Coenzyme A-linked Aldehyde Dehydrogenase from Escherichia coli

I. PARTIAL PURIFICATION, PROPERTIES, AND KINETIC STUDIES OF THE ENZYME*

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SUMMARY

Coenzyme A-linked aldehyde dehydrogenase from Escherichia coli strain B was purified 170-fold over cell-free extracts, and certain of its properties were investigated. The enzyme is essentially inactive in the absence of sulfhydryl compounds such as dithiothreitol and β-mercaptoethanol. Addition of a thiol reagent after incubation of the enzyme with substrates results in very slow reactivation. Incubation of aldehyde dehydrogenase with DPN plus mercaptan before addition of other substrates is required in order to obtain normal initial velocities.

The kinetics of the system was investigated from both sides of the reaction. Initial velocity experiments suggest the mechanism is “ping-pong.” These studies, although still preliminary, imply that acetyl-CoA adds to the dehydrogenase before addition of DPNH. With regard to the other side of the reaction, it is thought that DPN and acetaldehyde interact with the enzyme prior to the addition of CoA. The last step in the reaction sequence may be a transacylation between the enzyme and CoA to form acetyl-CoA.

Additional properties of the enzyme, such as optimum pH, substrate specificity, and reaction stoichiometry, were also studied.

EXPERIMENTAL PROCEDURE

Materials and Methods—Substrates for CoA-linked aldehyde dehydrogenase were obtained commercially. Reagent grade, redistilled acetaldehyde and DPN were obtained through J. T. Baker and Sigma, respectively. CoA and acetyl-CoA were products of Calbiochem. DPN was purified by the method of Dalziel (9) as modified by Zeve and Fromm (4). Purified DPNH was prepared from commercial (Sigma) DPNH (10). Acetaldehyde and DPN were assayed with yeast alcohol dehydrogenase and DPNH (11) and ethanol (12), respectively. Acetyl-CoA was purified by chromatography according to the procedure of Webster (13) and assayed with malic dehydrogenase and citrate-condensing enzyme (14). CoA was analyzed with excess DPN, acetaldehyde, and CoA-linked aldehyde dehydrogenase. The concentration of CoA was equivalent to the

In a recent report from this laboratory, it was suggested that competitive inhibitors and weak alternative substrates might be used advantageously in studying the mechanism of action of three-substrate enzyme systems (1). It had previously been shown how such compounds might be utilized to make a choice of mechanism among various possibilities for two substrate systems (2-4). In order to test the rate equations alluded to from purely theoretical considerations, it was desirable to choose a three-substrate system in which initial rate measurements could be made conveniently. The enzyme system finally chosen was coenzyme A linked aldehyde dehydrogenase (EC 1.2.1.10) from Escherichia coli.

In 1953, Burton and Stadtman (5) reported the purification and properties of aldehyde dehydrogenase from Clostridium kluyveri; however, poor yields of the enzyme were subsequently obtained from this source (6). Pinchot and Racker (7) suggested that the enzyme could be found in cell-free extracts of E. coli grown on an inorganic salt medium supplemented with glucose and Neopeptone (Difco). Dawes and Foster (8) prepared the CoA-linked enzyme from an ammonium sulfate-precipitated fraction of bacterial extracts and demonstrated the reversibility of the reaction.

This paper describes the partial purification of CoA-linked aldehyde dehydrogenase from E. coli B and certain of its properties. The reaction catalyzed by the enzyme is

\[ \text{CoA} + \text{DPN}^+ + \text{acetaldehyde} \rightleftharpoons \text{acetyl-CoA} + \text{DPNH} + \text{H}^+ \]

It will be shown that the dehydrogenase is a sulfhydryl enzyme and has a “ping-pong” mechanism, as suggested from initial rate studies. In many respects, aldehyde dehydrogenase from E. coli is quite similar to the enzyme from C. kluyveri (5).
The reverse reaction. The Cary model 15 spectrophotometer for studying the forward reaction, and 1-cm cells were used for the enzyme in an identical manner.

Lent concentrations based on thiol content, were found to affect prepared from once recrystallized salt and adjusted to pH 7.0 or oxidation. Incubation of enzyme and substrates was carried out at 28° in 2.0-ml reaction mixtures containing 0.5 mM DPN, 50 mM acetaldehyde, 23 mM CoA, 10 mM β-mercaptoethanol,1 and 50 mM potassium phosphate buffer, pH 7.0. The enzyme was incubated for 15 min with buffer, DPN, CoA, and β-mercaptoethanol, and the reaction was initiated with acetaldehyde (see below). Initial rates were determined in a Cary model 15 spectrophotometer (0 to 0.1 slide wire) in cells of 1-cm light path. Activity is reported as micromoles of DPNH formed (0 to 0.1 slide wire) was used to assay either DPNH formation (0 to 0.1 slide wire) in cells of 1-cm light path. Activity is reported as micromoles of DPNH formed (0 to 0.1 slide wire) was used to assay either DPNH formation per min. Protein was measured by the procedure of Lowry et al. (15).

Initial Rate Experiments—In the kinetic studies to be reported, initial rate measurements were made at 28° in 2.0-ml reaction mixtures containing 0.5 mM DPN, 50 mM acetaldehyde, 23 mM CoA, 10 mM β-mercaptoethanol, and 50 mM potassium phosphate buffer, pH 7.0. The enzyme was incubated for 15 min with buffer, DPN, CoA, and β-mercaptoethanol, and the reaction was initiated with acetaldehyde (see below). Initial rates were determined in a Cary model 15 spectrophotometer (0 to 0.1 slide wire) in cells of 1-cm light path. Activity is reported as micromoles of DPNH formed per min. Protein was measured by the procedure of Lowry et al. (15).

RESULTS

Purification of Aldehyde Dehydrogenase

All operations were carried out at 2° unless otherwise specified. Buffers were made up at room temperature and then cooled to 2°. Ammonium sulfate, saturated at room temperature, was prepared from once recrystallized salt and adjusted to pH 7.0.

1 β-Mercaptoethanol and dithiothreitol, when present in equivalent concentrations based on thiol content, were found to affect the enzyme in an identical manner.

with NH₂OH. All reagents were prepared with distilled water that had been passed through Amberlite MB-3 resin.

Growth of Bacteria and Preparation of Cell-free Extract—E. coli R were grown on an inorganic salt medium (16) supplemented with 0.4% glucose and 0.1% Neopeptone. The bacteria, which were grown on a nutrient agar medium, were transferred to a 500-ml starter culture and shaken vigorously for 8 hours at 37°. The starter culture was finally transferred to a 14-liter Microferm laboratory fermentor containing 8 liters of growth medium. The bacteria were grown for 18 to 20 hours at 37° under vigorous aeration. The E. coli cells were harvested by centrifugation and washed twice with 10 mM potassium phosphate buffer, pH 8.1. Cell-free extracts were prepared by passage of the bacteria, suspended in the same phosphate buffer, through a cold French press under a pressure of 20,000 p.s.i. This procedure was repeated, and the cell-free extract was diluted with buffer and permitted to stand overnight at 2°, after which it was centrifuged at 15,000 X g for 15 min.

Treatment with Streptomycin Sulfate—Streptomycin sulfate (10 g/100 ml of H₂O) was added to the cell-free extract in a 1:10 ratio slowly with stirring. After standing for 10 min, the suspension was centrifuged at 15,000 X g for 15 min, the precipitate was discarded, and the supernatant fluid was saved for further purification.

Ammonium Sulfate Fractionation—The solution obtained from the preceding step was adjusted to pH 7.0 with 0.2 M Tris-Cl, pH 8.1, after which the concentration of protein was determined by the biuret method with serum albumin as a standard. The solution was then diluted with ice-cold water to give a protein concentration of 12 mg per ml. To 700 ml of this solution were added 467 ml of saturated (NH₄)₂SO₄, pH 7.0, slowly with constant stirring. Stirring of the suspension was continued for 15 min after addition of the salt. The turbid suspension was then centrifuged for 15 min at 15,000 X g. All of the supernatant solution was collected, and the salt fractionation was repeated by adding 178 ml of saturated (NH₄)₂SO₄, pH 7.0. The protein precipitate from this step was then dissolved in 40 mM potassium phosphate buffer, pH 7.4, and dialyzed in a rocking dialyzer against 4 liters of this same buffer.

Diethylyaminomethyl Cellulose Chromatography—CoA-linked aldehyde dehydrogenase was chromatographed on a diethylaminomethyl cellulose column (3.5 x 46 cm) in the phosphate form that had been passed through Amberlite MB-3 resin.

Sephadex G-200 Chromatography—The concentrated enzyme from the previous step in the purification (10.5 ml) was added to a Sephadex G-200 column (2.5 x 36 cm) which had been equilibrated with 1 mM β-mercaptoethanol-10 mM potassium phos-
phate, pH 7.5. Elution was carried out with this same buffer, and 3.1-ml fractions were collected. The enzyme appeared in the first protein peak to emerge from the column.

Comments on Purification Procedure—In Table I are shown the results of the purification protocol for CoA-linked aldehyde dehydrogenase. The over-all enrichment of the enzyme in the course of numerous different purification attempts varied widely because of inconsistent values for the specific activity of the cell-free extracts. However, the specific activity after the chromatography steps in the purification procedure always approximated the values indicated in Table I.

The enzyme was found to be extremely unstable in the absence of a mercaptan after chromatography; even in the presence of thiol compounds, a slow loss of enzyme activity began a few days after purification. The enzyme is most stable at the ammonium sulfate step of the purification procedure and may be stored at 3° for several months at this point.

CoA-linked aldehyde dehydrogenase was found to be free of DPNH oxidase activity (assayed at 50 μM DPNH in 50 mM potassium phosphate buffer, pH 7.0; 2.0-ml reaction mixture), phosphotransacetylase (17), and alcohol dehydrogenase when assayed from the ethanol side of the reaction (18). On the other hand, the enzyme preparation always appeared to contain about 20% alcohol dehydrogenase activity (assayed at 50 μM DPNH, 50 mM acetaldehyde, and 50 mM potassium phosphate, pH 7.0; 2.0-ml reaction mixture) when the activities from the acetaldehyde side of the two reactions were considered.

We have not been able to separate these two activities by the following procedures: analytical polyacrylamide (10%) electrophoresis (pH 9.5), Sephadex G-200, microcrystalline calcium phosphate chromatography, Sepharose 4-B, isoelectric focusing, diethylaminoethyl cellulose, diethylaminoethyl cellulose-Sepherex, and triethylaminoethyl cellulose chromatography. The ratio of alcohol to CoA-linked aldehyde dehydrogenase activity remains relatively constant in the various eluate fractions in the DEAE-cellulose step of the purification procedure shown in Fig. 1. The two enzyme activities appear to be affected in a parallel manner by heat inactivation, 1,10-phenanthroline, and low pH treatment followed by dialysis. Both activities are markedly inhibited by chloroethanol. Although these observations suggest that the two enzyme activities may be associated with the same protein, it is not now possible to come to a definite conclusion on this point.

It is important that the findings of the experiments for aldehyde dehydrogenase, reported below, were not seriously affected by the presence of alcohol dehydrogenase. In the kinetic experiments, for example, in which initial velocities were measured, the amount of either DPNH formed (forward reaction) or acetaldehyde formed (reverse reaction) was too small for the effect of alcohol dehydrogenase to be of any significance. Essentially no alcohol dehydrogenase activity was observed when the concentration of DPNH was 10 μM or less, or when acetaldehyde was 1 mM or less. Similar comments could be made for other experiments cited in this report.

Identification of Reaction Products and Reaction Stoichiometry

The reaction products were identified as follows. A reaction mixture (2.2 ml) containing 0.18 μmole of CoA, 4.85 μmoles of DPN, 200 μmoles of acetaldehyde, 80 μmoles of β-mercaptoethanol, 800 μmoles of Tris-chloride buffer (pH 7.6), and 0.01 unit of enzyme was incubated at 35° for 90 min. The reaction was terminated by heating the reaction mixture in a boiling water bath for 5 min. In a control experiment, acetaldehyde, which had been omitted from the reaction mixture, was added after the boiling procedure.

Aliquots from the experimental and control reaction mixtures were analyzed for DPNH and acetyl-CoA as follows. DPNH was identified by its position and fluorescence relative to standard DPNH after paper chromatography in ethanol-ammonium acetate, pH 7.5 (19). The characteristic 340 μA absorbance due to DPNH disappeared after addition of excess acetaldehyde and yeast alcohol dehydrogenase.

Acetyl-CoA was determined enzymatically with malate dehydrogenase and condensing enzyme (14), spectrophotometrically by the increase in absorbance at 323 μA, and by modification of the ferric chloride-hydroxylamine test of Lipmann and Tuttle (20). In this last determination, control and experimental mixture samples were placed on Whatman No. 3 filter paper and dried under vacuum at 100° to remove acetaldehyde. Neutralized hydroxylamine was added, and after 10 min the paper was treated with ferric chloride reagent. The presence of acetyl-CoA was indicated by a red-brown spot. All tests for products were negative in the control samples.

The stoichiometry of the CoA-linked aldehyde dehydrogenase reaction was determined by measuring the increase in absorbance at 340 μA for DPNH and at 240 μA for acetyl-CoA. Reactions were carried out in 2.2-ml reaction mixtures as indicated above for the identification of the reaction products, but at 28°. Acetyl-CoA concentration was determined from the absorbance change at 240 μA; a suitable correction was made for the decrease in the absorbance at 240 μA when DPN was converted to DPNH (5). The concentrations of acetyl-CoA and DPNH were 0.17 and 0.18 μmole, respectively, after the reaction had essentially terminated. In a second experiment, in which only 30% of the CoA had been utilized, the ratio of acetyl-CoA to DPNH was 1.9:1.0. The initial rate of acetyl-CoA formation (measured from ΔA232) was found to be identical with the initial rate of DPNH formation (measured from ΔA340).

Effect of Sulfhydryl Reagents on Enzyme Activity

Burton and Stadtman (5), in studying aldehyde dehydrogenase from C. kluyveri, reported that the enzyme appeared to be inactive in the absence of β-mercaptoethanol. They suggested that the thiol was probably required for reduction of CoA and might not have an effect on the enzyme itself. In the early
The results depicted in Fig. 2 imply that aldehyde dehydrogenase itself has a thiol requirement. Maximum activation occurred between 7.5 and 15 mM mercaptan at pH 7.0 in 22.5 mM potassium phosphate buffer. This effect obviously requires additional and detailed investigation before definitive conclusions may be reached; however, the results from these studies suggest at least two possibilities for the role of thiol reagents. Because substrate DPN is required along with the thiol reagent to preclude the parabolic progress-velocity curve, it would appear that an otherwise inaccessible disulfide group might be exposed to the thiol in the presence of DPN. This could be merely a conformational change in the protein; however, a quaternary structure involvement is another possibility. The experiments reported here were carried out in Tris-HCl buffer, pH 8.1, and with $\beta$-mercaptoethanol. It was found that dithiothreitol could be substituted for the latter compound. Similar findings were observed with potassium phosphate buffer, pH 7.0, in place of Tris-Cl.

The time dependence of enzyme incubation with thiol reagent DPN, and CoA was also investigated. It was observed that at pH 7.0, the maximum initial velocity was obtained after incubation for 10 min. There appeared to be no significant change in initial rate during incubation between 10 and 45 min.

The effect of $\beta$-mercaptoethanol on the reverse reaction catalyzed by CoA-linked aldehyde dehydrogenase was also investigated. The enzyme was found to be essentially inactive in the absence of $\beta$-mercaptoethanol. Incubation of the enzyme with the mercaptoan for 10 min in the absence of acetyl-CoA and DPNH gave a normal velocity progress curve when substrates were added. These results support the view that $\beta$-mercaptoethanol has an effect on the E. coli enzyme directly, as contrasted to the dehydrogenase from C. kluweyori (5).

**Effect of pH**

Aldehyde dehydrogenase was found to be more active in potassium phosphate buffer than in either Tris-Cl or triethylamine buffer. The optimum pH in phosphate buffer was 7.0. At pH 6.2 the activity was only 67% of that exhibited at pH 7.0, while at pH 7.4 the enzyme was only 74% as active.

**Enzyme Specificity**

A number of compounds, including chloral, formaldehyde, benzaldehyde, 3-glycerylaldehyde, propionaldehyde, and butyraldehyde, were analyzed as substrates. The aldehyde concentrations were the same in all cases. The above compounds, except for propionaldehyde and butyraldehyde, did not serve as substrates in reaction mixtures containing 22.5 mM potassium phosphate buffer (pH 7.0), 14.9 mM CoA, 0.44 mM DPN, 10 mM dithiothreitol, and 3.2 mM aldehyde. Butyraldehyde was 30 to 40%, and propionaldehyde 60%, as active as acetaldehyde. TPN (0.42 mM) was found to be inactive when assayed with CoA and acetaldehyde.

Streptomycin sulfate at a concentration of 10 mg/2.0 ml of reaction mixture had no effect on the activity of the enzyme with the standard reaction mixture. This result implies that streptomycin sulfate, which contains an aldehyde group and is used for purification of the enzyme, neither inhibits nor serves as a substrate for aldehyde dehydrogenase.

**Kinetic Experiments**

Kinetic studies were initiated in order to investigate the mechanism of aldehyde dehydrogenase action. These experiments were carried out from both sides of the reaction. A pH of 7.0 was chosen, as it was found that initial velocities were most easy to survey at this pH.

A large number of mechanisms are possible for three-substrate enzyme systems (1, 21); however, they can be segregated into two classifications based upon initial rate experiments alone (1).
Two basic protocols may be used in these investigations. In the first, one substrate is varied while the other two substrates are maintained constant in the general concentration range of their Michaelis constants. This experiment is then repeated; however, a different concentration of fixed substrates is chosen, care being exercised to maintain the ratio of fixed substrates constant in both experiments. This procedure is then repeated until all substrates are varied. All those mechanisms involving quaternary complexes, i.e. when all substrates must be present on the enzyme before product formation can occur, will give Lineweaver-Burk plots that intersect at the left of the 1/v axis. On the other hand, a ping-pong mechanism will yield double reciprocal plots in which one or more sets of data describe parallel lines.

The second type of experiment that can be used for kinetic studies with three-substrate systems was first performed by Frieden (22) with glutamate dehydrogenase. Here one may hold one substrate constant and then vary the other two, as one normally does in the case of two-substrate systems. One advantage of the former approach is that only three experiments need be done, while in the latter case it may be necessary to carry out a maximum of six complete experiments if the double reciprocal plots yield parallel lines. This is a possibility if the concentration of the fixed substrate in a experiment is much greater than its Michaelis constant.

In Figs. 3 through 5 are shown results of initial rate experiments with aldehyde dehydrogenase. It can be seen from Figs. 3 and 4 that the double reciprocal plots, where 1/v is graphed against the reciprocal of either DPN or acetaldehyde, yield converging lines. On the other hand, the curves shown in Fig. 5 appear to be parallel. These data appear to support a ping-pong mechanism for the enzyme. There are only two basic ping-pong mechanisms consistent with these kinetic studies (1, 21).

In Schemes 1 and 2 are presented mechanisms that are in harmony with the data of Figs. 3 to 5.

![Scheme 1](image)

![Scheme 2](image)

The rate expressions accompanying the mechanisms are in the Dalziel form (23). For Scheme 2, the kinetic parameters are
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\[ \phi_0 = \frac{1}{k_0} + \frac{1}{k_9}, \quad \phi_1 = \frac{1}{k_1}, \]

\[ \phi_2 = \frac{(k_9 + k_k)}{k_9}, \quad \phi_3 = \frac{(k_9 + k_k)}{k_9}, \quad \text{and} \]

\[ \phi_4 = \frac{k_5(k_9 + k_k)}{k_9 k_9} \]

It is clear from Equations 1 and 2 that substrates A and C of Schemes 1 and 2, respectively, will give data of the type shown in Fig. 5. It is indeed difficult to rationalize how CoA could function in the mechanism depicted in Scheme 1. It is more reasonable to assume that CoA is represented in Scheme 2 by substrate C and that the reaction involves a transacylation from the enzyme to CoA to form acetyl-CoA. Although the latter hypothesis appears reasonable, it will require confirmation either from additional kinetic experiments or from isotope exchange studies.

It is possible from the kinetic data shown in Figs. 3 to 5 to evaluate the various Michaelis constants for the substrates by assuming the mechanism described by Scheme 2: \( V_{\text{max}} = \frac{E_0}{\phi_0} \), \( K_A = \phi_1/\phi_0 \), \( K_B = \phi_3/\phi_0 \), and \( K_C = \phi_4/\phi_0 \). By making secondary plots of the data shown in Figs. 3 and 4 for intercepts and slopes, it is possible to evaluate all \( \phi \) values. The Michaelis constants for CoA, DPN, and acetaldehyde are 10 \( \mu \)m, 50 \( \mu \)m, and 1.5 \( \mu \)m, respectively.

Initial rate experiments for the back-reaction, as illustrated in Figs. 6 and 7, depict ping-pong kinetics. Results of this type are mandatory if the mechanism illustrated in Scheme 2 is to be given any credence. For this pathway of enzyme and substrate interaction, the steady rate equation is

\[ \frac{V}{V_{\text{max}}} \times 10^{-4} \text{M} \]

\[ \text{FIG. 6. Plot of the reciprocal of initial reaction velocity (v) with respect to the reciprocal of the molar concentration of acetyl-CoA. The concentrations of acetyl-CoA were: ■, 42.5 \mu M, ○, 21.2 \mu M, Δ, 10.6 \mu M, ●, 7.1 \mu M, ★, 5.3 \mu M. The DPNH concentration varied from 0.21 mM to 0.05 mM. Other experimental details are described under "Experimental Procedure."} \]

\[ \frac{E_0}{V} = \phi_0 + \phi_1 P + \phi_2 Q \] (3)

The kinetic constants are defined as follows.

\[ \phi_0 = \frac{1}{k_0} + \frac{1}{k_9}, \quad \phi_1 = \frac{(k_9 + k_k)}{k_9 k_9}, \quad \phi_2 = \frac{(k_9 + k_k)}{k_9 k_9} \]

The Michaelis constants calculated from the results of Figs. 6 and 7 from secondary plots are 0.1 \( \mu \)m and 13 \( \mu \)m for DPNH and acetyl-CoA, respectively.

DISCUSSION

The present report describes the partial purification and some of the properties of CoA-linked aldehyde dehydrogenase from E. coli strain B. Two rather interesting aspects of the present investigation center on the sulfhydryl requirement of the enzyme and its mechanism of action. Additional investigations will be required before these points are clarified.

Although it is possible that the thiol reagents \( \beta \)-mercapto-
ethanol and dithiothreitol facilitate the aldehyde dehydrogenase reaction by keeping CoA in the reduced form, their major roles appear to involve reduction of some group, presumably a disulfide, on the enzyme. This is clear from the fact that incubation of either sulfhydryl compound with CoA does not preclude the parabolic progress curve illustrated in Fig. 2. It is also of interest that this effect is not eliminated when the enzyme is treated with thiol reagents in the absence of DPN. Exactly how DPN functions in this regard is not known; however, some suggestions for its possible role, other than its obvious function as a substrate, have been made. Additional support for the suggestion that the enzyme has a thiol requirement is provided from experiments from the acetyl-CoA side of the reaction; i.e. CoA-linked aldehyde dehydrogenase was found to be inactive in the absence of added β-mercaptoethanol.

The mechanism of action of aldehyde dehydrogenase appears to be of the ping-pong type, based on the initial rate experiments reported here. Although the pathway of enzyme and substrate interaction can only be speculated on, the most reasonable mechanism is shown in Scheme 2. Further initial rate experiments, such as those with product inhibitors and competitive substrate inhibitors, will be required before much credence can be placed in this mechanism. It is possible, however, to come to a tentative conclusion regarding the reaction mechanism, based on the experiments of this report and by analogy with such systems as glyceraldehyde 3-phosphate dehydrogenase. In Scheme 3 is presented a mechanism for aldehyde dehydrogenase which appears to be consistent with the findings of the current investigation.

Another possibility that appears to be in harmony with our data involves the addition of DPN rather than acetaldehyde to the enzyme first. A choice cannot be made between these two possibilities at present; however, the DPN requirement for thiol activation makes the latter hypothesis somewhat more attractive.

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