Steady-State and Pre-Steady-State Kinetic Studies on Mitochondrial Sheep Liver Aldehyde Dehydrogenase

A COMPARISON WITH THE CYTOPLASMIC ENZYME

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Kinetic studies were carried out on mitochondrial aldehyde dehydrogenase (EC 1.2.1.3) isolated from sheep liver. Steady-state studies over a wide range of acetaldehyde concentrations gave a non-linear double-reciprocal plot. The dissociation of NADH from the enzyme was a biphasic process with decay constants 0.6 s^{-1} and 0.09 s^{-1} . Pre-steady-state kinetic data with propionaldehyde as substrate could be fitted by using the same burst rate constant ($12 \pm 3\text{ s}^{-1}$) over a wide range of propionaldehyde concentrations. The quenching of protein fluorescence on the binding of NAD^+ to the enzyme was used to estimate apparent rate constants for binding ($2 \times 10^4\text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) and dissociation (4 s^{-1}). The kinetic properties of the mitochondrial enzyme, compared with those reported for the cytoplasmic aldehyde dehydrogenase from sheep liver, show significant differences, which may be important in the oxidation of aldehydes *in vivo*.

We have reported on the steady-state kinetics (MacGibbon *et al.*, 1977a) and on the results of stopped-flow studies on the pre-steady-state (burst) kinetics of the oxidation of aldehydes by cytoplasmic aldehyde dehydrogenase from sheep liver (MacGibbon *et al.*, 1977c) and the rates of binding of NADH to this enzyme (MacGibbon *et al.*, 1977b).

Because of the limitation of available NAD^+ in the cytoplasm (Marjanen, 1972), it has been suggested that the oxidation of the acetaldehyde produced during the metabolism of ethanol may take place principally in the mitochondria. However, the relative insensitivity of the aldehyde dehydrogenase isolated from the mitochondria of sheep liver to disulfiram (which is believed in humans to inhibit the oxidation of the acetaldehyde produced during the metabolism of ethanol by inactivating the appropriate enzyme) and the marked sensitivity of the cytoplasmic aldehyde dehydrogenase to this same chemical could argue against this assumption (Hart & Dickinson, 1977; Kitson, 1975). In the absence of information on the localization of aldehyde dehydrogenases in human liver the question of the relative importance of these enzymes to the metabolism of ethanol in humans must remain open.

The mitochondrial and cytoplasmic aldehyde dehydrogenases from sheep liver are of similar mol.wt. (205000 and 212000 respectively), each consisting of four identical subunits (Crow, 1975). Although steady-state kinetics of the mitochondrial enzyme have not been studied extensively, the Michaelis constant for NAD^+ binding has been

shown to be 6 times that for the cytoplasmic enzyme with glyceraldehyde as substrate (Crow *et al.*, 1974). It is therefore of interest to compare further the kinetic properties of these two enzymes, and in the present paper we report on kinetic studies carried out on aldehyde dehydrogenase isolated from the mitochondria of sheep liver.

Methods and Materials

Mitochondrial aldehyde dehydrogenase was purified as described by Crow *et al.* (1974). Acetaldehyde was obtained from BDH (Poole, Dorset, U.K.) and propionaldehyde from Koch–Light Laboratories (Colnbrook, Bucks., U.K.). The concentration of stock solutions of the aldehydes were determined by forming the semicarbazides and measuring the absorbance at near 220 nm. $[1\text{-}^2\text{H}]\text{Propionaldehyde}$ was prepared as described (MacGibbon *et al.*, 1977b). The NADH was obtained as preweighed vials and the concentration was checked by measurements of A_{340} , assuming a molar absorption coefficient of $6.22 \times 10^3\text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Horecker & Kornberg, 1948). NAD^+ (grade III) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and purified by the method of Whitaker *et al.* (1974). Deionized distilled water was used in the preparation of all reagent solutions.

Experimental conditions for carrying out the stopped-flow studies and analysing the data were the same as those described (MacGibbon *et al.*, 1977b,c).

Results

(a) Steady-state studies

The initial rate of reaction was measured by monitoring changes in nucleotide fluorescence for at least 3 min. A blank run in which buffer, enzyme and NAD^+ only were mixed showed no increase in nucleotide fluorescence, demonstrating the absence of aldehyde impurities from the assay mixture. Steady-state studies were carried out on the enzyme-catalysed oxidation of acetaldehyde in the presence of 1.35 mM- NAD^+ by using a wide range of acetaldehyde concentrations in 25 mM-sodium phosphate buffer, pH 7.6 at 25°C. The results of the duplicate runs were averaged and the data plotted as a double-reciprocal plot (Fig. 1). This plot was linear up to concentrations of 10 μM (Fig. 1a), with a Michaelis constant of 0.2 μM . However, at higher acetaldehyde concentrations deviations towards higher initial velocities were observed, so that at 20 mM-acetaldehyde the initial velocity was nine times that expected by extrapolation of the linear portion of the graph (Fig. 1b).

(b) NADH displacement

When NADH was displaced from the enzyme-NADH complex by mixing with excess of either NAD^+ or ADP-ribose, the fluorescence decreased as the NADH was displaced, demonstrating that significant enhancement of nucleotide fluorescence had occurred on binding of the coenzyme to the mitochondrial aldehyde dehydrogenase (a result confirmed by Hart & Dickinson, 1977).

A typical oscilloscope trace is shown in Fig. 2(a). The trace was biphasic and the data were treated as described previously (MacGibbon *et al.*, 1977b) and the appropriate plots of $\log \Delta F$ and $\log \Delta \Delta F$ versus time are shown in Figs. 2(b) and 2(c) respectively. Similar values for the decay constants for the fast and slow processes, namely 0.6s^{-1} and 0.09s^{-1} , were obtained with NAD^+ (1.0–2.9 mM) and with ADP-ribose (1.7 mM).

(c) Pre-steady-state measurements

When mitochondrial aldehyde dehydrogenase pre-mixed with NAD^+ was mixed with propionaldehyde,

Fig. 1. Double-reciprocal plot of initial velocity against acetaldehyde concentration for mitochondrial aldehyde dehydrogenase in 25 mM-sodium phosphate buffer, pH 7.6

The concentration of NAD^+ was constant at 1.35 mM. The initial-velocity data were measured fluorimetrically as described by MacGibbon *et al.* (1977a). The enzyme concentration was 0.1 μM and aldehyde concentrations were checked by formation of the semicarbazide (MacGibbon *et al.*, 1977c). (a) Low concentrations: K_m for acetaldehyde is 0.2 μM and $v_{\text{extrapolated}}$ is 0.07 fluorescence unit/min. (b) High concentrations: deviations from linearity commence at 10 μM and a maximum initial velocity of 0.66 fluorescence unit/min is reached at 20 mM. The straight line represents the extrapolation of the low-concentration data and the ratio of the intercepts is about 9.

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

(a) Reaction mixture contained $2.6\ \mu\text{M}$ -mitochondrial aldehyde dehydrogenase and $10.2\ \mu\text{M}$ -NADH from one syringe, and $1.81\ \text{mM}$ -NAD⁺ from another, in $25\ \text{mM}$ -sodium phosphate buffer, pH 7.6, at 25°C . Measurements were made in the fluorescence mode; the vertical scale is $0.5\ \text{V/division}$ (where $1\ \text{V}$ =fluorescence of $4.2\ \mu\text{M}$ -NADH), the horizontal scale is $5\ \text{s/division}$. The lower trace was obtained on retriggering the oscilloscope at the end of the reaction. (b) Graph of the logarithm of ΔF (in V), the difference between the voltage at each time and that 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) is $0.093\ \text{s}^{-1}$. (c) Graph showing a plot of the logarithm of $\Delta\Delta F$ (in V), the difference between the experimental points obtained during the first 4 s and the corresponding points obtained by extrapolating back the line through the later points against time. The rate constant derived from this plot is $0.6\ \text{s}^{-1}$.

a burst was observed, followed by the steady-state production of NADH (Fig. 3). The size of the fluorescence change in the burst with $0.4\ \text{mM}$ -propionaldehyde was only about one-sixth of the change observed for NADH displacement from an enzyme solution of the same concentration. However, over the range of propionaldehyde concentrations used the amplitude of the burst had increased by a factor of 3. The same burst rate constant ($12 \pm 3\ \text{s}^{-1}$) was obtained over this range (0.4 – $20\ \text{mM}$) of propionaldehyde concentrations. Because of the small size of the burst amplitude with $[1\text{-}^3\text{H}]$ propionaldehyde as substrate it was not clear whether there was a small isotope effect on the burst. However, the steady-state maximum velocity showed no isotope effect (V_H/V_{2H} 1.0).

(d) Quenching of protein fluorescence

There was significant quenching of protein fluorescence on binding of both NAD⁺ and NADH to the enzyme. Although such quenching may exhibit a non-linear relationship with the increase of fractional saturation of coenzyme-binding sites (Holbrook,

1972), as for the cytoplasmic enzyme (MacGibbon *et al.*, 1977c), when a range of NAD⁺ concentrations were mixed with enzyme in the stopped-flow spectrophotometer the decrease in fluorescence obeyed first-order kinetics and a plot of the calculated rate constant against NAD⁺ concentration was linear, with a slope of $2 \times 10^4\ \text{litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ and an intercept of approx. $4\ \text{s}^{-1}$. 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.

Discussion

It is clear that there are many similarities between the cytoplasmic and mitochondrial aldehyde dehydrogenases from sheep liver. For both enzymes there is a marked enhancement of nucleotide fluorescence on the binding of NADH and significant quenching of protein fluorescence on the binding of both coenzymes. The maximum burst rate constants with propionaldehyde as substrate were the same within experimental error (MacGibbon *et al.*, 1977c).

Fig. 3. Burst in the production of NADH during oxidation of propionaldehyde by mitochondrial aldehyde dehydrogenase

The reaction mixture contained $5.6\mu\text{M}$ -sheep liver mitochondrial aldehyde dehydrogenase and 6mM - NAD^+ from one syringe, and propionaldehyde (41.6mM) from another, in 25mM -sodium phosphate buffer, $\text{pH} 7.6$ at 25°C . Measurements were made in the fluorescence mode by monitoring nucleotide fluorescence through Wratten 2B and 47B filters; the vertical scale is $0.2\text{V}/\text{division}$ (where 1V =fluorescence from $5.3\mu\text{M}$ -NADH), the horizontal scale is $50\text{ms}/\text{division}$. The rate constant derived from the photograph was 11s^{-1} . The upper two traces show the steady-state production of NADH.

There is no isotope effect on the steady-state maximum velocities, demonstrating that hydride transfer is not rate-limiting in the steady state. The Lineweaver-Burk plots derived from the dependence of the steady-state rate on acetaldehyde concentration show similar deviations from linearity at high acetaldehyde concentrations, and the displacement of NADH from both enzymes obeyed biphasic kinetics characteristic of two first-order processes.

However, there are significant quantitative differences between the kinetic parameters derived from these studies. Thus the K_m values obtained by extrapolating the linear Lineweaver-Burk plots obtained at low acetaldehyde concentrations are $0.2\mu\text{M}$ and $0.67\mu\text{M}$ for the mitochondrial and cytoplasmic enzymes respectively. The deviation from linearity in the Lineweaver-Burk plot is less marked for the mitochondrial enzyme, occurring at about $10\mu\text{M}$ -acetaldehyde, compared with $1.3\mu\text{M}$ for the cytoplasmic enzyme.

Since an ordered or partially random mechanism has been reported for all aldehyde dehydrogenases for which detailed steady-state kinetic results are available, it seems unlikely that the mitochondrial enzyme is different. Possible explanations for non-

linear double-reciprocal plots, based on these mechanisms, have been discussed by MacGibbon *et al.* (1977a).

The decay constants obtained from the studies on the rate of dissociation of NADH from the enzymes are the same within experimental error for the faster process, although the decay constant calculated for the slow process for the mitochondrial enzyme is less than half that observed for the cytoplasmic enzyme. Another difference is that the amplitude of the slow process was about 60% of the total fluorescence change for the mitochondrial enzyme, compared with $40(\pm 10)\%$ for the cytoplasmic enzyme (MacGibbon *et al.*, 1977b).

The biphasic semi-logarithmic plots obtained by monitoring the nucleotide fluorescence on dissociation of NADH from the mitochondrial enzyme as a function of time could arise from the presence of different binding sites (either on the same enzyme or on isoenzymes) or from a two-step dissociation of the NADH from the enzyme. MacGibbon *et al.* (1977b) showed that the last of these possibilities applied to the cytoplasmic enzyme. Since the fluorimetric titration of the binding of NADH to the mitochondrial enzyme was fitted assuming a single tight NADH-binding site ($K_{E-NADH}=0.5\mu\text{M}$; Hart & Dickinson, 1977) a two-step dissociation process may again be involved. If this is so, then the catalytic-centre activity of the mitochondrial enzyme should be less than that of the cytoplasmic enzyme, as the maximum rate of the mitochondrial enzyme-catalysed oxidation of aldehydes could not be greater than the slower NADH-dissociation rate (namely 0.09s^{-1}).

The burst amplitude for the mitochondrial enzyme was significantly less than would be expected on the basis of the amplitudes observed in the NADH-dissociation experiments; thus either other non-fluorescent enzyme-containing intermediates must be present in appreciable concentrations, or the fluorescing species being observed in the burst shows an enhancement of NADH fluorescence much less than that observed for the enzyme-NADH complex. Support for the first of these explanations comes from the 3-fold increase in amplitude of the burst that was observed when the propionaldehyde concentration was increased from 0.4 to 20mM . It should be noted that for the cytoplasmic enzyme the amplitude of the burst was almost exactly what would have been expected from the known active-site concentration (MacGibbon *et al.*, 1977c).

From the protein-fluorescence-quenching studies a comparison can be made of the apparent rate constants for NAD^+ binding to the cytoplasmic ($1.8 \times 10^5 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$; MacGibbon *et al.*, 1977c) and mitochondrial ($2 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$) enzymes. However, although the apparent rate constant for binding is about 10 times as great for the cytoplasmic

enzyme, the apparent dissociation rate constants are fairly similar, differing only by a factor of 2.

If an ordered mechanism, with NAD^+ binding first, is assumed for the mitochondrial enzyme (as was shown for the cytoplasmic enzyme by MacGibbon *et al.*, 1977a) the difference in apparent binding rate constants could provide an explanation for the 6-fold difference in the Michaelis constant of NAD^+ for the two enzymes. For the three-intermediate scheme:

where Ald is aldehyde and the Michaelis constant for NAD^+ is given by the expression:

$$K_m^{\text{NAD}} = \frac{k_{+3}k_{+4}}{k_{+1}(k_{+3} + k_{+4})}$$

So, provided that the rate constants for the other steps are comparable for the two enzymes, then the smaller size of the k_{+1} term would give rise to a correspondingly larger value of the Michaelis constant for the mitochondrial enzyme, as is observed experimentally.

The proportion of the total aldehyde dehydrogenase activity in the mitochondria and cytoplasm of sheep liver is similar for both enzymes (Crow *et al.*, 1974). If it is assumed that the rate of dissociation of NADH contributes significantly to the steady-state rate for the mitochondrial enzyme, then the actual concentrations of the enzymes in the two compartments should be comparable. When substrate concentrations are limiting *in vivo*, it is clear from the differences described above that the proportion of the aldehyde that is metabolized in each compartment

will depend not only on the availability of NAD^+ and aldehyde, but also on the magnitude of the $[\text{NAD}^+]/[\text{NADH}]$ ratio, since not only is the rate of binding of NAD^+ to the mitochondrial enzyme ten times slower, but also the dissociation constant for NADH is one-twentieth of that for the cytoplasmic enzyme. Under limiting NAD^+ concentrations therefore, when similar $[\text{NAD}^+]/[\text{NADH}]$ ratios are present in the two compartments, the mitochondrial enzyme will more slowly metabolize available aldehyde because of both the slower rate of NAD^+ binding and the greater portion of the enzyme that will be bound by available NADH.

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