value for propionaldehyde.

The data obtained by using $[1,2,2,2\text{-}^2\text{H}]$ acetaldehyde are also shown in Fig. 4, which indicates that a kinetic isotope effect ($k_b^{\text{H}}/k_b^{\text{D}}$) was observed at low acetaldehyde concentrations, but not at saturating concentrations. Thus the two plots have the same intercept and hence the same value of k_{+3} , but there is an isotope effect on the slope $[(k_{+3} + k_{-2})/k_{+2}]$ according to eqn. (7), suggesting a kinetic isotope effect on the association and dissociation of acetaldehyde from the enzyme.

Eckfeldt & Yonetani (1976) studied the pre-steady-state kinetics of horse liver aldehyde dehydrogenase with acetaldehyde by following the change in A_{350} . From the double-reciprocal plot of k_b and aldehyde concentration the isotope effect on k_{+3} was found to be $1.9 \pm 1.5 \text{ s}^{-1}$, and that on the slope was $2.8 \pm 0.1 \text{ s}^{-1}$. Although these results were interpreted in terms of k_{+3} relating to the hydride-transfer step, when the large error in k_{+3} is taken into account these same results could better fit the situation found for the sheep liver enzyme where an isotope effect is observed on the steps designated k_{+2} and k_{-2} in eqn. (1). This agrees with the generalization made by Cleland (1975) that the chemical steps in enzyme-catalysed reactions are not usually the major rate-determining ones, and in fact alcohol dehydrogenase is one of the few dehydrogenases to show an isotope effect on the burst (Shore & Gutfreund, 1970).

Similar maximum burst rate constants for acetaldehyde were found for the cytoplasmic enzyme from sheep liver and the cytoplasmic (F1) isoenzyme of horse liver, namely 23 s^{-1} and 33 s^{-1} (Eckfeldt & Yonetani, 1976) respectively. The kinetic trace shown in the paper of Weiner *et al.* (1976) can be used to estimate a burst rate constant of 33 s^{-1} , when NAD^+ and propionaldehyde at concentrations of $850 \mu\text{M}$ and $300 \mu\text{M}$ respectively are mixed with the mitochondrial (F2) isoenzyme of horse liver, a value that is significantly greater than the maximum burst rate constant of 11 s^{-1} obtained for propionaldehyde by using the sheep liver cytoplasmic enzyme.

Although reaction eqn. (1) is a good approximation to the pre-steady-state kinetic behaviour with propionaldehyde and acetaldehyde as substrates, it does not account for some facets of the steady-state behaviour. For example it may be necessary to include some isomerization steps to account for the biphasic nature of the NADH displacement (MacGibbon *et al.*, 1977b) and for the low coenzyme-binding rates.

As shown in Table 2, *p*-nitrobenzaldehyde was the only aldehyde for which no burst was observed. In general the burst rate constants for the aldehydes listed in Table 2 were similar, but they were significantly lower than for acetaldehyde or propionaldehyde. For the aromatic aldehydes there is a general tendency for the burst rate constant to increase with increasing electron-withdrawing power of the substituent, which is consistent with a nucleophilic attack on the carbonyl carbon atom after the hydride-transfer step. The lack of a burst for *p*-nitrobenzaldehyde can be interpreted in terms of a change-over to a rate-limiting hydride transfer, since increasing electronegativity of the *para* substituents would make this step less favourable. It is noteworthy that the burst rate constants obtained with benzaldehyde as substrate (Table 3) also increased with pH and approached the value obtained with propionaldehyde at pH 9.8.

It is not possible to identify unambiguously the rate-limiting step of the burst in eqn. (1), although since a kinetic isotope effect was not observed on k_{+3} the hydride-transfer step obviously cannot contribute significantly.

The rate-limiting step in the steady state appears to be solely NADH displacement at high aldehyde concentrations, since the slow step in the displacement of NADH ($0.22 \pm 0.05 \text{ s}^{-1}$) is identical within experimental error with $V/[E]$ for both propionaldehyde and acetaldehyde (MacGibbon *et al.*, 1977a,b). At lower concentrations, however, some additional step has a significant effect on the reaction, since $V/[E]$ is decreased (0.08 s^{-1} for $50 \mu\text{M}$ -acetaldehyde) and is dependent on aldehyde structure (MacGibbon, 1976). The nature of this additional step cannot be deduced from the present results.

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