Steady-State and Pre-Steady-State Kinetics of Coenzyme A Linked Aldehyde Dehydrogenase from *Escherichia coli*†

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ABSTRACT: Coenzyme A linked aldehyde dehydrogenase from *Escherichia coli* strain B has been purified to a specific activity of 14 units/mg of protein, and initial rate and substrate analogue inhibition experiments have been performed. On the basis of these steady-state measurements, a bi-uni-uni-uni ping-pong mechanism is proposed in which NAD^+ binds to the free enzyme followed by acetaldehyde. The product NADH is then released before coenzyme A (CoA) can bind, and acetyl-CoA is the final product released. A pre-steady-state time-dependent activation of the enzyme was observed when assays were initiated with NAD^+. This lag phase of the reaction was studied as a function of the NAD^+ concentration and found to be first order. Furthermore, the presence of NAD^+ was demonstrated to be necessary to maintain the enzyme in the active conformation. Evidence that the enzyme contains two distinct NAD^+ binding sites, an activator site and a catalytic site, has been obtained from pre-steady-state experiments with the NAD^+ analogues AMP and 3-pyridinecarboxaldehyde adenine dinucleotide. AMP, a potent competitive inhibitor with respect to NAD^+ under steady-state conditions, did not affect the rate of enzyme activation during pre-steady-state measurements. The analogue 3-pyridinecarboxaldehyde adenine dinucleotide, a potent activator of the aldehyde dehydrogenase, was a poor substrate compared with NAD^+. At concentrations of this analogue that fully activated the enzyme, no alternate substrate inhibition was observed with respect to NAD^+. A model incorporating two binding sites for NAD^+ has been put forward to explain these observations.

Escherichia coli coenzyme A (CoA) linked aldehyde dehydrogenase was first isolated in a crude state by Dawes & Foster (1956) and subsequently has been partially purified by Rudolph et al. (1968), who studied the initial rate kinetics of the enzyme. The aldehyde dehydrogenase is associated with a multienzyme complex of approximately 40 S in size (Schmitt, 1975) and catalyzes the following reaction:

$$CH_3CHO + NAD^+ + CoA \rightleftharpoons \text{acetyl-CoA} + NADH + H^+$$

The enzyme is dependent on the presence of a thiol such as 2-mercaptoethanol or dithiothreitol for its activity.

On the basis of their initial rate studies, Rudolph et al. (1968) have tentatively proposed a bi-uni-uni-uni ping-pong mechanism for the aldehyde dehydrogenase in which the product NADH must be released before CoA can bind to the enzyme. An almost identical mechanism has been proposed for the CoA-linked aldehyde dehydrogenase of *Clostridium kluyveri* in a recent study by Smith & Kaplan (1980) in which the enzyme mechanism was elucidated from the results of initial rate and product inhibition experiments.

The aldehyde dehydrogenase from *E. coli* has been shown to be a hysteretic enzyme in that slow activation occurs in the presence of NAD^+ and thiol (Rudolph et al., 1968). Steady-state rates are obtained only after an incubation period with both a thiol and NAD^+. This slow enzyme response to rapid changes in ligand concentration is not shown by the aldehyde dehydrogenase from *C. kluyveri* (Smith & Kaplan, 1980).

In the present paper, an improved purification procedure is described for the *E. coli* aldehyde dehydrogenase, and the kinetic mechanism is examined further with substrate analogue inhibition studies. A study of the pre-steady-state kinetics provides evidence for two NAD^+ binding sites on the enzyme, a high-affinity activator site and a lower affinity catalytic site.

Experimental Procedures

Materials. Acetaldehyde was the product of Baker Chemical Co. NAD^+, NADH, AMP, and 3-pyridinecarboxaldehyde adenine dinucleotide were obtained from Sigma. Acetyl-CoA, CoA, and benzoyl-CoA were from P-L Biochemicals, Inc. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) and 2-mercaptoethanol were products of Calbiochem. Benzaldehyde was obtained from Fisher Scientific Co. and 2-hydroxyethyl disulfide from Aldrich Chemical Co. DEAE-cellulose was from Whatman and Sephadex G200 from Pharmacia.

NAD^+ was assayed with yeast alcohol dehydrogenase and ethanol (Klingenberg, 1974), and acetaldehyde was assayed with this enzyme and NADH (Bernt & Bergmeyer, 1974). Coenzyme A was estimated with acetyl phosphate and phosphotransacetylase (Michal & Bergmeyer, 1974), and acetyl-CoA was assayed by using malic dehydrogenase and citrate synthase (Decker, 1974).

Enzyme Assays. Coenzyme A linked aldehyde dehydrogenase was assayed during purification in 2-mL assays containing 25 μM CoA, 0.5 mM NAD^+, 50 mM acetaldehyde, 20 mM 2-mercaptoethanol, and 23 mM potassium phosphate buffer, pH 7.0, as described previously (Rudolph et al., 1968). Enzyme activity is reported in micromoles of NADH formed per minute at 28 °C.

NADH oxidase was assayed with 50 μM NADH and 50 mM potassium phosphate buffer, pH 7.0. Phosphotransacetylase activity was assayed with CoA and acetyl phosphate (Bergmeyer et al., 1974) and alcohol dehydrogenase at 50 μM acetaldehyde and 50 μM NADH in 50 mM potassium phosphate buffer, pH 7.0.

Purification of the CoA-Linked Aldehyde Dehydrogenase. CoA-linked aldehyde dehydrogenase was purified by the procedure of Rudolph et al. (1968) with some modification. *E. coli* B strain was grown aerobically at 37 °C for 8 h in 6 L of salt medium (Lieberman, 1956) supplemented with 0.5% glucose and neopeptone. After aerobic growth, the volume

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was made up to 12 L with media, and the bacteria were grown under anaerobic conditions for a further 30 h. The cells were harvested, washed, and broken in the French press as described (Rudolph et al., 1968), and the cell-free extract was made up to 1 L with 10 mM potassium phosphate buffer, pH 8.1, containing 1 mM 2-hydroxyethyl disulfide (2-HED). Streptomycin sulfate (10 g in 100 mL of H_2O) was added to the crude extract, in the ratio 1:100, slowly with stirring. After the suspension stood for 10 min, it was centrifuged at 15000*g* for 15 min and the pellet discarded. The supernatant fluid was brought to 20% saturation with ammonium sulfate by slow addition of a saturated solution at pH 7.0, and the resulting solution was stirred for 15 min and then centrifuged at 15000*g* for 15 min. The pellet was discarded, the supernatant fluid made 50% saturated with ammonium sulfate by the same procedure and centrifuged, and the pellet retained for further purification. Ion-exchange chromatography was performed on a column (3.5 × 40 cm) of DEAE-cellulose equilibrated in 20 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol and 1 mM 2-HED. When the absorbance at 280 nm of the eluate dropped below 0.3, the enzyme was eluted with a 1200-mL gradient of from 20 to 90 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol and 1 mM 2-HED. The fractions containing the highest specific activity were pooled, concentrated to 10 mL by ultrafiltration, and then chromatographed on a column of Sephadex G200 (2.5 × 90 cm) equilibrated in 20 mM potassium phosphate buffer, pH 7.4, containing 10 mM glycerol and 1 mM 2-HED. The highest activity fractions were collected and concentrated by ultrafiltration.

Steady-State Kinetic Studies. Initial rate and substrate analogue inhibition studies were performed at 28 °C in 23 mM potassium phosphate buffer, pH 7.0, containing 20 mM 2-mercaptoethanol. Enzyme was incubated for 20 min with CoA and NAD^+ and initiated with acetaldehyde for studies in the forward reaction; for studies in the reverse reaction, enzyme was incubated with NADH and initiated with acetyl-CoA. NADH formed, or oxidized, was monitored with a Cary Model 118 recording spectrophotometer with the absorbance scale setting at 0.02 full scale.

Initial rate data were analyzed and fit to a model by the least-squares method, assuming equal variance of velocities, by a computer program written in the OMNITAB language (Siano et al., 1975).

Pre-Steady-State Kinetic Studies. Pre-steady-state studies were performed by using preactivated enzyme. Enzyme solution (0.1 mL, 15 units/mL) was added to 0.2 mL of 23 mM potassium phosphate buffer, pH 7.0, containing 0.6 mM NAD^+ and 30 mM 2-mercaptoethanol and incubated for 20 min at 28 °C. The enzyme solution was made up to 0.8 mL with 20 mM potassium phosphate buffer, pH 7.0, containing 10% glycerol and 20 mM 2-mercaptoethanol and then diluted with the same buffer containing 150 μM NAD^+ as required by the experiment. The preactivated enzyme solution was stored at 0 °C during the course of the experiment.

Pre-steady-state kinetic studies were performed at 28 °C in assays (2 mL) containing 23 mM potassium phosphate buffer, pH 7.0.

Results

The results of a typical purification procedure for the aldehyde dehydrogenase are shown in Table I. Grown under anaerobic conditions, *E. coli* gave enzyme in higher yield and greater specific activity than that previously obtained by Rudolph et al. (1968) using aerobically grown bacteria. The specific activity at the final stage of the purification was about 14 units/mg, a value approximately 3-fold greater than that obtainable from aerobically grown *E. coli*. The aldehyde dehydrogenase, in the presence of 2-mercaptoethanol, is a labile enzyme (Rudolph et al., 1968). Substitution of this thiol with 1 mM 2-HED, an oxidizing agent, rendered the enzyme less labile, particularly up to the ion-exchange step in the purification, at which point the enzyme retained about 85% of its activity after 1 month. The stabilizing effect of 2-HED on the aldehyde dehydrogenase was considerably less after the final gel filtration step, however, with approximately 30–40% of the enzyme activity being retained after 1 month.

Table I: Purification of the Aldehyde Dehydrogenase

purification stage	volume (mL)	protein concn (mg/mL)	total units	sp act. (units/mg)
cell-free extract	450	19	550	0.064
streptomycin	1000	6.5	525	0.081
ammonium sulfate[a]	125	23	605	0.21
DEAE-cellulose	250	0.25	285	4.5
Sephadex G200	40	0.29	165	14.1

[a] The increase in the total units at this stage reflects the removal of NADH oxidase from the enzyme solution.

The aldehyde dehydrogenase thus prepared was found to be free of NADH oxidase activity and phosphotransacetylase activity but did contain a contaminating alcohol dehydrogenase activity. The alcohol dehydrogenase activity constituted about 15–20% of the aldehyde dehydrogenase activity when assayed from the acetaldehyde side of the reaction. Attempts to purify the enzyme further by employing various denaturing salts (LiBr, NaI, NaSCN, guanidine hydrochloride, urea, $CaCl_2$) or detergents [sodium deoxycholate, Triton X-100, sodium dodecyl sulfate ($NaDodSO_4$), octyl β-D-glucopyranoside] to break the enzyme complex failed, the enzyme proving to be too labile under these conditions.

Steady-State Kinetics. Of the large number of possible mechanisms for three substrate enzyme systems (Fromm, 1979), most can be eliminated by inspection of the initial rate data. The experimental protocol of Fromm (1967) in which each of the three substrates is varied in turn at many fixed concentrations of the other two substrates (the concentrations of the fixed substrates being held in constant ratio) provides an efficient method for performing initial rate experiments on three-substrate systems. The initial rate experiments for *E. coli* CoA-linked aldehyde dehydrogenase have been performed previously by Rudolph et al. (1968) using this protocol. In the present study, the initial rate experiments have been repeated, yielding qualitatively similar results and a more accurate evaluation of the Michaelis constants.

In the initial rate experiments when either NAD^+ or acetaldehyde is the varied substrate, a double-reciprocal plot of velocity against the varied substrate results in converging patterns of lines. The slope and intercept replots are linear for both substrates. In the experiment where CoA is the varied substrate, however, a double-reciprocal plot of this substrate against velocity yields a parallel set of lines. The intercept replot in this instance is parabolic concave-up. These initial rate data are consistant with a ping-pong type mechanism (Fromm, 1967) in which the binding of CoA is preceded by the release of a product. Of the two mechanistic alternatives, the bi-uni-uni-uni ping-pong and the uni-uni-bi-uni ping-pong, it is difficult to see how the latter mechanism could operate since it predicts the release of a product after the binding of CoA to the free enzyme. As pointed out by Rudolph et al. (1968), the most feasible mechanism on the basis of the initial rate data seems to be of the bi-uni-uni-uni ping-pong type in

Scheme I

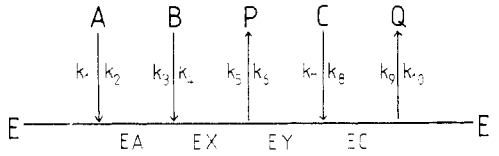


Table II: Michaelis Constants for the Aldehyde Dehydrogenase

substrate	Michaelis constant (mM) ± SD
NAD^+	0.080 ± 0.004
CoA	0.008 ± 0.002
acetaldehyde	10.0 ± 0.600
NADH	0.025 ± 0.007
acetyl-CoA	0.0070 ± 0.0004

which the release of the first product is preceded by binding of both NAD^+ and acetaldehyde (Scheme I). The rate equation for this mechanism, expressed in Dalziel form (Dalziel, 1957), is given in eq 1 in which C represents the substrate CoA and A and B represent the other two substrates:

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{(A)} + \frac{\phi_2}{(B)} + \frac{\phi_3}{(C)} + \frac{\phi_4}{(A)(B)} \quad (1)$$

The kinetic parameters for eq 1 are

$$\phi_0 = \frac{1}{k_5} + \frac{1}{k_9} \qquad \phi_1 = \frac{1}{k_1} \qquad \phi_2 = \frac{k_4 + k_5}{k_3 k_5}$$

$$\phi_3 = \frac{k_8 + k_9}{k_7 k_9} \qquad \phi_4 = \frac{k_2(k_4 + k_5)}{k_1 k_3 k_5}$$

The Michaelis constants could not be determined accurately from the initial rate experiments since the values of ϕ_0 in eq 1 were always small and in some experiments statistically insignificant. To estimate the K_m values more accurately, we carried out experiments in which each of the three substrates was varied at saturating concentrations of the other two. This approach allowed the use of lower enzyme concentrations from which a more accurate value of ϕ_0 and the Michaelis constants could be determined (Table II).

In the reverse reaction of the aldehyde dehydrogenase, double-reciprocal plots of either NADH or acetyl-CoA concentration against velocity resulted in parallel sets of lines, an essential result if any credence is to be given to the proposed bi-uni-uni-uni ping-pong mechanism. Michaelis constants for the reverse reaction substrates are given in Table II.

Substrate analogue inhibition studies were conducted to determine the binding order, if any, of the substrates NAD^+ and acetaldehyde. Benzaldehyde was found to be a good analogue for acetaldehyde and was not a substrate for the aldehyde dehydrogenase. Benzaldehyde was a competitive inhibitor with respect to acetaldehyde and an uncompetitive inhibitor with respect to both NAD^+ and CoA (data not shown). The uncompetitive nature of benzaldehyde inhibition with respect to NAD^+ is indicative of an ordered mechanism for the aldehyde dehydrogenase with the substrate NAD^+ adding first to the enzyme.

A specific inhibitor for NAD^+ could not be found. The various commercially available analogues for NAD^+ (3-acetylpyridine adenine dinucleotide, thionicotinamide adenine dinucleotide, nicotinamide-hypoxanthine adenine dinucleotide, and 3-pyridinealdehyde adenine dinucleotide) were poor inhibitors of the aldehyde dehydrogenase and unsuitable as substrate analogues. AMP and ADP-ribose are good inhibitors of the enzyme, but the inhibition with respect to NAD^+ is competitive only at saturating concentrations of CoA. At nonsaturating levels of CoA, the inhibition is noncompetitive.

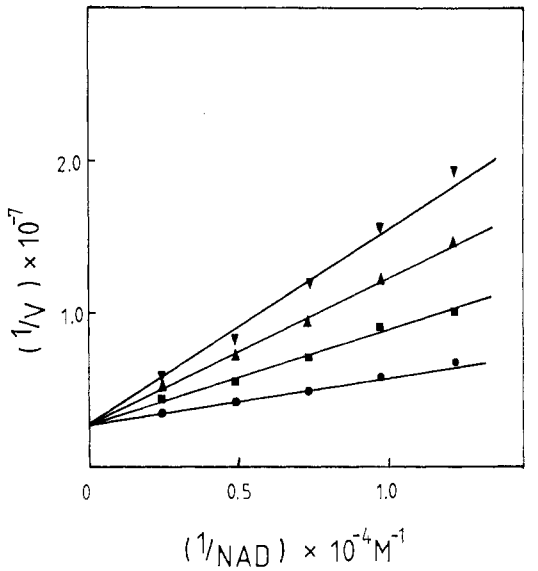


FIGURE 1: Plot of the reciprocal of the initial reaction velocity (V) with respect to the reciprocal of the molar concentration of NAD^+ in the absence (●) and presence of 0.3 (■), 0.6 (▲), and 0.9 mM (▼) AMP. The acetaldehyde concentration was held at 2.8 mM and the CoA concentration held saturating at 40 μM. NAD^+ was varied from 0.09 to 0.45 mM. Other experimental detals are as described under Experimental Procedures.

Similarly, the inhibition of AMP and ADP-ribose is competitive with respect to CoA only at saturating concentrations of NAD^+, becoming noncompetitive at subsaturating NAD^+ levels. It seems clear from these data that AMP and ADP-ribose inhibit the enzyme by competing for both the NAD^+ and CoA binding sites. The inhibition patterns obtained are in accordance with those predicted by the velocity equation (eq 2) for this type of inhibition where $K_i = (E)(I)/(EI)$ and

$$\frac{E_0}{v} = \phi_0 + \frac{\phi_1}{(A)}\left[1 + \frac{(I)}{K_i}\right] + \frac{\phi_2}{(B)} + \frac{\phi_3}{(C)}\left[1 + \frac{(I)}{K_{ii}}\right] + \frac{\phi_4}{(A)(B)}\left[1 + \frac{(I)}{K_i}\right] \quad (2)$$

$K_{ii} = (EY)(I)/(EY{\cdot}I)$. With no specific NAD^+ inhibitor available, AMP was used as a substrate analogue in the presence of saturating concentrations of CoA. Under these conditions, AMP was found to be a competitive inhibitor with respect to NAD^+ (Figure 1) and noncompetitive with respect to acetaldehyde (Figure 2).

Attempts to find a specific analogue for CoA also failed. The aldehyde dehydrogenase was not inhibited by the CoA analogues pantothenate and benzoyl-CoA. AMP was therefore used as an inhibitor in the presence of saturating concentrations of NAD^+. Under these conditions, AMP was competitive with respect to CoA (Figure 3) and uncompetitive with respect to acetaldehyde (Figure 4).

These results of the substrate analogue inhibition experiments are completely in accordance with an ordered bi-uni-uni-uni ping-pong mechanism (Fromm, 1967), with the substrate NAD^+ adding first to the enzyme.

Pre-Steady-State Kinetics. The hysteretic nature of the CoA-linked aldehyde dehydrogenase from *E. coli* has been demonstrated previously by Rudolph et al. (1968), who showed that incubation with both NAD^+ and 2-mercaptoethanol was necessary to eliminate the lag phase in the reaction before the attainment of a steady-state rate. The function of the thiol is unknown, but it seems likely that the reduction of a group on the enzyme, possibly a disulfide, is involved. That incu-

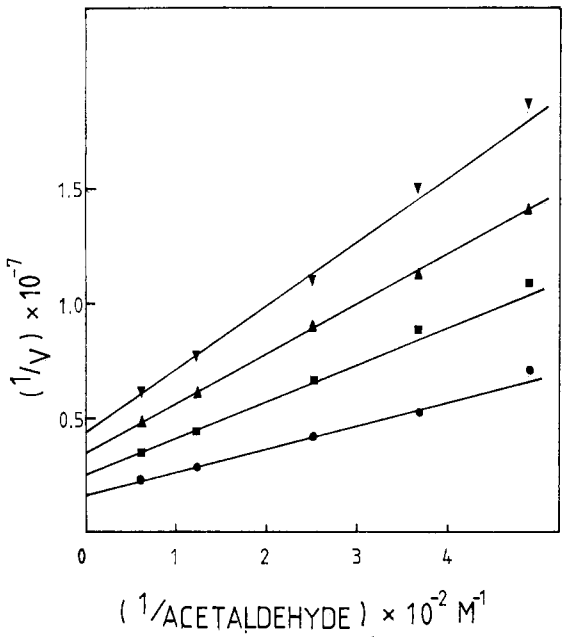


FIGURE 2: Plot of the reciprocal of the initial reaction velocity (V) with respect to the reciprocal of the molar concentration of acetaldehyde in the absence (●) and presence of 0.22 (■), 0.44 (▲), and 0.66 mM (▼) AMP. The NAD^+ concentration was held at 0.14 mM and the CoA concentration held saturating at 40 μM. Acetaldehyde was varied from 2.05 to 16.4 mM. Other experimental details are as described under Experimental Procedures.

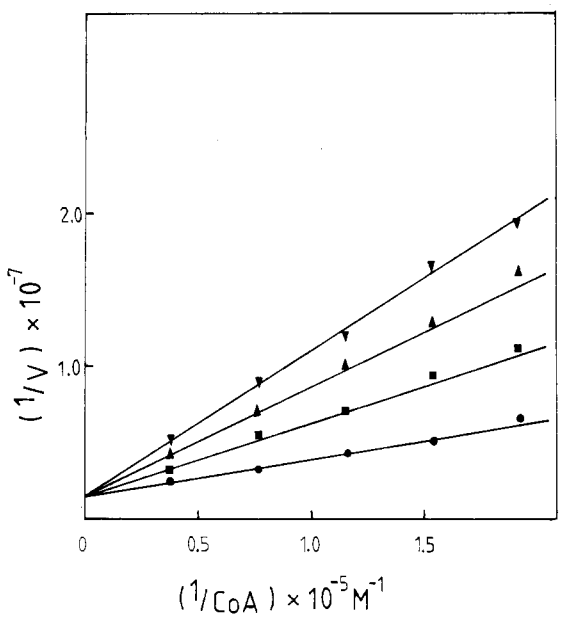


FIGURE 3: Plot of the reciprocal of the initial reaction velocity (V) with respect to the reciprocal of the molar concentration of CoA in the absence (●) and presence of 0.87 (■), 1.73 (▲), and 2.6 mM (▼) AMP. The acetaldehyde concentration was 3.1 mM, and the NAD^+ concentration was held saturating at 1.9 mM. CoA was varied from 5.2 to 26 μM. Other experimental details are as described under Experimental Procedures.

bation with 2-mercaptoethanol alone is insufficient to eliminate the lag phase imposes a role on NAD^+ in the activation of the enzyme. One possible explanation for these data is that NAD^+ induces a conformational change in the enzyme, exposing a reducible group previously inaccessible to the thiol. In an attempt to clarify the role of NAD^+ in the enzyme activation, further studies of the pre-steady-state kinetics of the aldehyde dehydrogenase have been undertaken.

If the role of the thiol is in the reduction of a group on the enzyme, then this could well be the limiting factor in the

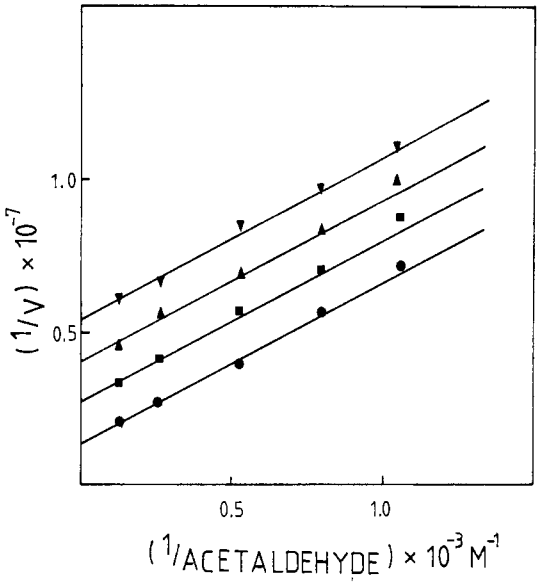
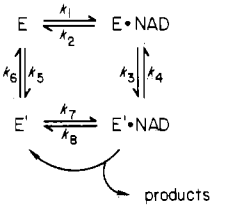


FIGURE 4: Plot of the reciprocal of the initial reaction velocity (V) with respect to the reciprocal of the molar concentration of acetaldehyde in the absence (●) and presence of 0.8 (■), 1.6 (▲), and 2.4 mM (▼) AMP. The concentration of CoA was held at 10 μM and the NAD^+ concentration held saturating at 1.8 mM. Acetaldehyde was varied from 0.94 to 7.5 mM. Other experimental details are as described under Experimental Procedures.

duration of the lag phase. The activation of the enzyme by NAD^+ was therefore studied by using enzyme stock solution that had been preactivated with NAD^+ and 2-mercaptoethanol as outlined under Experimental Procedures. The enzyme was maintained in its active conformation by the presence of 150 μM NAD^+ and 20 mM 2-mercaptoethanol in the stock solution.

An aliquot of the preactivated enzyme was incubated in an assay containing CoA (40 μM), acetaldehyde (40 mM), and 2-mercaptoethanol (20 mM) in 23 mM potassium phosphate buffer, pH 7.0. The enzyme aliquot was made small enough so that the final concentration of NAD^+ in the assay was less than 1 μM. After a 10-min incubation at 28 °C, the assay was initiated by the addition of NAD^+ to a final concentration of 40 μM. A lag was observed in the enzyme reaction before the attainment of a steady-state rate (Figure 5). The duration of the lag phase remained constant when the incubation period with CoA and acetaldehyde was increased to 15 and 20 min. If, however, the preactivated enzyme was used to initiate an identical assay already containing NAD^+, the lag phase was eliminated. Linear velocity traces were also obtained when enzyme was used to initiate assays containing higher concentrations of NAD^+, up to 200 μM, indicating that the enzyme in the stock solution was fully activated. These results show that the presence of NAD^+ is necessary to maintain the enzyme in an active conformation and that the changes invoked by this ligand are slow compared with other steps in the reaction.

By treatment of the CoA-linked aldehyde dehydrogenase as a single substrate enzyme (i.e., at saturating concentrations of the substrates CoA and acetaldehyde), the simplest model capable of explaining the enzyme activation is mechanism I:



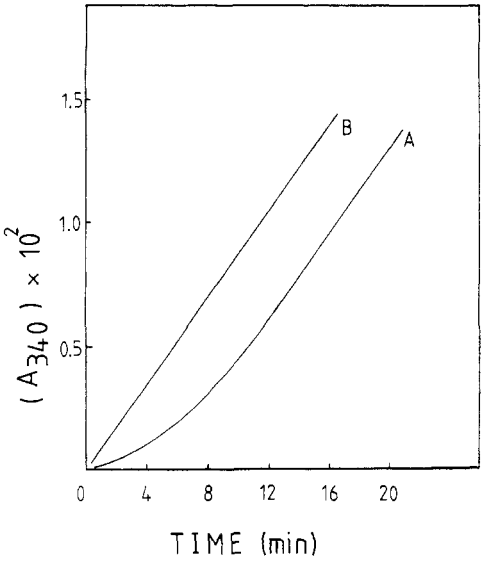


FIGURE 5: Plot of absorbance (A_{340}) as a function of time for the aldehyde dehydrogenase. Assay mixtures contained preactivated enzyme (see Experimental Procedures), 40 μM NAD^+, 40 μM CoA, 40 mM acetaldehyde, and 23 mM potassium phosphate buffer, pH 7.0. In curve A, the enzyme was incubated for 10 min in assays from which NAD^+ had been omitted. The reaction was initiated with NAD^+. In curve B, the preactivated enzyme was added to the complete assay to initiate the reaction.

where E and E′ are the inactive and active forms of the enzyme, respectively, and are interconvertible via the slow steps k_3, k_4 and k_5, k_6. This model is similar to those put forward by Frieden (1970) and Ainslie et al. (1972). Since it has been shown that the enzyme is maintained in a fully activated form by 150 μM NAD^+, it must be assumed that the enzyme is almost entirely in the form E′·NAD, which necessarily implies that NAD^+ is saturating at this concentration and that $k_3 \gg k_4$. In addition, since the enzyme seems to be inactive in the absence of NAD^+, one must also predict that $k_6 \gg k_5$.

If it is assumed that during the course of the reaction the substrate concentration does not significantly change and that the buildup of product does not affect the enzyme, then the time dependence of product formation can be shown to be

$$P = v_\infty + (v_0 - v_\infty)\frac{1}{K_{app}}(1 - e^{-K_{app}t}) \qquad (3)$$

where v_∞ is the velocity at time $t = \infty$, v_0 is the velocity at $t = 0$, and K_{app} is the apparent rate constant for the approach to a steady-state rate. Under the conditions imposed by mechanism I, K_{app} is dependent on the substrate concentration and has been shown (Frieden, 1970) to assume the form

$$K_{app} = \frac{k_3k_1(A) + k_5k_2}{k_2 + k_1(A)} + \frac{k_4k_7(A) + k_6k_8}{k_8 + k_7(A)} \qquad (4)$$

Values of K_{app} may be calculated from velocity traces such as Figure 5 by the graphical procedure of Hatfield et al. (1970). A plot of log $[(\Delta OD_\infty - \Delta OD_t)/\Delta OD_\infty]$ vs. time was found to be linear (Figure 6), as is to be expected if the activation process is first order with respect to NAD^+. A slight negative deviation from linearity was frequently observed at higher values of t.

Figure 7 shows the dependence of K_{app} on the concentration of NAD^+. The value for K_{app} increases proportionally with respect to NAD^+ concentration between 0 and 70 μM; at higher concentrations, the rate of activation levels off. The activation rate of the enzyme became rapid above 100 μM, and K_{app} values at NAD^+ concentrations above 130 μM were difficult to measure accurately. Plots of K_{app} vs. NAD^+

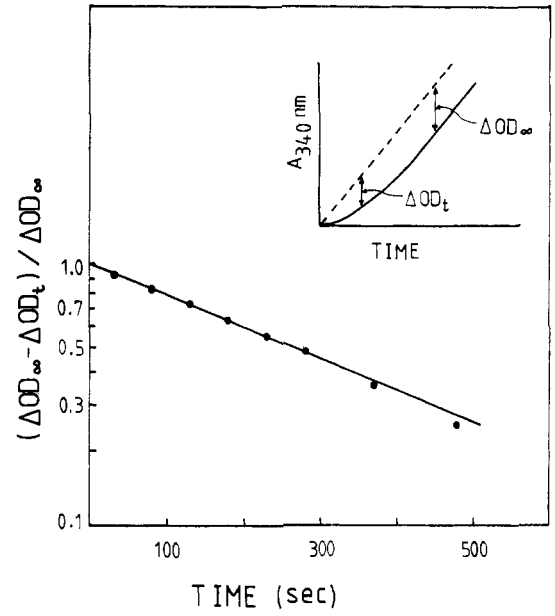


FIGURE 6: Graphical determination of K_{app} from a plot of log $[(\Delta OD_\infty - \Delta OD_t)/\Delta OD_\infty]$ with respect to time (t) (Hatfield et al., 1970). The best straight line was drawn through the points, and a value of K_{app} is given by $K_{app} = -2.3 \times$ the slope of this line. The inset details the measurement of values of ΔOD_∞ and ΔOD_t. The dashed line was drawn parallel to the steady-state portion of the velocity trace through the origin. The vertical distance ΔOD_t was measured at numerous values of t.

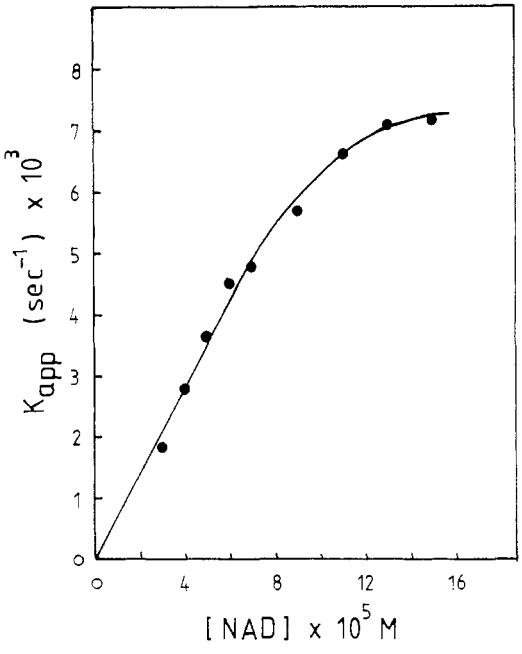


FIGURE 7: Plot of the rate constant for the approach to steady state, K_{app}, with respect to the molar concentration of NAD^+. Assay conditions were as for curve A in Figure 5. The NAD^+ concentration was varied from 30 to 150 μM.

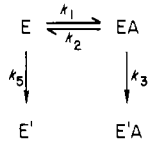
concentration gave values of zero at zero substrate concentrations, a result that would seem to be at variance with eq 4, which predicts an intercept value of $k_5 + k_6$. In the initial stages of the enzyme activation, however, the concentrations of E′ and E′A will be small relative to the concentrations of E and EA, and in addition, the concentrations of E′ and E′A will be depleted by the formation of other intermediates of the reaction. Plots of log $[(\Delta OD_\infty - \Delta OD_t)/\Delta OD_\infty]$ vs. t, such as Figure 6, should only be linear at small values of t, and indeed, deviations from linearity were frequently observed at high t values, which may reflect the buildup of E′ and E′A during

Table III: Effect of AMP on the Activation Rate of the Aldehyde Dehydrogenase. Final Steady-State Rates of the AMP-Inhibited Enzyme (See Figure 8)

NAD^+ concn (μM)	final steady-state velocities (ΔA_{340}/min × 10^3) at AMP concn (mM)			
	0	0.2	0.4	0.6
40	1.28	0.88	0.71	0.48
55	1.60	0.92	0.80	0.68
70	2.08	1.24	0.93	0.70

the course of the enzyme activation.

With the assumption that the rate constants k_4, k_6, k_7, and k_8 are near zero, mechanism I simplifies to mechanism II:



and K_{app} becomes

$$K_{app} = \frac{k_3k_1(A) + k_5k_2}{k_1(A) + k_2} \quad (5)$$

If it is further assumed that k_5 also approaches zero, then eq 5 simplifies to

$$K_{app} = \frac{k_3k_1(A)}{k_1(A) + k_2} \quad (6)$$

The data in Figure 7 are in compliance with eq 6, which predicts K_{app} to range from a value of zero when the NAD^+ concentration is zero to a maximum value of k_3 when NAD^+ concentration becomes saturating. Values of k_3 were found to be somewhat variable, and three determinations of the transient rate constant gave values of 6.7×10^{-3}, 7.2×10^{-3}, and 7.6×10^{-3} s^{-1} from which an average value of the half-time ($t_{1/2}$) for the transient (given by $t_{1/2} = 0.693/K_{app}$) was determined to be 97 s. This value of $t_{1/2}$ represents the maximum rate of enzyme activation.

Figure 7 shows that the enzyme becomes saturated with NAD^+ as the concentration of this ligand approaches 150 μM. Data from initial rate steady-state experiments, however, gave a K_m value for NAD^+ of approximately 80 μM and demonstrated that saturation of the catalytic site does not occur below 0.5 mM. The data would seem to suggest that the NAD^+-activation site and the site of catalysis are two distinct sites on the enzyme.

AMP Inhibition. AMP has been demonstrated to be an effective competitive inhibitor of NAD^+ under steady-state conditions; therefore, for further investigation of the possibility of two-NAD^+ binding sites on the CoA-linked aldehyde dehydrogenase, the effect of AMP on the rate of enzyme activation was examined.

Enzyme in the oxidized form could not be activated by incubation with AMP (1 mM) and 20 mM 2-mercaptoethanol for 20 min, and the inhibitor was found to be equally ineffective at maintaining the active state of NAD^+ preactivated enzyme under similar incubation conditions. Values of K_{app} were determined in the presence of 0, 0.2, 0.4, and 0.6 mM AMP at various concentrations of NAD^+. Figure 8 shows the results of such an experiment. The value of K_{app} was found to be independent of the concentration of AMP; values of the transient rate constant were unaffected by the inhibitor even when the final steady-state rate was up to 70% inhibited (Table III).

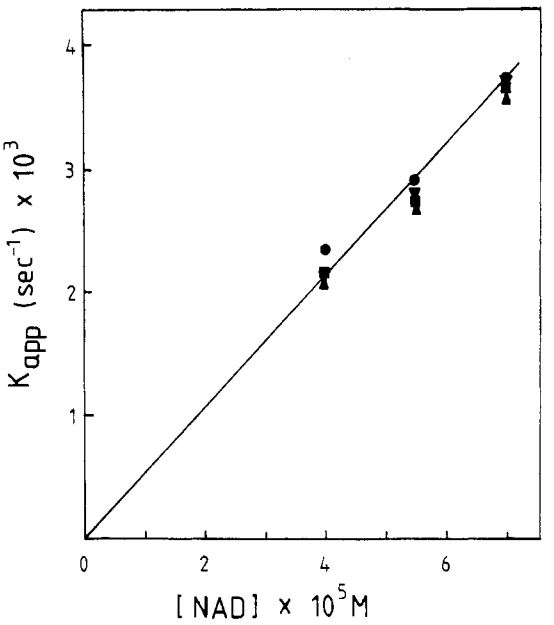
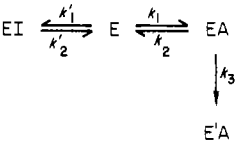


FIGURE 8: Plot of the rate constant for the approach to steady state, K_{app}, with respect to the molar concentration of NAD^+ in the absence (●) and presence of 0.2 (▲), 0.4 (▼), and 0.6 mM (■) AMP. Other experiment details were as for curve A in Figure 5. The NAD^+ concentrations were 40, 55, and 70 μM.

Mechanism III below shows the result of an inhibitor (I) competing for the activator site:



When this occurs, the expression for K_{app} becomes

$$K_{app} = \frac{k_3k'_2k_1(A)}{k_2k'_1(I) + k_2k'_2 + k'_2k_1(A)} \quad (7)$$

Equation 7 indicates that K_{app} should decrease with increasing concentrations of I. The invariance of K_{app} with increasing AMP concentration is inconsistent with this prediction and demonstrates the inability of AMP to bind to the activator site of the aldehyde dehydrogenase. The data in Table III clearly show inhibition of the steady-state rate by AMP, and, since CoA was held at saturating levels during the experiment, this inhibition must be attributed to AMP acting as a competitive inhibitor for NAD^+ at the catalytic site. It is difficult to rationalize these data in terms of a single NAD^+-binding site, and the results of the AMP inhibition study suggest two such sites, a catalytic site to which AMP can bind and an activator site that is insensitive to the inhibitor.

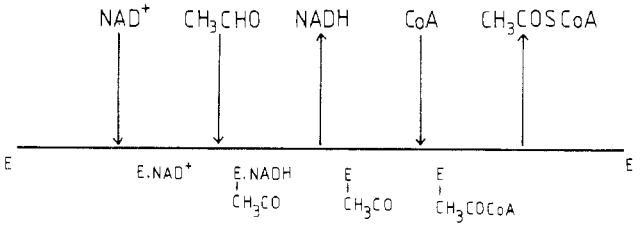
Activation with 3-Pyridinecarboxaldehyde Adenine Dinucleotide. The NAD^+ analogue 3-pyridinecarboxaldehyde adenine dinucleotide (3-PAD) was found to be an efficient activator of the CoA-linked aldehyde dehydrogenase. Enzyme, preactivated with NAD^+, was incubated in assays (under the conditions described in Figure 5) containing 30 μM 3-PAD. After a 10-min incubation, the reaction was initiated with NAD^+ (80 μM). No lag in the enzyme rate was observed, indicating the enzyme to be fully active in the incubation mixture. A similar experiment was performed with NAD^+ in which enzyme was incubated in assays containing 30 μM NAD^+. The amount of substrate used in the reaction during this incubation period was small enough to be ignored. After a 10-min incubation, the reaction was initiated with a further addition of NAD^+ to a final concentration of 80 μM. A lag

Table IV: Effect of 3-Pyridinecarboxaldehyde Adenine Dinucleotide on the Activation Rate of Aldehyde Dehydrogenase

ligand	ligand concn in incubation mixture (μM)[a]	K_{app} ($s^{-1} \times 10^3$)	final steady-state rate at 80 μM NAD$^+$ (ΔA_{340}/min $\times 10^3$)
NAD$^+$	20	5.3	1.98
	30	5.6	2.0
3-PAD	20	very slight lag	2.1
	30	lag absent	2.2

[a] Incubation conditions were as described under Figure 5, curve A.

Scheme II



in the enzyme rate was observed (Table IV).

The preceding observations would seem to indicate that 3-PAD is able to bind the activator site of the aldehyde dehydrogenase in an analogous manner to NAD$^+$ in mechanism II to form an active E'I complex. In addition, 3-PAD seems to bind the enzyme more strongly than NAD$^+$, the concentration of 3-PAD required to maintain the enzyme in its active conformation being approximately one-fifth of that for NAD$^+$.

The analogue 3-PAD was found to be a poor substrate for the enzyme, with approximately 10% the activity of NAD$^+$. If the catalytic and activator sites were assumed to be a single site, then one would predict 3-PAD to be a good alternative substrate inhibitor with respect to NAD$^+$ for the steady-state reaction at a concentration of 30 μM since the activation kinetics suggest that the NAD$^+$ site is saturated at this concentration. Table IV shows the steady-state rate to be unaffected by 3-PAD, lending further evidence toward the proposal of two NAD$^+$ sites on the enzyme. On the basis of these data, the NAD$^+$ analogue 3-PAD seems to bind quite strongly to the activator site and relatively weakly to the catalytic site.

Discussion

In this study, CoA-linked aldehyde dehydrogenase isolated from anaerobically grown *E. coli* B strain has been purified to a specific activity of 14 units/mg. The enzyme in this state is a component of a multienzyme complex that up to the present has eluded further purification. Attempts to purify the aldehyde dehydrogenase by employing denaturing salts and detergents have failed because of the rather labile nature of the enzyme.

Initial rate and substrate analogue inhibition experiments suggest that enzyme catalysis proceeds via a bi-uni-uni-uni ping-pong mechanism. The data suggest that NAD$^+$ binds to the free enzyme followed by acetaldehyde to form what may be a covalent enzyme intermediate. The product NADH is then released before CoA can bind to the enzyme (Scheme II). The nature of any possible covalent intermediate is unknown, but in view of the thiol requirement, it is tempting to speculate that an enzyme–*S*-acetyl intermediate may be involved. Intermediates of this nature have been demonstrated in several enzymes including liver aldehyde dehydrogenase (Feldman & Weiner, 1972) and glyceraldehyde-3-phosphate

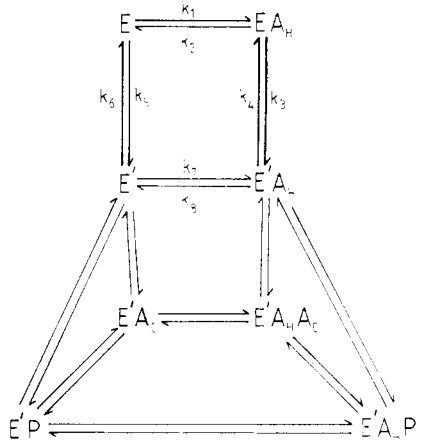


FIGURE 9: Model for interconversion of the active and inactive forms of the CoA-linked aldehyde dehydrogenase from *E. coli* in which E and E' are the inactive and active forms of the enzyme, respectively, and k_3, k_4 and k_5, k_6 the slow steps in the reaction. A_H represents the ligand NAD$^+$ bound at the activator site, and A_C represents NAD$^+$ bound at the catalytic site. For simplicity, the enzyme is treated as a single-substrate system, assuming the substrates CoA and acetaldehyde are at saturating concentrations.

dehydrogenase (Krimsky & Racker, 1955). These possibilities for the mechanism and nature of the intermediate for the CoA-linked aldehyde dehydrogenase have been suggested previously by Rudolph et al. (1968), and although the present substrate analogue data lend further credence to such proposals, these suggestions must remain tentative until the extenence of a covalent intermediate has been demonstrated experimentally.

An almost identical ping-pong mechanism has been proposed recently for a CoA-linked aldehyde dehydrogenase from *C. kluyveri* (Smith & Kaplan, 1980). There are, however, several differences between these enzymes in that the *C. kluyveri* enzyme shows marked substrate inhibition with CoA and does not show any hysteretic properties.

The study of the pre-steady-state kinetics of the aldehyde dehydrogenase presented in this paper demonstrates a direct role for NAD$^+$ in the activation of the enzyme. This ligand was shown to induce a slow change in the enzyme state, presumably a conformation change, from an inactive to an active form. The activation process was found to be first order with respect to NAD$^+$, and furthermore, it was shown that the presence of this substrate is necessary to maintain the enzyme in the active conformation. Such substrate-induced slow enzyme activation has been demonstrated in several enzymes such as threonine deaminase (Hatfield et al., 1970) and glutamine-PP–ribose-P amidotransferase (Rowe et al., 1970).

The apparent rate constant for the approach to a steady-state rate was found to increase linearly with increasing NAD$^+$ concentration up to approximately 70 μM. Above 150 μM NAD$^+$, however, the value of K_{app} reached a maximum as the activator site became saturated with the substrate. In view of this discrepancy between the concentration of NAD$^+$ required to saturate the enzyme as an activator and that required to saturate the enzyme as a substrate (>0.5 mM), two NAD$^+$ binding sites have been postulated on the enzyme. A possible mechanism for the activation of the CoA-linked aldehyde dehydrogenase (simplified to a single-substrate enzyme) is depicted in Figure 9. Above 150 μM NAD$^+$, the NAD$^+$-activator site will be saturated, and the E'A_H forms of the enzyme will dominate the reaction; furthermore, since the enzyme can be fully activated by incubation with 150 μM NAD$^+$ and thiol, it is necessary to predict that, in Figure 9, $k_3 \gg k_4$. If this were not the case then, upon completion of

the reaction mixture, a further lag would be observed as the enzyme forms $E'A_HP$ accumulated, resulting in a perturbation of the $EA \rightleftharpoons E'A$ equilibrium. By analogous reasoning for the slow step involving the free enzyme forms, E and E', it may be concluded that $k_6 \gg k_5$ since the enzyme is inactive in the absence of NAD^+.

If, in Figure 9, the concentration of NAD^+ falls below 100 μM and becomes subsaturating for the NAD^+-activator site, then the E and E' forms of the enzyme will become significant. Under these conditions, a proportion of the total enzyme will return to the inactive form E, the ratio of the active to the inactive forms being determined by the concentration of NAD^+. The mechanism in Figure 9 assumes that the $E'A_C$ and E'P enzyme forms are maintained in the active conformation by the various intermediate substrates and products of the reaction. There is, however, no evidence in the present study to suggest that this is the case, and it is quite possible that these enzyme intermediates are constantly reverting to the inactive form E during the course of the reaction. Under these conditions, the rate of formation of E from the E' forms will be increased, and as a consequence, the contribution of the E' intermediates to catalysis will be less than that predicted by the mechanism in Figure 9.

The NAD^+ analogues AMP and 3-pyridinecarboxaldehyde adenine dinucleotide were shown to have contrasting effects on the transition from the inactive to the active enzyme form. AMP was shown to be a good inhibitor of the steady-state enzyme rate but had no observable effect on the rate of attainment of this steady state. Equation 7 shows that when an inhibitor competes with NAD^+ for the activator site on the enzyme, then at a given NAD^+ concentration K_{app} is a function of 1/(I). The absence of any effect of AMP concentration on the value of K_{app} would preclude the binding of the inhibitor to the enzyme activator site. In terms of the mechanism in Figure 9, AMP seems to compete effectively with A_C for the catalytic site but very poorly with A_H for the activator site.

In contrast to AMP, 3-PAD was found to be an effective activator of the enzyme; indeed, the concentration of the analogue required to maintain the enzyme in a completely active form was on the order of 5-fold less than that for NAD^+. In terms of Figure 9, 3-PAD seems to mimic the effect of A_H at the activator site. The analogue, however, was a poor substrate for the enzyme, seemingly because of a low affinity for the catalytic site since the analogue was not an alternate substrate inhibitor with respect to NAD^+.

It is difficult to rationalize these data in terms of a single site for NAD^+ on the enzyme. In the case for AMP, it would have to be proposed that NAD^+ could still function in its role as an activator while AMP occupied the binding site, a rather unlikely proposal. An analogous assumption with even less credence would have to be made for the analogue 3-PAD. Inasmuch as the aldehyde dehydrogenase is a component of a multienzyme complex, there exists the possibility that the NAD^+-activator site is not situated on the aldehyde dehydrogenase but on an adjacent enzyme and that conformational changes are mediated via protein–protein interactions. Interprotein interactions have indeed been shown to be essential for the CoA/NAD-dependent oxidative carboxylation of pyruvate by the pyruvate dehydrogenase complex from bovine kidney (Kresze & Steber, 1979). With regard to the aldehyde dehydrogenase, however, until the enzyme has been successfully purified, such possibilities must remain conjecture.

References

Ainslie, G. R., Jr., Shill, J. P., & Neet, K. E. (1972) *J. Biol. Chem. 247*, 7088–7096.

Bergmeyer, H. U., Gawehn, K., & Grassl, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd English ed., Vol. 1, pp 507–508, Academic Press, New York.

Bernt, E., & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd English ed., Vol. 3, pp 1506–1509, Academic Press, New York.

Dalziel, K. (1957) *Acta Chem. Scand. 11*, 1706–1723.

Dawes, E. A., & Foster, S. M. (1956) *Biochim. Biophys. Acta 22*, 253–265.

Decker, K. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd English ed., Vol. 4, pp 1988–1993, Academic Press, New York.

Feldman, R. I., & Weiner, H. (1972) *J. Biol. Chem. 247*, 267–272.

Frieden, C. (1970) *J. Biol. Chem. 245*, 5788–5799.

Fromm, H. J. (1967) *Biochim. Biophys. Acta 139*, 221–230.

Fromm, H. J. (1979) *Methods Enzymol. 63*, 42–53.

Hatfield, G. W., Ray, W. J., Jr., & Umbarger, H. E. (1970) *J. Biol. Chem. 245*, 1748–1754.

Klingenberg, M. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd English ed., Vol. 4, pp 2045–2059, Academic Press, New York.

Kresze, G.-B., & Steber, L. (1979) *Eur. J. Biochem. 95*, 569–578.

Krimsky, I., & Racker, E. (1955) *Science (Washington, D.C.) 122*, 319–321.

Lieberman, I. (1956) *J. Biol. Chem. 223*, 327–339.

Michal, G., & Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) 2nd English ed., Vol. 4, pp 1967–1981, Academic Press, New York.

Rowe, P. B., Coleman, M. D., & Wyngaarden, J. B. (1970) *Biochemistry 9*, 1498–1505.

Rudolph, F. B., Purich, D. L., & Fromm, H. J. (1968) *J. Biol. Chem. 243*, 5539–5545.

Schmitt, B. (1975) *Biochimie 57*, 1001–1004.

Siano, D. B., Zyskind, J. W., & Fromm, H. J. (1975) *Arch. Biochem. Biophys. 170*, 587–600.

Smith, L. T., & Kaplan, N. O. (1980) *Arch. Biochem. Biophys. 203*, 663–675.