Evidence for Two-Step Binding of Reduced Nicotinamide–Adenine Dinucleotide to Aldehyde Dehydrogenase

By ALASTAIR K. H. MACGIBBON, PAUL D. BUCKLEY and LEONARD F. BLACKWELL

*Department of Chemistry, Biochemistry and Biophysics, Massey University,
 Palmerston North, New Zealand*

(Received 6 December 1976)

The displacement of NADH from cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3) from sheep liver was studied by using NAD⁺, 1,10-phenanthroline, ADP-ribose, deamino-NAD⁺ and pyridine-3-aldehyde–adenine dinucleotide as displacing agents, by following the decrease in fluorescence as a function of time. The data obtained could be fitted by assuming two first-order processes were occurring, a faster process with an apparent rate constant of $0.85 \pm 0.20 \text{ s}^{-1}$ and a relative amplitude of $60 \pm 10\%$ and a slower process with an apparent rate constant of $0.20 \pm 0.05 \text{ s}^{-1}$ and a relative amplitude of $40 \pm 10\%$ (except for pyridine-3-aldehyde–adenine dinucleotide, where the apparent rate constant for the slow process was 0.05 s^{-1}). The displacement rates did not change significantly when the pH was varied from 6.0 to 9.0. Kinetic data are also reported for the dependence of the rate of binding of NADH to the enzyme on the total concentration of NADH. Detailed arguments are presented based on the isolation and purification procedures, the equilibrium coenzyme-binding studies and the kinetic data, which lead to the following model for the release of NADH from the enzyme:

The parameters that best fit the data are: $k_{+1} = 0.2 \text{ s}^{-1}$; $k_{-1} = 0.05 \text{ s}^{-1}$, $k_{+2} = 0.8 \text{ s}^{-1}$ and $k_{-2} = 5 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$. The slow phase of the NADH release is similar to the steady-state turnover number for substrates such as acetaldehyde and propionaldehyde and appears to contribute significantly to the limitation of the steady-state rate.

A number of tissue aldehyde dehydrogenases (EC 1.2.1.3) (Freda & Stoppani, 1970; Duncan & Tipton, 1971; Feldman & Weiner, 1972) have been isolated, and steady-state studies on these systems have yielded valuable information on the mechanisms of action of these enzymes. Eckfeldt & Yonetani (1976) reported the first rapid kinetic study to be carried out on an aldehyde dehydrogenase; despite the extreme sensitivity to oxidation of the F1 isoenzyme of horse liver, they were able to prepare sufficient quantities of this enzyme to carry out stopped-flow studies. They reported an NADH dissociation rate that was close to the steady-state turnover number, and suggested that the rate of NADH release contributed significantly to limitation of the enzyme catalytic activity.

We report here the results of a study of the dissociation of NADH from cytoplasmic aldehyde dehydrogenase from sheep liver.

Materials and Methods

Materials

Cytoplasmic aldehyde dehydrogenase from sheep liver was purified as described previously (Crow *et al.*,

1974). NADH (grade III), NAD⁺ (grade III), deamino-NAD⁺, pyridine-3-aldehyde–adenine dinucleotide and ADP-ribose were Sigma (St. Louis, MO, U.S.A.) chemicals used without purification, except where otherwise specified. 1,10-Phenanthroline was purchased from BDH (Poole, Dorset, U.K.).

Methods

Stopped-flow spectrophotometric studies were carried out with a Durrum–Gibson D110 stopped-flow spectrophotometer in the fluorescence mode. The excitation monochromator, which contained a tungsten light-source, was set on 340 nm with a 9 nm bandwidth, and the fluorescence was observed at 90° to the exciting light through Wratten 47B and 2B filters sandwiched between glass discs. These filters together have a maximum transmission at 435 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 35 mm film. The traces were projected on to graph paper, from which the data

were obtained. The enzyme concentrations quoted represent the concentration of NADH-binding sites determined from a fluorimetric titration similar to that described by Luisi *et al.* (1973), based on the enhancement of nucleotide fluorescence (approx. 5.6-fold) which results when NADH binds to the enzyme (MacGibbon, 1976). Plots of $1/(1-R)$ versus $[NADH]/R$, where R represents the fraction of occupied binding sites, were linear, showing no evidence for subunit interactions and giving a value of $1.2\mu\text{M}$ for the E-NADH dissociation constant, similar to that determined from steady-state-inhibition data (MacGibbon *et al.*, 1977).

Absorbance measurements were made using the combined fluorescence-absorbance cuvette (1.7 cm path-length) at 340 nm, where there is an increase of $0.66\text{mm}^{-1}\cdot\text{cm}^{-1}$ in the absorption coefficient for NADH on displacement from the enzyme.

Aldehyde dehydrogenase was assayed by the method of Crow *et al.* (1974), and protein determinations were carried out by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The mol.wt. of the enzyme, which consists of four identical subunits, is 212000 ± 8000 (Crow, 1975).

Displacement experiments

The NADH was displaced from the enzyme-NADH complex by mixing with a ligand, X, which was capable of binding to the same enzyme site as NADH according to Scheme 1:

Scheme 1

If the concentrations of X are such that $k_{+x}[X]$ is much greater than k_{+1} and also much greater than $k_{-1}[NADH]$, then the rate of decrease in fluorescence caused by the conversion of the enzyme-NADH complex into the less fluorescent free NADH will be determined by the rate of dissociation of the enzyme-NADH complex, and a first-order process with rate constant k_{-1} should be observed.

Results

NAD⁺ as the displacing ligand

Although the mechanism in Scheme 1 requires that a single first-order process should be observed for a displacement reaction under the conditions specified, the experimental behaviour was more complicated, as is shown by a typical stopped-flow trace in Fig. 1(a). An attempt to plot these data as a single first-order process is also shown (Fig. 1b). However, a biphasic plot, typical of consecutive first-order processes, was obtained.

Fig. 1. Displacement of NADH from aldehyde dehydrogenase by NAD⁺

(a) The reaction mixture contained $2.1\mu\text{M}$ sheep liver aldehyde dehydrogenase and $8.4\mu\text{M}$ -NADH from one syringe, and 8.6mM -NAD⁺ from another, in 0.025M -sodium phosphate buffer, pH 7.6, at 25°C . Measurements were made in the fluorescence mode; the vertical scale is 0.2V/division , the horizontal scale is 2s/division (where $1\text{V} = \text{fluorescence of } 6.25\mu\text{M-NADH}$ solution). The lower trace was obtained on re-triggering at the completion of the reaction. (b) Graph of the logarithm of ΔF , the difference between the voltage at each time and the voltage at infinite time plotted against time, showing the biphasic plot typical of consecutive first-order processes. The rate constant (calculated by the least-squares method) derived from the line shown is 0.14s^{-1} . (c) Graph showing a plot of the logarithm of $\Delta\Delta F$, the difference between the experimental points obtained during the first 2 s and the corresponding points obtained by extrapolating back the line through the later points. The rate constant derived from this plot is 0.9s^{-1} .

To ensure that the biphasic behaviour was not an artifact of the fluorescence measurements, the displacement reaction was repeated by following changes in A_{340} under similar conditions. Biphasic plots were again obtained. To check the instrumentation further, the binding of proflavin ($110\mu\text{M}$) to

Table 1. Rate constants for the displacement of NADH from the enzyme-NADH complex

The reaction mixture contained aldehyde dehydrogenase ($2\mu\text{M}$) and NADH ($8.5\mu\text{M}$) from one syringe and the displacing agent at the concentrations listed from another syringe in 0.025M -sodium phosphate buffer, pH 7.6, at 25°C . The rate constants were calculated as shown in Fig. 1. The specific activity of the enzyme samples ranged from 377 to 619 units/mg in the assay of Crow *et al.* (1974) at 20°C . Results quoted for NAD^+ displacement are averages of 30 determinations over several enzyme preparations. The error quoted is the s.d. All other values are averages of at least ten separate determinations.

Displacing agent	Rate constant (s^{-1})	
	Fast process	Slow process
NAD^+ ($0.52\text{--}10\text{mM}$)	0.85 ± 0.20	0.22 ± 0.05
1,10-Phenanthroline ($0.045\text{--}0.71\text{mM}$)	1.0	0.25
Deamino- NAD^+ ($0.6\text{--}1.6\text{mM}$)	1.0	0.24
ADP-ribose ($0.36\text{--}1.8\text{mM}$)	1.1	0.29
Pyridine-3-aldehyde-adenine dinucleotide (1.9mM)	0.9	0.053

α -chymotrypsin ($10.4\mu\text{M}$) in 0.05M -sodium phosphate buffer, pH 7.3, was followed at 465nm under the conditions described by Fersht & Requena (1971). The oscilloscope trace obtained was a single exponential, with a rate constant of 3.4s^{-1} , in good agreement with the literature value (Fersht & Requena, 1971). The biphasic behaviour observed for the NADH displacements was not therefore a machine artifact.

NAD^+ that had been purified (Whitaker *et al.*, 1974) immediately before use gave the same biphasic plots for the displacement as obtained with the Sigma (grade III) NAD^+ used without purification.

For a typical run, after the displacement reaction was recorded on the storage oscilloscope, the instrument was retriggered to obtain the infinity reading for the fluorescence at the end of the displacement. The logarithm of the difference between the fluorescence at each time and the fluorescence at infinite time was plotted against time. A straight line could be drawn through the later points and a value of the first-order rate constant for the slower of the two processes calculated from the slope. The rate constant for the fast process was obtained by extrapolating back to zero time the line through the later points and plotting the logarithm of the difference between this extrapolated line and the actual experimental points at convenient time intervals. Such a difference plot for the fast process is also shown in Fig. 1(c). The rate constants were calculated from a least-squares analysis of the data. Rate constants quoted are the average of at least ten separate runs.

Table 2. Effect of pH on the displacement of NADH from the enzyme-NADH complex

The reaction mixture contained aldehyde dehydrogenase ($2\mu\text{M}$) and NADH ($8.5\mu\text{M}$) from one syringe and NAD^+ (2mM) from another syringe in 0.025M -sodium pyrophosphate buffer. The pH value was measured after mixing and the rate constants were calculated as shown in Fig. 1. The specific activity of the enzyme was 377 units/mg in the assay of Crow *et al.* (1974) at 20°C .

pH	Rate constant (s^{-1})	
	Fast process	Slow process
6.3	0.79	0.073
9.0	0.62	0.054
9.9	0.59	0.057
7.6*	0.85 ± 0.2	0.22 ± 0.05

* 0.025M -Sodium phosphate buffer; see Table 1 for details.

The amplitudes of the fast and slow phases were approximately equal ($60 \pm 10\%$ fast phase and $40 \pm 10\%$ slow phase) and the sum of the two was always approx. 90% of the active-site concentration.

The concentration of NAD^+ was varied to demonstrate that the rate constants for the fast and slow processes were independent of NAD^+ concentration, and reflected only the displacement of NADH from the enzyme. The rate constants for different NAD^+ concentrations were identical within experimental error, with values of $0.85 \pm 0.20\text{s}^{-1}$ for the fast process and $0.22 \pm 0.05\text{s}^{-1}$ for the slow process (Table 1).

The displacement reaction was also carried out in a range of pyrophosphate buffers (Table 2). Little variation in displacement rates was observed with pH, although the rate constant for the slow process in pyrophosphate buffers is less than in phosphate buffers.

Other displacing ligands

Similar biphasic plots were obtained when pyridine-3-aldehyde-adenine dinucleotide, ADP-ribose, deamino- NAD^+ , and 1,10-phenanthroline (a competitive inhibitor of NAD^+) were used as the displacing ligands, and the rate constants were calculated as described above (Table 1). Except for pyridine-3-aldehyde-adenine dinucleotide, all the displacing ligands gave rate constants in the same range as those found for NAD^+ . With pyridine-3-aldehyde-adenine dinucleotide the rate constant for the fast process was comparable with that obtained for the other ligands, but the rate constant for the slow process was less (0.05s^{-1}).

An attempt was made to form ternary complexes by premixing NADH and the enzyme in the presence

of a 2000-fold excess of propionaldehyde and then displacing the NADH with 1,10-phenanthroline. The resulting oscilloscope trace was biphasic, with the same rate constants as when propionaldehyde was absent. A similar experiment using α -bromophenylacetic acid (a known inhibitor of the enzymic reaction) in place of the propionaldehyde also provided no evidence of ternary-complex formation.

Displacement of deamino-NADH

Since deamino-NAD⁺ functioned successfully as a cofactor for the oxidation of propionaldehyde to propionic acid, by treating limiting amounts of propionaldehyde ($5.4\mu\text{M}$) and a small excess of deamino-NAD⁺ ($8\mu\text{M}$) in the presence of the enzyme ($1.7\mu\text{M}$), significant amounts of the enzyme-deamino-NADH complex could be produced. The deamino-NADH could then be displaced from the enzyme by mixing with excess of NAD⁺. In this case a single exponential trace only was obtained, with a rate constant of 0.17s^{-1} (Fig. 2). An analogous experiment carried out by using limiting amounts of NAD⁺ instead of deamino-NAD⁺ to produce the NADH-enzyme complex, gave the usual biphasic plot with rate constants 0.6 and 0.15s^{-1} .

Association of NADH with aldehyde dehydrogenase

To obtain more information about the binding of NADH to aldehyde dehydrogenase, an excess of NADH was mixed with the enzyme in the stopped-flow spectrophotometer. The traces were again clearly biphasic (Fig. 3), and, provided that a sufficient excess of NADH was used to ensure pseudo-first-order kinetics for the NADH-binding step, the data could be analysed in the same way as discussed for the displacement experiments. Only the faster process shows a small dependence on the NADH concentration, as shown by the plot of the observed rate constants versus NADH concentration (Fig. 4) at pH 7.6. The analogous data collected at pH 6.0 were identical within experimental error.

Quantitative interpretation of the association data may be complicated by the self-quenching of nucleotide fluorescence that occurs at concentrations greater than about $10\mu\text{M}$ -NADH.

Concentration-jump experiments

When a mixture of enzyme and NADH initially at equilibrium was diluted by mixing with an equal concentration of buffer in the stopped-flow spectrophotometer, the approach of the system to the new equilibrium position could be conveniently followed by the changes in fluorescence. The oscilloscope traces were again biphasic and could be analysed in terms of two exponentials, as discussed for the

Fig. 2. Displacement of deamino-NADH from aldehyde dehydrogenase by NAD⁺

(a) The reaction mixture contained premixed aldehyde dehydrogenase ($1.7\mu\text{M}$), deamino-NAD⁺ ($8\mu\text{M}$) and propionaldehyde ($5.2\mu\text{M}$) from one syringe and NAD⁺ (4.3mM) from another in 0.025M-sodium phosphate buffer, pH 7.6, at 25°C . Measurements are made in the fluorescence mode; the vertical scale is 0.1 V/division, the horizontal scale is 2 s/division. The lower trace represents the final fluorescence value. (b) The rate constant derived from the best-fit straight line by the least-squares procedure is 0.17s^{-1} .

displacement experiments (Fig. 5). When solutions with initial concentrations of enzyme and NADH of 3 and $4.8\mu\text{M}$ respectively were prepared, the first-order rate constants describing the approach to equilibrium after dilution were 3.0 and 0.8s^{-1} respectively.

Fig. 3. Association of NADH with aldehyde dehydrogenase (a) The reaction mixture contained aldehyde dehydrogenase ($1.8 \mu\text{M}$) from one syringe, and NADH ($16 \mu\text{M}$) from another, in 0.025M -sodium phosphate buffer, pH 7.6, at 25°C . Measurements are made in the fluorescence mode; the vertical scale is $0.2\text{V}/\text{division}$ and the horizontal scale is $0.2\text{s}/\text{division}$ ($1\text{V} = \text{fluorescence of } 5.7 \mu\text{M NADH}$). The upper trace represents the final fluorescence value. (b) Graph of the logarithm of the difference, ΔF , between the voltage at infinite time and the voltage at each time plotted against time. The rate constant derived from the line shown is 0.85s^{-1} . The inset shows a plot of the logarithm of the difference between the experimental points obtained during the first 0.5s and the corresponding points obtained by extrapolating back the line through the later points. The rate constant derived from this plot is 4.8s^{-1} . (The amplitude is equal to the enzyme concentration.)

Fig. 4. Dependence of the rate constants for the association of NADH with aldehyde dehydrogenase on the concentration of NADH

(a) A graph of the measured values of the rate constant for the fast process, observed when NADH is mixed with aldehyde dehydrogenase, derived as shown in Fig. 3, as a function of the initial concentration of NADH after mixing. (b) A graph of the measured values of the rate constant for the slow process observed when NADH is mixed with aldehyde dehydrogenase as a function of the initial concentration of NADH after mixing. (The broken lines represent the best-fit lines as determined by a least-squares procedure.)

Discussion

There are several possible explanations for the biphasic nature of the NADH displacement reactions.

(1) Two isoenzymes of aldehyde dehydrogenase are present which have different rates of NADH dissociation.

(2) Although a single enzyme is present, there are two different types of binding site for NADH on the enzyme; the sites may be different but independent, or the dissociation of the NADH from one site may alter the rate of dissociation of NADH from the second site.

(3) All the binding sites on the enzyme are identical, but the NADH may dissociate in two steps: a first step involving either a change in the conformation of the NADH in the binding site, or a conformational change of the enzyme-NADH complex, which is followed in either case by the dissociation of NADH.

We have been unable to obtain any evidence for the existence of isoenzymes of cytoplasmic aldehyde dehydrogenase. As described previously (Crow *et al.*, 1974), the cytoplasmic, mitochondrial and microsomal aldehyde dehydrogenases purified from sheep liver have been separately isolated. The cytoplasmic enzyme discussed in the present study

Fig. 5. Dilution of a mixture of aldehyde dehydrogenase and NADH that was initially at equilibrium

(a) The reaction mixture contained aldehyde dehydrogenase ($7.5\mu\text{M}$) and NADH ($10.0\mu\text{M}$) in 0.025M -sodium phosphate buffer, pH 7.6, from one syringe, and 0.025M -sodium phosphate buffer, pH 7.6, from the other at 25°C . Measurements are made in the fluorescence mode; the vertical scale is 0.2V/division and the horizontal scale is 0.5s/division . (1V = fluorescence emission from $4\mu\text{M}$ -NADH.) The amplitude of the change corresponds to 15% of the total enzyme concentration. (The lower trace represents infinity fluorescence). (b) Graph of the logarithm of the difference, ΔF , between the voltage at each time and the voltage at infinite time plotted against time. The rate constant derived from the line shown is 0.83s^{-1} . The inset shows a plot of the logarithm of the difference between the experimental points obtained during the first 2s and the corresponding points obtained by extrapolating back the line through the later points against time. The rate constant derived from this plot is 3.04s^{-1} .

showed a single band on gel electrophoresis and behaved as a single species in isoelectric-focusing experiments (Crow, 1975). Although such results cannot positively exclude the existence of isoenzymes, the evidence does not support explanation (1).

The titration curve obtained when the increase in fluorescence is monitored as batches of NADH are added to a fixed amount of enzyme can be fitted by assuming a single binding site (MacGibbon, 1976). This observation rules out NADH-binding sites with different dissociation constants, whether such sites are present on isoenzymes of aldehyde dehydrogenase or represent different sites on a single enzyme. Significant numbers of such binding sites are excluded whether the fluorescent enhancement is the same or different for the two sites. A Hill plot of the titration data gives a straight line with a slope of 1.0 ± 0.03 , which implies no allosteric effects. It should also be noted that the approximately equal amplitudes (Fig. 1) of the fast and slow processes observed during displacement experiments excludes the existence of a small number of different binding sites, and any attempt to produce the experimentally observed amplitudes by arbitrarily postulating a much greater fluorescence enhancement on the binding of NADH to this very small number of sites would simultaneously produce deviations from single-site behaviour in the titration curve.

On the basis of such experimental observations, explanations (1) and (2) can be excluded.

Having excluded non-identical-site models we are left with the model proposed in explanation (3) and shown in Scheme 2:

Scheme 2

Provided the $k_{+x}[\text{X}]$ is much greater than $k_{-2}[\text{NADH}]$, the displacement reaction represented by Scheme 2 will, in general, be a biphasic process, where the measured first-order decay constants are given by the expression (Frost & Pearson, 1961):

$$\lambda_F, \lambda_S = \frac{1}{2} \{ (k_{+1} + k_{-1} + k_{+2}) \pm [(k_{+1} + k_{-1} + k_{+2})^2 - 4k_{+1}k_{+2}]^{1/2} \}$$

Values of k_{+1} , k_{-1} and k_{+2} were chosen for the individual steps in Scheme 2, and the associated values of λ_F and λ_S calculated from the above equation. These calculated values for the decay constants were compared with the measured values of $0.85 \pm 0.20\text{s}^{-1}$ and $0.22 \pm 0.05\text{s}^{-1}$. By systematically varying k_{+1} , k_{-1} and k_{+2} , all possible combinations of these rate constants that lead to calculated decay constants which agree within experimental error with the measured values can be tabulated.

It is also necessary to calculate the relative amplitudes of the two decay processes, and to compare these with the experimental amplitudes ($60 \pm 10\%$ fast phase, $40 \pm 10\%$ slow process).

Although it is possible to calculate the amplitudes of the two phases from the known initial-equilibrium concentrations (Frost & Pearson, 1961), we found it convenient to use the continuous system-modelling package that is available on the Burroughs B6700 computer, to simulate the displacement reactions. We could include the appropriate fluorescence-enhancement factors (MacGibbon, 1976) and determine the relative amplitudes of the two phases directly from the computer print-out of the logarithm of the difference of the fluorescence as a function of time for the particular model being considered.

The fitting of the NADH-titration data assuming a single binding site places further restrictions on the model shown in scheme 2, and leads to the following possibilities:

Case (a), either $E^* \cdot \text{NADH}$ or $E \cdot \text{NADH}$ is present in very small concentrations.

Case (b), the $E \cdot \text{NADH}$ complex has the same fluorescence properties as free NADH, but the fluorescence of $E^* \cdot \text{NADH}$ is enhanced with respect to free NADH.

Case (c), the $E^* \cdot \text{NADH}$ complex has the same fluorescence properties as free NADH, but the fluorescence of $E \cdot \text{NADH}$ is enhanced with respect to free NADH.

Case (d), $E^* \cdot \text{NADH}$ and $E \cdot \text{NADH}$ show the same fluorescence enhancement properties.

For cases (b), (c) and (d) an apparent dissociation constant will be obtained from the NADH-titration data which is in each case given by the expression:

$$K_D = \frac{[E][\text{NADH}]}{[E^* \cdot \text{NADH}] + [E \cdot \text{NADH}]}$$

where K_D represents the ratio of free and bound NADH.

It should also be noted that the fluorescence difference will be proportional, in case (b) to the concentration of $E^* \cdot \text{NADH}$, in case (c) to the concentration of $E \cdot \text{NADH}$ and in case (d) to the sum of the concentrations of $E^* \cdot \text{NADH}$ and $E \cdot \text{NADH}$.

Each possible case will be considered in turn.

Case (a)

If $E \cdot \text{NADH}$ is the complex present in much larger concentrations than $E^* \cdot \text{NADH}$ at equilibrium, then as expected intuitively, and confirmed by computer simulation, only a single process would be observed, with a characteristic decay constant equal to k_{+2} .

If $E^* \cdot \text{NADH}$ is the complex present in much larger concentration at equilibrium, then necessarily k_{-1} must be much larger than k_{+1} . When choosing k_{-1} values which must not exceed a value of about

1.1 s^{-1} , in order to prevent λ_F from becoming significantly greater than the decay constant for the faster process, it is found that the small values of k_{+1} necessary to meet the equilibrium requirement lead to values of λ_S which are much less than the measured value. It is not possible therefore to choose values of k_{+1} , k_{-1} and k_{+2} that simultaneously meet this thermodynamic requirement and which give reasonable values of both λ_F and λ_S that agree within experimental error with the measured decay constants.

Case (b)

Although suitable combinations of k_{+1} , k_{-1} and k_{+2} can be found to give values of λ_F and λ_S which agree with the experimental decay constants, when computer simulations were carried out using these parameters it was found that the concentration of $E^* \cdot \text{NADH}$ (and hence the fluorescence difference) decays as either a single exponential or else displays a pronounced lag phase. Both types of behaviour are contrary to what is observed experimentally (Fig. 1), and case (b) can therefore be excluded.

Case (c)

It was found that for suitable choices of the parameters k_{+1} , k_{-1} and k_{+2} , the decay of $E \cdot \text{NADH}$ with time gave biphasic plots that have both amplitudes and decay constants that agree with those observed experimentally in the displacement experiments. However, when the association experiments were simulated, it was found that the concentration of $E \cdot \text{NADH}$ passed through a maximum. As shown in Fig. 4, the fluorescence actually increased throughout the association reaction, and case (c) can also be excluded.

By using the method of Holmes,* it is concluded that case (d) best describes the displacement reaction.

The combination of rate constants that give the best fit to the data are: $k_{+1} = 0.2 \text{ s}^{-1}$, $k_{-1} = 0.05 \text{ s}^{-1}$ and $k_{+2} = 0.8 \text{ s}^{-1}$.

With the values of k_{+1} and k_{-1} chosen, the concentration of the $E \cdot \text{NADH}$ species is relatively high, and the fast process can be visualized in terms of the dissociation of NADH from this species, and the significant step in the slower process is interconversion of enzyme-NADH complexes.

Although by using the average values of the observed decay constants and the relative amplitudes of the fast and slow processes the rate constants needed to give the best fit could be unambiguously assigned within reasonably narrow limits when the experimental error in the measured rate constants

* As said by Sherlock Holmes in *The Sign of the Four* (written by Sir Arthur Conan Doyle in 1890): 'How often have I said to you that when you have eliminated the impossible, whatever remains, however improbable, must be the truth?'

and amplitudes is taken into account, there is obviously some latitude in the values of the chosen rate constants.

The apparent dissociation constant for NADH may be expressed in terms of the rate constants for the individual steps:

$$K_D = k_{+2}/k_{-2}[1/(1 + k_{-1}/k_{+1})]$$

Since values of K_D (MacGibbon *et al.*, 1977) k_{+1} , k_{-1} and k_{+2} are known, a value of $5 \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$ may be calculated for the rate constant k_{-2} . With this value for the rate constant of the NADH-binding step, values for the rate constants of the concentration-jump experiment can be calculated to be 2.6 and 0.27 s^{-1} , which are in good agreement with the experimental values of 3.0 and 0.8 s^{-1} .

From these same constants it is possible to calculate the decay constants for the association experiments. At $10 \mu\text{M}$ -NADH the constants for the two processes would be 5.8 and 0.25 s^{-1} , and at $20 \mu\text{M}$ -NADH they would be 10.8 and 0.25 s^{-1} . It should be noted that the results are in good qualitative agreement with the experimental data shown in Fig. 4, namely a fast NADH-dependent process and a slow NADH-independent process. The calculated results show a lower value for the slow process and a greater NADH-dependence for the fast process, although, because of the difficulty in determining accurate decay constants, for the association, there is a large amount of scatter in the data shown in Fig. 4. It should be noted that the NADH-independence of the slower process is further strong supporting evidence against the existence of parallel binding on different NADH-binding sites. Such a model would require both processes to be NADH-dependent.

The rate constants for displacement of NADH from aldehyde dehydrogenase were insensitive to changes in displacing agent, as expected for a process where release of NADH from the enzyme is rate-determining. Although with pyridine-3-aldehyde-adenine dinucleotide a significantly different slower process was observed, this coenzyme analogue did not act as a coenzyme with propionaldehyde in the steady-state reaction (although it did act as an aldehyde substrate with NAD^+ as coenzyme), and may not be suitable as a displacing agent (MacGibbon, 1976).

In contrast with NADH, when deamino-NADH was displaced from the enzyme only a single-phase process was observed at a rate similar to the slow process of NADH. A possible explanation, which seems unlikely, is that the step involving k_{+2} is now too fast to be observed. The result obtained could also mean that only a single enzyme-deamino-NADH complex is present in significant concentrations. However, it was clear from the simulations that, even when significant amounts of two enzyme-

NADH complexes are present, if k_{+1} is a good deal larger than k_{+2} or if k_{+1} , k_{-1} and k_{+2} are all of similar size, only a single process (the slower one) will be observed. Such an explanation could apply for the displacement of deamino-NADH from aldehyde dehydrogenase.

The pH-independence of the rate constants for displacement and association argues against the presence of a step involving proton release similar to that suggested for malate dehydrogenase (Czerlinski & Schreck, 1964).

It is significant that the slow phase of the NADH displacement (0.22 s^{-1}) is similar to the k_{cat} . ($V_{\text{max}}/[E]_0$) values found for high concentrations of propionaldehyde and acetaldehyde in the steady state, of 0.25 and 0.27 s^{-1} respectively (MacGibbon *et al.*, 1977). Thus for sheep liver cytoplasmic aldehyde dehydrogenase, as was reported for the F1 isoenzyme of horse liver (Eckfeldt & Yonetani, 1976) the slow rate of NADH release contributes significantly to the limitation of enzyme catalytic-centre velocity, a result that may prove to be general for all tissue aldehyde dehydrogenases.

We thank Mr. Terry Braggins for his expert technical assistance. We also thank the New Zealand University Grants Committee for the purchase of the stopped-flow spectrophotometer.

References

- Crow, K. E. (1975) Ph.D. Thesis, Massey University, Palmerston North, New Zealand
- Crow, K. E., Kitson, T. M., MacGibbon, A. K. H. & Batt, R. D. (1974) *Biochim. Biophys. Acta* **350**, 121–128
- Czerlinski, G. H. & Schreck, G. (1964) *Biochemistry* **3**, 89–100
- Duncan, R. J. S. & Tipton, K. F. (1971) *Eur. J. Biochem.* **22**, 538–543
- Eckfeldt, J. H. & Yonetani, T. (1976) *Arch. Biochem. Biophys.* **173**, 273–281
- Feldman, R. I. & Weiner, H. (1972) *J. Biol. Chem.* **247**, 260–266
- Fersht, A. R. & Requena, Y. (1971) *J. Mol. Biol.* **60**, 279–290
- Freda, C. E. & Stoppani, A. O. M. (1970) *Enzymologia* **38**, 225–242
- Frost, A. A. & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd edn., pp. 175–177, John Wiley and Sons, New York
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Luisi, P. L., Olomucki, A., Baici, A. & Karlovic, D. (1973) *Biochemistry* **12**, 4100–4105
- MacGibbon, A. K. H. (1976) Ph.D. Thesis, Massey University, Palmerston North, New Zealand
- MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977) *Eur. J. Biochem.* in the press
- Whitaker, J. R., Yates, D. W., Bennett, N. G., Holbrook, J. J. & Gutfreund, H. (1974) *Biochem. J.* **139**, 677–697