ENZYMEOLOGY OF BUTANOL FORMATION IN CLOSTRIDIUM BEIJERINCKII NRRL B592

by

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(ABSTRACT)

The present study encompasses an investigation of the expression of solvent-forming enzymes and purification and characterization of butanol-forming enzymes. More sensitive and accurate procedures for the determination of acids and solvents in cultures have been developed, which led to the recognition of the onset of solvent production at the mid-exponential phase, about two h earlier than previously reported. Activities of solvent-forming enzymes started to increase about one h before the onset of measurable solvent production and the activities of solvent-forming enzymes did not increase simultaneously. CoA-acylating aldehyde dehydrogenase (ALDH) was purified to near homogeneity. The ALDH showed a native $M_r$ of 100,000, and a subunit $M_r$ of 55,000. ALDH could use either NAD(H) or NADP(H) as the coenzyme. ALDH was oxygen-labile. The $O_2$-inactivated enzyme could be reactivated by incubating the enzyme with CoA. Both NADH- and NADPH-dependent alcohol dehydrogenase activities were present in crude extracts. The ratio of NADPH-dependent activity
to NADH-dependent activity (the P/D ratio) varied in crude extracts. The P/D ratio was affected by O₂, ionic strength, pH, growth stage of cell, Fe in culture medium and temperature. Two ADHs have been identified in crude extracts. The NADPH-dependent ADH (P-ADH) could be separated from the NADH/NADPH-dependent ADH (D/P-ADH). The D/P-ADH has been extensively purified. The D/P-ADH showed a native $M_r$ of 70,000 and subunits with $M_r$ of 45,300 and 40,000. The D/P-ADH activity could be inactivated by $\alpha,\alpha'$-dipyridyl and restored by Fe$^{2+}$. 
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ORGANIZATION OF THE DISSERTATION

Clostridium beijerinckii NRRL B592, which produces acetone, butanol, and ethanol (solvents), was used in this study. The dissertation contains six chapters. Introduction and background constitute Chapter I. A study of the expression of solvent-forming enzymes [acetoacetate decarboxylase, CoA-acylating aldehyde dehydrogenase (ALDH) and alcohol dehydrogenases (ADHs)] and the onset of solvent formation is presented in Chapter II. Purification and characterization of butanol-forming enzymes, ALDH and ADH, are described in Chapter III and Chapter IV, respectively. The finding in acetaldehyde samples of NAD(P)H-reacting substance(s), which reacts with NAD(P)H and interferes with the assay of ADH, is presented in Chapter V. The last Chapter contains conclusions and suggestions for future research as a result of this study.

Chapter II is excerpted from the following publication:


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Chapter I

INTRODUCTION AND BACKGROUND

Introduction

The genus Clostridium contains several species that can produce a large quantity of acetone or 2-propanol and butanol (solvents) from a variety of carbohydrates. Before 1960, the acetone-butanol fermentation was a major industrial process (Walton and Martin, 1979; Jones and Woods, 1986). The first successful commercial process was established in England during the years 1913 and 1914. Soon thereafter, plants were placed in operation in Canada, the United States and India. During the first part of this century it ranked second in importance only to ethanol fermentation. After 1960, this fermentative process has largely been replaced by synthetic processes in countries with a ready supply of petroleum. However, the situation may change because petroleum is a nonrenewable resource that eventually will be depleted. The usage of solvents as feedstock chemicals and fuel additives has been projected to rise rapidly. These considerations and recent developments in the field of biotechnology have resulted in a renewal of interest in this fermentation as a possible route for acetone-butanol-2-propanol production (Spivey, 1978; Tong, 1978; Lenz and Moreira, 1980; Zeikus, 1980; Lipinsky, 1981; Gottschalk and Bahl, 1981; Linden and Moreira, 1982; Rogers, 1984; Beck, 1985; Busche, 1985). During the last ten years, there has been an escalation in research aimed at obtaining a greater understanding of this complex and interesting fermentation.

Background

Usage of solvents. During World War I, acetone was required in large amounts for the manufacture of munitions, which was the driving force for the development of a
fermentation that produced acetone and also butanol as a byproduct (Killeffer, 1927; Gabriel, 1928; Gabriel and Crawford, 1930). Thereafter, butanol and its ester, butyl acetate, were required in large amounts as solvents for quick-drying nitrocellulose lacquer for car bodies (Gabriel and Crawford, 1930). Today, acetone and butanol are used mainly as feedstock chemicals, which account for more than 50% of their uses; less than 30% is used as solvents. 2-propanol has been proposed as one of the four short-chain aliphatic alcohols that may become the major feedstocks for the future chemical industry (Palsson et al., 1981). Besides the current uses for these chemicals, butanol and 2-propanol can be used as additives to replace lead to enhance the octane number of gasoline.

**Solvent-producing bacteria.** Several *Clostridium* species can produce significant amounts of neutral solvents. However, the type and the ratio of the solvents produced differ significantly. Beesch (1952) tabulated the ratios of butanol-ethanol-acetone-2-propanol produced by bacteria used in twenty-one U.S. patents, and the table contained the names of twenty-one different bacteria. This illustrates the multitude of names given to organisms that produce "solvents". In scientific publications, *Clostridium acetobutylicum* and "*Clostridium beijerinckii*" have been the two most frequently used names. "*C. beijerinckii*" is no longer a species on the 1980 "Approved Lists of Bacterial Names" (Skerman et al., 1980), and it was found to be synonymous to *Clostridium beijerinckii* (George et al., 1983). Based on the results of a study of cell wall composition and DNA homology, it was shown that *C. acetobutylicum* and *C. beijerinckii* are distinct species (Cummins and Johnson, 1971).

*C. acetobutylicum* produces acetone, butanol and low amounts of ethanol. *C. beijerinckii* produces solvents in approximately the same ratio as *C. acetobutylicum*, but 2-propanol is produced in place of acetone in 2-propanol producing strains (George et al.,
Fifty-two strains of *C. beijerinckii* were tested for their ability to produce acetone, 1-butanol, and 2-propanol (Chen and Hiu, 1986). Under the test condition used, 12 strains produced 60 mM or more of 1-butanol. Among the 21 strains producing 25 mM or more of 1-butanol, 9 also produced 10 mM or more of 2-propanol whereas 6 produced 10 mM or more of acetone, indicating that the molar ratio of 2-propanol to butanol varied for these strains (Chen and Hiu, 1986). The tested strains of *Clostridium aurantibutyricum* produce higher concentrations of acetone and 2-propanol in relation to 1-butanol than *C. beijerinckii* (George et al., 1983). *Clostridium tetanomorphum* produces almost equimolar amounts of 1-butanol and ethanol but does not produce acetone or 2-propanol (Gottwald et al., 1984). *Clostridium saccharoperbutylicum* produces ethanol, acetone and 1-butanol (Hatashida and Yoshino, 1990). Thermophilic *Clostridium thermosaccharolyticum* produces 1-butanol (up to 40 mM) and a much higher amount of ethanol (up to 90 mM), but does not produce acetone or 2-propanol (Freier-Schroder et al., 1989). *Bacillus macerans* produces higher amount of ethanol than acetone but does not produce 1-butanol or 2-propanol (Weimer, 1984).

**Factors with a possible role in the metabolic transition for solvent production.** In batch cultures, solvent-producing *Clostridium* produces H₂, CO₂, acetate and butyrate during the early exponential phase of growth. As the culture is entering into the stationary phase, the metabolism of the cells undergoes a shift from acid production to solvent production (Reilly et al., 1920; Speakman, 1920; Johnson et al., 1931; Davies and Stephenson, 1941; Jones, et al., 1982; George and Chen, 1983; Chen and Hiu, 1986). However, the onset of expression of solvent-forming enzymes and the onset of solvent production are much earlier than the accumulation of solvents (this work; see Chapter II). An understanding of the control...
mechanism for the transition into solvent production is likely to have practical applications in future process development and strain improvement. For example, strains of *Clostridium* lose the ability to produce solvents after repeated subcultures in the vegetative state. This degenerative process could involve regulatory aberrations that result in the failure of the cell to respond to factor(s) triggering the onset of the metabolic transition. This type of cellular defect may be overcome if the triggering mechanism is known. A number of factors have been studied as possible signals for triggering the metabolic transition.

(A) **pH.** The culture pH was recognized as a key factor in the initiation of solvent production, for many of the early reports on the industrial production of solvents indicated that the initiation of solvent production occurred only after the external pH had decreased to around 4.5 to 5.0 (Davies and Stephenson, 1941; Beesch, 1953; Prescott and Dunn, 1959; Ross, 1961; Thimann, 1963). However, George and Chen (1983) have found that solvents could be produced by a strain of *C. beijerinckii* maintained at pH 6.8. The production of solvents in cultures maintained at neutral pH has since also been reported in a strain of *C. acetobutylicum* (Holt et al., 1984), and *C. thermosaccharolyticum* (Freier-Schroder et al., 1989), but a higher level of acetic and butyric acids seems to be required for the onset at neutral pH. Although a low pH can facilitate the shift, pH itself is not the trigger.

(B) **Acid concentration.** The onset of solvent production under no pH control is normally associated with a fall in the pH of the medium resulting from the accumulation of acids. Gottschal and Morris (1981) reported that addition of acetate and butyrate to batch cultures of *C. acetobutylicum* maintained at pH 5.0 resulted in a rapid induction of solvent formation. However, a close relationship between the concentration of undissociated butyric acid and the induction of solvent production has not been established in some
experimental systems. Although there is mounting evidence suggesting that acetic and butyric acids play a role in triggering the onset (Bahl et al., 1982; George and Chen, 1983; Holt et al., 1984; Monot et al., 1984; Fond et al., 1985; Huang et al., 1985), it is still unknown how the acids exert their regulatory effect.

(C) CoA and phosphate concentrations. Gottwald and Gottschalk (1985) have pointed out that the elevated butyrate and acetate concentrations in the cell is important. The concentration is primarily affected by the external butyrate concentration and pH across the cytoplasmic membrane. Since the reactions which lead from butyryl CoA via butyryl phosphate to butyrate are reversible, an elevated concentration of butyrate may result in elevated levels of butyryl phosphate and butyryl CoA in the cell. Eventually this must result in a drastic decrease in both CoA and phosphate pools. They suggested that this situation would provide the signal for the shift.

(D) ATP and NADH concentrations. In solvent fermentation the amount of ATP produced per mole of substrate consumed is strongly dependent on product distribution (Papoutsakis, 1983). The only other source of ATP besides the glycolytic pathway is through the production of acetic and butyric acids. Production of solvents results in significantly less ATP formation. The efficiency of ATP generation can therefore be regulated through control of product selectivity. Under conditions of limited supply of substrates or high demand for ATP, production of acids would predominate while conditions of excess supply or low demand for ATP would result in solvent formation. Primarily acid production was observed in glucose-limited continuous cultures (Meyer and Papoutsakis, 1989). Low ATP demand would result from a decreased growth rate and would lead to enhanced solvent production. Another effect that has been reported for
initiating solvent production involves the use of CO. It was reported that batch cultures
grown in the presence of CO produced enhanced levels of butanol and ethanol (Kim et al.
1984; Datta and Zeikus 1985). In completely acidogenic continuous cultures, butanol and
ethanol production could be initiated by sparging with CO while acetone production
remained negligible (Meyer et al. 1986). It was hypothesized that by inhibiting hydrogenase,
CO had caused an increased availability of reduction energy (NAD(P)H), which was
required for butanol and ethanol formation. Acetone does not require NAD(P)H for its
synthesis and its production was not affected. Meyer and Papoutsakis (1989) point out that
increased levels of ATP and NADH are associated with increased solvent production in
continuous cultures of \textit{C. acetobutylicum}, but neither ATP nor NADH levels appear to
correlate with normal solventogenesis in batch cultures. However, a mutant defective in
NADH generation isolated from \textit{C. saccharoperbutylacetonicum} and produces lower
amounts of 1-butanol and ethanol than the wild type in batch cultures, whereas the acetone
production is similar. Much higher amounts of acids and H\textsubscript{2} are produced in this mutant
(Hayashida and Yoshino, 1990).

Although these factors have been investigated, the external signals triggering the
metabolic shift and the mechanism of the shift have not been determined.

**Solvent-forming Enzymes.** The biochemical pathways (Figure I-1) utilized for the
conversion of carbohydrates to solvents by \textit{C. acetobutylicum} and \textit{C. beijerinckii} have been
largely established.

Pyruvate resulting from glycolysis is oxidized by pyruvate:ferredoxin oxidoreductase in
the presence of CoA to yield CO\textsubscript{2}, acetyl-CoA and reduced ferredoxin (Meinecke et al.,
1989). In the solvent producing phase, acetaldehyde is produced from acetyl CoA by a
Figure I-1. Metabolic pathway of acid and solvent production in *Clostridium beijerinckii*. *C. beijerinckii* NRRL B593 carries out all of the reactions depicted in the figure. *C. beijerinckii* NRRL B592 does not carry out reaction 14. Enzymes catalyzing the numbered reactions are as follows (reaction number in parenthesis):

Pyruvate:ferredoxin oxidoreductase (1)

Thiolase (2)

3-hydroxybutyryl-CoA dehydrogenase (3)
Crotonase (4)
Butyryl-CoA dehydrogenase (5)
Phosphotransacetylase (6)
Acetate kinase (7)
Phosphotransbutyrylase (8)
Butyrate kinase (9)
Aldehyde dehydrogenase (10, 15)
Alcohol dehydrogenase (11, 14, 16)
Acetoacetyl-CoA:acetate/butyrate-CoA transferase (12)
Acetoacetate decarboxylase (13)

CoA-acylating aldehyde dehydrogenase and is reduced to ethanol by alcohol dehydrogenase.

The four enzymes responsible for the formation of butyryl-CoA from acetyl-CoA are thiolase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase (Chen, 1991).

Thiolase carries out the condensation of two acetyl-CoA to one molecule of acetoacetyl-CoA, and it has been purified from C. acetobutylicum ATCC824 (Wiesenborn et al., 1988). Thiolase activity is competitively inhibited by CoA. Since the variation in specific activity can be significantly different from the variation in specific product rate and a high specific activity is present in all fermentations, the key regulation of flux through this branch [i.e. the
ratio of butanol plus acetone (2-propanol) to ethanol] occurs at the enzyme level (Wiesenborn et al., 1988).

3-Hydroxybutyryl-CoA dehydrogenase catalyzes the formation of 3-hydroxybutyryl-CoA from acetoacetyl-CoA. NADH-linked activity of the enzyme has been measured in C. acetobutylicum ATCC 824 (Hartmanis and Gatenbeck, 1984). The enzyme has been purified from C. beijerinckii NRRL B593, and it showed activity with both NADH and NADPH (Colby, G. D. and Chen, J.-S., unpublished results). NADPH-specific 3-hydroxybutyryl-CoA dehydrogenase has been purified from C. kluveri (Madan et al., 1973; Sliwkowski and Hartmanis, 1984). The structural gene for the enzyme has been cloned from C. acetobutylicum P262 (Youngleson et al., 1989).

Crotonase, which catalyzes the formation of crotonyl-CoA from 3-hydroxybutyryl-CoA, has been purified from C. acetobutylicum (Waterson et al., 1972).

Butyryl-CoA dehydrogenase, which catalyzes the formation of butyryl-CoA from crotonyl-CoA, has not been isolated from solvent-producing clostridia, and no NADH or NADPH linked activity has been demonstrated (Hartmanis and Gatenbeck, 1984). Using dye-linked assays, activity of the enzyme was detected in this group of anaerobes (Hartmanis and Gatenbeck, 1984).

A CoA-acylating aldehyde dehydrogenase, which catalyzes the formation of acetaldehyde and butyraldehyde from corresponding acyl CoA, has been purified from C. acetobutylicum NRRL B643 (Palosaari and Rogers, 1988) and from C. beijerinckii NRRL B592 (this work). An alcohol dehydrogenase, which catalyzes the last step of alcohol formation, has been purified from C. beijerinckii NRRL B593 (the strain producing 2-propanol). In C. beijerinckii NRRL B593, the enzyme is a primary and secondary alcohol dehydrogenase
which is NADPH specific and can catalyze the production of ethanol, butanol, and 2-propanol (Hsu et al., 1987; Zhu et al., 1991). However, 2-propanol is not a product of alcohol dehydrogenases from *C. beijerinckii* NRRL B592 (the strain not producing 2-propanol) or *C. acetobutylicum*. Two alcohol dehydrogenases are present in *C. acetobutylicum* (Durre et al., 1987; Welch et al., 1989), and an NADH- and NADPH-dependent enzyme has been purified from *C. acetobutylicum* ATCC 824 (Welch et al., 1989). The gene encoding an NADPH-dependent alcohol dehydrogenase from *C. acetobutylicum* P262 has been cloned and sequenced (Youngleson et al. 1989). The deduced amino acid sequence of the NADPH-dependent ADH of *C. acetobutylicum* exhibits 39% homology with the Fe-activated alcohol dehydrogenase from *Zymomonas mobilis*.

The branch leading to acetone or 2-propanol formation involves two steps (acetone being the end product) or three steps (isopropanol being the end product). CoA transferase (acetoacetyl CoA:acetate/butyrate CoA-transferase), which catalyzes the formation of acetoacetate from acetoacetyl CoA, has been purified from *C. acetobutylicum* ATCC 824 in the presence of ammonium sulfate and glycerol (Weisenborn et al., 1989). The Km values for butyrate (0.66 M) and acetate (1.2 M) are higher than the intracellular concentration of these acids. The genes encoding the two subunits of this enzyme have been cloned and expressed in *E. coli* (Cary et al., 1990). Acetoacetyl CoA-reacting phosphotransbutyrylase, which possibly catalyzes the formation of acetoacetyl phosphate from acetoacetyl CoA, has been purified from *C. beijerinckii* NRRL B592 (Thompson and Chen, 1990). Acetoacetate decarboxylase, which catalyzes the decarboxylation of acetoacetate to form acetone, has been purified from *C. acetobutylicum* ATCC 824 (Zeren et al. 1966; Petersen
and Bennett, 1990), and the gene encoding this enzyme has been cloned (Petersen and Bennett, 1990; Gerischer and Durre, 1990) and sequenced (Gerischer and Durre, 1990). The enzyme is much more stable than many of the other enzymes of the fermentation pathway and it is insensitive to O₂ and can withstand acetone.
REFERENCES


17. **George, H. A., and J.-S. Chen.** 1983. Acidic conditions are not obligatory for onset of butanol formation by *Clostridium beijerinckii* (synonym, *C. butylicum*).


47. Reilly, J., W. J. Hichinbothom, F. R. Henley, and A. C. Thaysea. 1920. The products of
the "acetone:n-butyl alcohol" fermentation of carbohydrate material with special reference to some of the intermediate substances produced.


Microbiol. 34:423-464.


Chapter II

Expression of solvent-forming enzymes and onset of solvent production during growth in batch cultures of Clostridium beijerinckii NRRL B592

INTRODUCTION

Acetone, ethanol, and 1-butanol (solvents) are characteristic products of several Clostridium species (Compere and Griffith, 1979; George et al., 1983; Gottwald et al., 1984; Chen and Hiu, 1986). While certain strains of Clostridium beijerinckii characteristically produce 2-propanol, 1-butanol, and ethanol, Clostridium acetobutylicum and some strains of Clostridium beijerinckii (C. butylicum) produce acetone, 1-butanol, and ethanol as the final products (Chen and Hiu, 1986). Alcohols produced by these fermentations are useful both as chemical feedstocks and as fuel additives.

Solvent production is a regulated metabolic process. In a normal batch culture, solvent-producing Clostridium species produce acetate and butyrate during the exponential growth phase. Only during the late growth phase does the metabolism shifts to rapid solvent production. This was first shown by Peterson and Fred (1932) and Davies and Stephenson (1941) and also by more recent studies using C. acetobutylicum (Jones et al., 1982; Andersch et al., 1983; Fond et al., 1985; Afshar et al., 1986; Rogers, 1986). The metabolic shift is also observed in C. beijerinckii (George and Chen, 1983; Chen and Hiu, 1986). Therefore, solvent fermentation is generally divided into two phases: the acid-producing or acidogenic phase and the solvent-producing or solventogenic phase. It is expected that the shift in metabolic activity, which occurs when cells switch from the acid-producing phase to the solvent-producing phase, is accompanied by a corresponding shift in the cellular content.
Figure II-1. Metabolic pathway of acid and solvent production in *Clostridium beijerinckii*. *C. beijerinckii* NRRL B593 carries out all of the reactions depicted in the figure. *C. beijerinckii* NRRL B592 does not carry out reaction 14. Enzymes catalyzing the numbered reactions are as follows (reaction number in parenthesis):

Pyruvate:ferredoxin oxidoreductase (1)

Thiolase (2)
3-hydroxybutyryl-CoA dehydrogenase (3)
Crotonase (4)
Butyryl-CoA dehydrogenase (5)
Phosphotransacetylase (6)
Acetate kinase (7)
Phosphotransbutyrylase (8)
Butyrate kinase (9)
Aldehyde dehydrogenase (10, 15)
Alcohol dehydrogenase (11, 14, 16)
Acetoacetyl-CoA:acetate/butyrate-CoA transferase (12)
Acetoacetate decarboxylase (13)

of enzymes involved in the acid- and solvent-producing pathways (Fig. II-1). The enzymes specifically involved in solvent production are expected to be synthesized or activated or both before the shift to solvent production can occur. The external signals that trigger the metabolic shift have not been determined (for a recent review, see Jones and Woods, 1986).

The onset of the solventogenic phase is empirically assigned, and the accuracy of the assignment is limited by the sensitivity of assays that detect the first sign of solvent accumulation. During the period when a culture is shifting to solvent production, the
culture conditions undergo rapid changes. Therefore, the assigned time of onset significantly affects the culture conditions to be considered as triggering signals. It is possible that the threshold external signals which trigger the metabolic switch differ from the more easily identified culture conditions (in batch or continuous cultures) that are conducive to sustained solvent production. Knowledge of the more precise moment of the beginning of the metabolic transition allows the identification of more pertinent external conditions and physiological states as possible factors involved in the triggering of the metabolic transition. For this purpose, activities of solvent-forming enzymes can serve as earlier indicators of the onset of solventogenic switch. It is also important to determine whether these enzyme activities appear (or increase) in coordination or independently, since this information could bear on the elucidation of the control mechanism for the solventogenic switch and could affect approaches to be taken to improve the fermentation.

Changes in acid- and solvent-forming enzyme activities during growth of \textit{C. acetobutylicum} have been reported (Andersch et al., 1983; Hartmanis et al., 1984; Rogers, 1986; Durre et al., 1987), but different patterns of activity changes were observed among laboratories. During this study, we first controlled the growth stage of the inocula and then measured the cellular levels of enzymes responsible for acetone and butanol formation (reactions 12, 13, 15, and 16 in Fig. II-1) in \textit{C. beijerinckii} NRRL B592 throughout the entire growth period. (The presence of these solvent-forming enzymes in \textit{C. beijerinckii} has been reported previously [George and Chen, 1983; Hiu et al., 1987; S. F. Hiu, W.-L. Fan, W. R. Aimutis, and J.-S. Chen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, o-83, p. 249]). In addition, we measured the cellular level of thiolase and glucose-6-phosphate isomerase for comparison. Cell density, culture pH, and the level of fermentation products
in the growth medium were monitored at frequent intervals to determine the culture conditions at the onset of solvent production.
MATERIALS AND METHODS

Materials. Tryptone and yeast extract were obtained from Difco Laboratories, Detroit, Mich.; dithiothreitol (DTT), DNase I (DN-25), NAD⁺, NADP⁺, NADPH, glycylglycine, coenzyme A (CoA), glucose-6-phosphate dehydrogenase (from Baker's yeast), fructose-6-phosphate (disodium salt), acetoacetyl CoA, acetoacetic acid (lithium salt), n-butyric acid, biotin, and Trisma base were obtained from Sigma Chemical Co., St. Louis, Mo.; the dye-binding protein assay kit was obtained from BioRad Laboratories, Richmond, Calif.; α-toluenesulfonyl fluoride was obtained from Eastman-Kodak Co.; 1-butanol, acetone, butyraldehyde, glacial acetic acid were obtained from Fisher Scientific Co., Pittsburgh, Pa.; methanol was obtained from Burdick and Jackson Laboratories Inc., Muskegon, Mich.

Organisms and Growth Conditions. C. beijerinckii NRRL B592 (VPI 13436; the strain not producing 2-propanol) was used in this study. For each growth experiment, 6 ml of a spore suspension was heated in boiling water for 1 min, transferred to 50 ml of a chopped-meat-carbohydrate (CMC, meat removed) medium (George and Chen, 1983) under N₂ and incubated at 35°C with shaking for 13-18 h. The culture, which was at the exponential growth stage, was transferred to 1 liter of a tryptone-yeast extract-sucrose (TYS) medium (George and Chen, 1983), which was then incubated at 30°C with stirring for 3.75 to 5.75 h. Finally, the 1-liter culture (in a 1-liter Erlenmeyer flask fitted with gassing and sampling ports), which was at the exponential growth stage, was used to inoculate 7 liters of TYS medium, and this 8-liter culture (in a 9-liter glass serum bottle fitted with gassing and sampling ports) was incubated at 30°C with stirring and monitored for 29 to 30 h (entire growth period) or 6 h (early phase). During growth, frequent samples were taken for the measurement of cell density, culture pH, and product concentrations, and up to seven
samples were taken for the measurement of enzyme levels. The 1- and 8-liter cultures were initially kept under N₂ and were then vented through a bubbler during growth to maintain a 1-atm (101.3-kPa) gas phase. During sampling, N₂ was used to maintain the gas phase slightly above 1 atm to displace a desired volume of culture through glass tubing. Cells were sedimented by centrifugation, and culture supernatants were stored at -20°C until analyzed. For enzyme analysis, sedimented cells were washed once in 50 mM Tris chloride buffer (pH 8) and stored at -80°C for up to 4 days before cell extracts were prepared. Growth was monitored at 550 nm with a Hitachi Model 100-40 spectrophotometer.

Preparation of cell extracts. Cell paste was thawed under argon in 50 mM Tris chloride, pH 8, (3 ml/g cell paste) containing DNase I (0.1 mg/ml) and α-toluenesulfonyl fluoride (0.3 mg/ml) as a protease inhibitor. All operations were carried out under argon. Cells were disrupted by two passages through a French pressure cell at 18,000 lb/in², and suspensions of broken cells were incubated at room temperature for 15 min to allow DNase I to decrease the viscosity. Cell debris were then removed by centrifugation at 37,000 x g for 30 min at 4°C. The supernatant (cell extract) was stored as frozen pellets in liquid nitrogen.

Analytical procedures. Solvents and acids in culture supernatants were measured using a Gow-Mac series 750 FID gas chromatograph (Gow-Mac Instrument Co., Bridgewater, N.J.) linked to an HP 3390A integrator (Hewlett Packard Co., Avondale, Pa). Quantification of acids in culture supernatants by gas chromatography was prone to large variations. Therefore, each sample for acid determination was measured up to 20 times (10 times on the average), so that the coefficients of variation (standard deviation divided by mean) were below 0.15.

Solvent concentrations were determined by using a glass column of 2 m by 4 mm
containing 80/100 mesh Carbopack C/0.1% SP-1000 (Supelec, Inc., Bellefonte, Pa.). The column temperature was 80°C; the injector and detector temperatures were 150°C. The flow rate of the carrier gas, nitrogen, was 30 ml/min. For samples containing low concentrations of solvents (less than 1 mM butanol), 5 μl of the sample was used, and for samples with higher concentrations of solvents, 1 μl of the sample was used. In more recent experiments, better resolution was obtained by injecting 1 μl of sample onto a glass column of 2 m by 2 mm containing the same packing, with the column temperature at 75°C, the injector and detector temperatures were 150°C.

Acid concentrations were determined by using a glass column (2 m by 2 mm) containing Chromosorb 101. The column temperature was 165°C; the injector and detector temperatures were 210°C. The flow rate of the carrier gas (N₂) was 25 ml/min. The samples were acidified first by adding 20 μl 1 N HCl to 100 μl of each sample before injection of 1 μl of samples.

Protein was determined by the dye-binding assay (Bradford, 1976) with bovine serum albumin as a standard.

**Enzyme Assay.** NAD(P)H-linked enzymes in crude extracts were assayed under anaerobic conditions to circumvent the interference of diaphorase activities (Hiu et al., 1987). Other enzymes were stable in air and were assayed under aerobic conditions. The volume of reaction mixture for spectrophotometric assays was 1 ml. Alcohol dehydrogenase activities were determined in the direction of butyraldehyde reduction (Hiu et al., 1987). Butyraldehyde dissolves slowly in water, and a clear aqueous solution is not readily formed. To solve this problem, we dissolved butyraldehyde in methanol before use (Hiu et al., 1987). Butyraldehyde dehydrogenase activity was measured by an assay modified from Rogers
(1986). The assay mixture contained 50 mM glycy1glycine buffer (adjusted to pH 9.0 with NaOH), 1 mM DTT, 0.5 mM coenzyme A, 3 mM NAD⁺, and 10 to 40 μl of cell extracts. The reaction was initiated by the addition of butyraldehyde (11 mM; butyraldehyde was first diluted 10-fold with methanol).

The enzyme(s) which catalyzes acetoacetate formation from acetoacetyl CoA was measured by following the disappearance of acetoacetyl CoA. The assay mixture contained 175 mM Tris chloride buffer (pH 7.5), 20 mM MgCl₂, 0.1 mM acetoacetyl CoA, 100 mM potassium acetate or potassium butyrate, and 20 ul of cell-free extracts. Potassium acetate or butyrate was added last during the assay. Two activities (acetoacetyl CoA:acetate/butyrate CoA transferase and acetoacetyl CoA hydrolase) have been found in C. beijerinckii, and the hydrolase activity was potassium-activated (Yan and Chen, Federation. Proc., 1987). Since acetate, butyrate, and potassium are present in cell-free extracts, the activities (after corrections for non-enzymic hydrolysis of acetoacetyl CoA) observed before the addition of potassium acetate or potassium butyrate could be attributed to acetoacetyl CoA:acetate/butyrate CoA-transferase and acetoacetyl CoA hydrolase activities from endogenous substrates and were thus not subtracted from the final activities. Acetoacetate decarboxylase activity was measured by manometry at 30°C as described by Davies (1943). In the assay mixture, 5-300 μl of cell extracts was used. Thiolase activity was assayed in the direction of acetyl CoA formation from acetoacetyl CoA and CoA. The assay mixture contained 105 mM Tris chloride buffer (pH 7.5), 24 mM MgCl₂, 90 μM acetoacetyl CoA, and 1 μl of cell extracts. The reaction was initiated by addition of 60 μM CoA. Glucose-6-phosphate isomerase activity was assayed as described by Bergmeyer (1974), except that 50 mM Tris chloride buffer (pH 7.5) was used.
RESULTS

When solvent-producing clostridia are grown in batch cultures, it is possible that the cellular level of solvent-forming enzymes is subjected to different types of controls: the level could increase when cells switch from acid production to solvent production, or the level could decrease or remain unchanged when cells at the solvent-producing stage are transferred into fresh growth medium. To minimize the last complications, only cultures at the early exponential growth phase, which showed no solvent production, were used as inocula during this study. In order to locate the very earliest moment of the solventogenic switch, the assays used in this study for enzyme activities and fermentation products were adapted for sensitivity and reproducibility. Whenever possible, enzyme activities were measured in the physiological direction. Changes in the activity levels of enzymes involved in acetone and butanol formation (Fig. II-1, reactions 12, 13, 15, 16) during the growth of C. beijerinckii strains NRRL B592 are shown in Fig. II-2. To resolve the order of the appearance of (or increases in) these enzyme activities and to deduce the limiting enzyme(s) for the start of solvent production, an experiment was performed in which seven samples for enzyme analysis were taken during the transition period (Fig. II-3).

The improved assay procedures permitted reliable measurements of solvents at concentrations below 0.1 mM, which led to the recognition of the beginning of solvent production about 2 h earlier (in relation to growth) than the previously assigned onset time (George et al., 1983). The culture pH at the onset of solvent production was 5.3 (Fig. II-3), which is significantly higher than what is usually considered necessary for rapid solvent production (for a review, see Jones and Woods, 1986). The total acid (dissociated plus undissociated acetate and butyrate) concentration in cultures at the onset of solvent
Figure II-2. Growth, culture pH, solvent production, and specific activity of solvent-forming enzymes of *C. beijerinckii* NRRL B592 in TYS medium. (A) A550 (○), acetone (◇), butanol (△), and pH (●). (B) Acetoacetate decarboxylase (■), butyraldehyde dehydrogenase (◇), butanol dehydrogenase (△), and glucose-6-phosphate isomerase (●). (C) Acetoacetate-forming (acetoacetyl-CoA-utilizing) enzymes in the presence of potassium acetate (○) or potassium butyrate (●), and thiolase (△).
Figure II-3. Growth, culture pH, acid and solvent production, and specific activity of solvent-forming enzymes during the early phase of growth of *C. beijerinckii* NRRL B592. (A) A550 (○), acetate (□), butyrate (■), acetone (◇), butanol (△), and pH (●). (B) Acetoacetate decarboxylase (■), butyraldehyde dehydrogenase (◇), and butanol dehydrogenase (△).
production was 12 mM (6.5 mM of acetate and 5.5 mM of butyrate) (Fig. II-3A), which is lower than the previously reported value (about 20 mM) based on a later onset time (George and Chen, 1983).

It may be expected that the level of at least some of these solvent-forming enzymes would increase before solvent production begins, and this was indeed the case as shown in Fig. II-2 and II-3. Increases in these enzyme activities started at least 40 min earlier than the apparent onset of solvent production (Fig. II-3). However, activities of all solvent-forming enzymes did not rise simultaneously. The extent and the pattern of increases in these enzyme activities were also different.

For the acetone-producing pathway of C. beijerinckii strain NRRL B592, the enzyme(s) which catalyzes acetoacetate formation from acetoacetyl CoA was present in acid-producing (exponentially growing) cells (Fig. II-2A and C). The specific activity of acetoacetate-forming enzyme(s) increased only twofold (Fig. II-2C; Table II-1), and the time of rapid increase paralleled the active solvent-producing period. The activity then decreased to below the level present in acid-producing cells (Fig. II-2C). Acetoacetate decarboxylase was also present in acid-producing cells (Fig. II-2A and B), and the activity started to increase when the activity of acetoacetate-forming enzyme(s) began to increase (Fig. II-2B and C). The specific activity of acetoacetate decarboxylase increased 124-fold during the active solvent-producing period. When more frequent samples were taken in a separate experiment (Fig. II-3B), acetoacetate decarboxylase activity was not detected in the first two samples, and the activity was rising as soon as it was detected. Acetoacetate decarboxylase activity was assayed by manometry with a detection limit of 1.4 nmol min⁻¹ mg⁻¹ protein. Therefore, it increased at least 165-fold during the 6-h experimental period (Fig. II-3A and
Table II-1. Increase of specific activities of solvent-forming enzymes between the solvent-producing stage and the acid-producing stage in C. beijerinckii NRRL B592.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Increase (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetoacetate-forming enzyme(s)</td>
<td>2</td>
</tr>
<tr>
<td>Acetoacetate decarboxylase</td>
<td>165</td>
</tr>
<tr>
<td>CoA-acylating butyraldehyde dehydrogenase</td>
<td>25</td>
</tr>
<tr>
<td>NADPH-dependent butanol dehydrogenase</td>
<td>5</td>
</tr>
</tbody>
</table>

B; Table II-1). (The manometric assay could not reliably measure a rate of gas production below 10 ul/30 min. With the protein concentration of cell extracts at about 30 mg/ml, the limit of the assay was 1.4 nmol min⁻¹ mg⁻¹ protein.)

For the butanol-producing pathway of C. beijerinckii strain NRRL B592, CoA-acylating butyraldehyde dehydrogenase and NADPH-dependent butanol dehydrogenase activities were present in acid-producing cells (Fig. II-2A and B and II-3A and B). The specific activity of butyraldehyde dehydrogenase decreased (Fig. II-2B and II-3B) before it started to increase about 40 min before the onset of butanol production (Fig. II-3B). The specific activity of NADPH-dependent butanol dehydrogenase, however, started to increase near the mid-exponential phase of growth (Fig. II-2A and B and II-3A and B), at least 1 h earlier than the onset of butanol formation. The degree of increase for butyraldehyde
dehydrogenase activity (25-fold) was much higher than that for butanol dehydrogenase (5-fold) (Table II-1).

Thiolase activity was also measured in this study, because it might interfere with the assay for acetoacetate formation from acetoacetyl-CoA. Thiolase activity was high, and the level changed little in \textit{C. beijerinckii} NRRL B592 during growth (Fig. II-2C). The distinct patterns of the activity levels of thiolase and the acetoacetate-forming enzyme(s) indicate that the latter pattern was not complicated by thiolase activity. The activity of a glycolytic enzyme, glucose-6-phosphate isomerase, was measured for comparison, and this activity remained relatively constant throughout the entire experimental period (Fig. II-2B). Thus, the observed activity changes in solvent-forming enzymes were not caused by cellular changes that might affect the extraction and assay of enzymes.

The culture pH and acid concentrations were examined with respect to the earlier point of observed activity increase of solvent-forming enzymes in \textit{C. beijerinckii} NRRL B592. This point for butanol dehydrogenase was at pH >5.7, and the acetate and butyrate (dissociated and undissociated) concentrations were 5.2 and 3.6 mM, respectively (Fig. II-3A and B). The point for acetoacetate-forming enzyme(s), acetoacetate decarboxylase and butyraldehyde dehydrogenase was at pH >5.3 (Fig. II-2A to C). The previously recognized low pH (<5) for rapid solvent production is apparently not required for the onset of expression of solvent-forming enzymes or solvent production in \textit{C. beijerinckii}. 
DISCUSSION

All of the enzymes required for acetone and butanol synthesis (Fig. II-1) have been detected in C. beijerinckii NRRL B592. The activity of butyraldehyde dehydrogenase in cell extract was NAD(H)-specific and CoA was required in the non-physiological direction. NADP⁺-linked activity was not detectable under the same assay conditions. NAD(P)⁺-linked activity of butyraldehyde dehydrogenase in strain NRRL B593 was not detected by the same procedure. However, it could be detected by measuring butyryl-CoA (see Chapter III). It is possible that the butyraldehyde dehydrogenase in strain NRRL B593 differs from that in NRRL B592. Since butanol dehydrogenase in these two strains differs significantly (Hiu et al., 1988; Yan and Chen, unpublished, see Chapter IV), it may be speculated that their sequential metabolic enzyme (Srere, 1987; Srivastava et al., 1987), butyraldehyde dehydrogenase, also differs significantly, so that the two dehydrogenases can interact closely to permit an efficient transfer of the slowly soluble metabolic intermediate, butyraldehyde. Another possibility is that butyraldehyde dehydrogenase in the two strains is similar, and the discrepancy is caused by reoxidation of NAD(P)H by other enzymes in crude extracts so that it is not detectable in crude extracts of NRRL B593 when the formation of NAD(P)H is assayed (see Discussion in Chapter III).

The first step in the acetone-producing pathway is the formation of acetoacetate from acetoacetyl-CoA. Since it has been reported after this growth study that CoA transferase from C. acetobutylicum was unstable without ammonium sulfate and glycerin (Weisenborn et al., 1989), and similar results were obtained with C. beijerinckii (Colby and Chen, unpublished results), the results of the expression of acetoacetate-forming enzyme(s) was not conclusive based on the activity measured under the conditions where the enzyme was
not stabilized (see Chapter VI). However, it is not clear how the pattern of the expression of the acetoacetate-forming enzyme(s) might change under conditions where the enzyme(s) is stablized.

It has been reported that acetoacetate decarboxylase in C. acetobutylicum can be induced by linear acids from C\textsubscript{1} to C\textsubscript{4} (at external concentrations above 5 mM and at pH 4.8), with acetoacetic acid being the most effective (Bollongue et al., 1985). However, it is not clear if acetoacetic acid at pertinent intracellular concentrations is as effective. Our results show that (i) the formation of acetone follows the appearance of (or a large increase in ) acetoacetate decarboxylase activity in C. beijerinckii, (ii) active acetone production paralleled a sustained increase in acetoacetate decarboxylase activity, and (iii) the activity increase in the acetoacetate-forming enzyme(s) was transient under the conditions for crude extract preparation and enzyme assay in this study. These findings seem to suggest that the start of acetone production is controlled by the expression of acetoacetate decarboxylase activity and that the latter is influenced by the activity of acetoacetate-forming enzyme(s). Thus, an increased expression of the acetoacetate-forming enzyme(s) is perhaps an early event of the solventogenic switch (for the acetone branch).

Butyraldehyde dehydrogenase and butanol dehydrogenase are enzymes specific for the butanol-forming pathway. With C. acetobutylicum, butyraldehyde dehydrogenase was not detectable in acid-producing cells (Andersch et al., 1983; Rogers, 1986; Durre et al., 1987), but low levels of butanol dehydrogenase were detected in acid-producing cells (Durre et al., 1987). Both butyraldehyde dehydrogenase and NADP-dependent butanol dehydrogenase were detected in acid-producing C. beijerinckii cells. A low level of butanol dehydrogenase (4% of the highest activity) was detected in exponentially growing cells in CMC cultures of
C. beijerinckii NRRL B592 two generations after outgrowth from spores (data not shown).
The point at which the specific activity of butanol dehydrogenases began to increase was earlier than that of butyraldehyde dehydrogenase, although butanol dehydrogenase catalyzes the last step for butanol formation. It appears that in C. beijerinckii NRRL B592, butanol production occurs after the level of butyraldehyde dehydrogenase has increased, and the mode of regulation for butyraldehyde dehydrogenase and butanol dehydrogenases was different. In C. acetobutylicum DSM 1732, a difference in the pattern of activity changes for the two dehydrogenases was reported (Durre et al., 1987), but the scatter of the measured enzyme activities made the assignment of the onset of the activity increases difficult.
SUMMARY

Clostridium beijerinckii NRRL B592 was grown in batch cultures without pH control. More sensitive and accurate procedures for the determination of acids and solvents in cultures have been developed, which led to the recognition of the onset of solvent production at the mid-exponential phase, about two h earlier than the previously reported point and at a higher culture pH (>5.3). Reliable assay procedures for enzyme activities required for solvent production in cell-free extracts have also been developed. The results showed that activities of these enzymes started to increase about one h before the measured onset of solvent production and that the increase in activities of solvent-forming enzymes was not simultaneous. The degree of increase in these enzyme activities also varied, under the enzyme assay conditions used, with the acetoacetate-forming enzyme(s) and NADPH-dependent alcohol dehydrogenase showing the least (2-fold and 5-fold, respectively) and acetoacetate decarboxylase (>165-fold) showing the greatest increases (Table II-1).

Although the increase in acetoacetate-forming enzyme activity was not large, it could represent an early event of the solventogenic switch and its expression might affect the expression of acetoacetate decarboxylase. These findings will be useful in selection of solvent-forming genes to be studied in an effort to characterize promoters specific for solvent fermentation. Since the onset of expression of solvent-forming enzymes and the onset of solvent production are much earlier (in relation to growth) than the previously recognized time, investigations of extra- and intracellular conditions as possible triggering signals for the metabolic transition can now be carried out with cultures or cells at a more pertinent stage of growth. It may also be worthwhile to determine whether a differentiation can be made between conditions that are most effective for triggering the onset of solvent
production (enzyme expression) and conditions that are optimal for the ensuing solvent production. This information is also useful for enzyme purification since we can harvest the culture when the enzyme level is high and the cell is still sensitive to lysozyme.
REFERENCES


Chapter III

Coenzyme A-Acylating Aldehyde Dehydrogenase from *Clostridium beijerinckii*

NRRL B592

INTRODUCTION

Aldehyde dehydrogenase (ALDH) activity is present in both eukaryotic and prokaryotic organisms, and several forms of the ALDH have been found in different subcellular locations of mammalian tissues (Jakoby, 1963; Tipton, 1985) or in bacterial cells grown under different conditions (Jones and Turner, 1985). ALDH may be distinguished on the basis of their coenzyme or substrate requirements: (i) the CoA-independent but NAD(P)^+^-dependent ALDH, which is present in animals, plants, yeasts, and bacteria (Steinman and Jakoby, 1968; Takeuchi and Uritani, 1981; Peterson and LaRue, 1982; Tipton, 1985; Janssen et al., 1987), (ii) the CoA- and NAD(P)^+^-independent ALDH, which is present in some bacteria (Patel et al., 1980; Muraoka et al., 1982; Poels et al., 1987), and (iii) the CoA-acylating, NAD(P)-linked ALDH, which has been found in several bacteria (Burton and Stadtman, 1953; Rudolph et al., 1968; Kazahaya et al., 1972; Hosoi et al., 1979; Smith and Kaplan, 1980; Byers and Meighen, 1984; Palosaari and Rogers, 1988; Yan et al., 1988), a green alga (Kreuzberg, 1985), and a fish (Griffith et al., 1981).

CoA-acylating ALDH (EC 1.2.1.10) was first detected in *Clostridium kluyveri* (Burton and Stadtman, 1953), which catalyzes the following reaction:
Aldehyde + CoA + NAD(P)^+ \rightleftharpoons \text{Acyl CoA} + \text{NAD(P)H} + H^+

The enzyme has been partially purified (about 40% pure) from \textit{C. kluveri} strain K-1, and the preparation contained little alcohol dehydrogenase activity (Smith and Kaplan, 1980). However, ALDH has also been isolated as a complex with alcohol dehydrogenase from \textit{C. kluveri} strain DSM 555 (Lurz et al., 1979) and from \textit{Leuconostoc mesenteroides} (Kazahaya et al., 1972). A CoA-acylating ALDH has been extensively purified from \textit{Escherichia coli}, and the preparation contained a significant level of alcohol dehydrogenase activity (Rudolph et al., 1968; Shone and Fromm, 1981). These ALDHs require a thiol compound (besides CoA) to show activity. In addition, the oxidized \textit{E. coli} enzyme further requires incubation with a thiol compound and NAD^+ to be activated (Rudolph et al., 1968; Shone and Fromm, 1981). Activity of CoA-acylating ALDH from other sources has also been measured after a preincubation with a thiol compound and substrates (Kazahaya et al., 1972; Hosoi et al., 1979; Palosaari and Rogers, 1988), but whether the preincubation was necessary was not reported.

The physiological role for the various forms of ALDH has not been fully defined (Weiner, 1979), but an ALDH is usually involved in alcohol metabolism. In solvent-producing \textit{Clostridium acetobutylicum} and \textit{Clostridium beijerinckii}, ALDH catalyzes the first specific reaction in the pathways leading to the formation of ethanol and \(n\)-butanol (Fig. II-1). A CoA-acylating ALDH, which is induced when cells switch from acid production to solvent production (Andersch
et al., 1983; Palosaari and Rogers, 1988), has been purified from *C. acetobutylicum* NRRL B643 (Palosaari and Rogers, 1988), and the preparation contained no alcohol dehydrogenase activity.

In our study of the expression of solvent-forming enzymes in *C. beijerinckii* NRRL B592, the CoA-acylating ALDH was found to be a regulated enzyme (Yan et al., 1988). The activity level of ALDH increased 25-fold, whereas that of alcohol dehydrogenase increased 5-fold, during the switch from acid production to solvent production. In this paper, we report the purification and properties of the CoA-acylating ALDH from this organism.
MATERIALS AND METHODS

Materials. Tryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI); DNase I, lysozyme, coenzyme A (CoA), acetyl CoA, butyryl CoA, NAD⁺, NADP⁺, NADH, NADPH, immunoglobulin G (human), RNase A, bovine serum albumin, thyroglobulin (bovine), Cibacron Blue 3GA-Agarose (Type 3000), 2(N-morpholino)ethanesulfonic acid (MES), 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), and Trizma base were obtained from Sigma Chemical Co. (St. Louis, Mo); 3-(N-morpholino)-propanesulfonic acid (MOPS) was obtained from Calbiochem Co. (La Jolla, CA); dye-binding protein assay kit, sodium dodecyl sulfate (SDS), and SDS-polyacrylamide gel electrophoresis (PAGE) standards, low range (Mᵋ 10,000-100,000) were obtained from Bio-Rad Laboratories (Richmond, CA); Matrex Gels Red A and ultrafiltration membranes were obtained from Amicon Corp. (Lexington, MA); Coomassie brilliant blue G250, α-toluenesulfonyl fluoride, and butyaldehyde were obtained from Eastman Kodak Co. (Rochester, NY); acetaldehyde was obtained from Fisher Scientific Co. (Pittsburgh, PA); Centricon-30 Microconcentrator was obtained from Amicon Division, W.R. Grace & Co. (Danvers, MA).

Organisms and growth conditions. C. beijerinckii ("Clostridium butylicum") NRRL B592 (VPI 13436) was used in this study. Growth conditions were as described in Chapter II except that cells for enzyme purification were harvested
when A_{550 nm} reached 7. Cell paste was stored in liquid nitrogen.

**Preparation of crude extracts.** Cell paste was thawed under argon in 50 mM Tris acetate (pH 7) at the ratio of 1 g cells per 2 ml of buffer. DNase I (0.1 mg/ml), dithiothreitol (5 mM), lysozyme (2 mg/ml), and α-toluenesulfonyl fluoride (0.3 mg/ml every 30 min) were added to the cell suspension. The cell suspension was incubated at room temperature for 2 hours. Cell debris was removed by centrifugation at 37,000 x g for 30 min at 4°C. The supernatant (crude extract) was stored as frozen pellets in liquid nitrogen if not immediately used.

**Protein determination.** Protein was determined by the dye-binding assay (Bradford, 1976), with bovine plasma gamma globulin as a standard.

**Enzyme assays.** Crude extracts of *C. beijerinckii* had a high NAD(P)H-oxidizing (diaphorase) activity in air, which necessitated the use of anaerobic conditions for assaying ALDH activities in unfractionated crude extracts. Anaerobically purified ALDH (containing 5 mM DTT) could be assayed in air. In routine assays, ALDH activity was measured anaerobically in the non-physiological direction. The assay mixture (1 ml final volume) contained 50 mM potassium CHES buffer (pH 8.6), 5 mM DTT, 0.5 mM CoA, 2 mM NAD^+, 0.01 to 0.05 U ALDH, and either 11 mM butyraldehyde (diluted 10-fold with methanol) or 18 mM acetaldehyde (diluted 10-fold with deoxygenated water kept on ice). Acetaldehyde used was without the NAD(P)H-reacting substance(s)
(Yan et al., 1987). The reaction was initiated by the addition of aldehyde.

ALDH activity was also determined by measuring the formation of butyryl-CoA under the condition of routine assays; butyryl-CoA was measured after conversion into hydroxamic acid (Stadtman, 1957). Alcohol dehydrogenase activity was assayed in the physiological direction. The assay mixture (1 ml final volume) contained 5.5 mM butyraldehyde (diluted 10-fold with methanol) and either 50 mM Tris chloride buffer (pH 7.5) and 0.2 mM NADPH or 50 mM potassium MES buffer (pH 6.0) and 0.2 mM NADH. The reaction was initiated with butyraldehyde.

**Purification of ALDH.** A 50 mM Tris acetate buffer (pH 7) containing 5 mM DTT and 20% (vol/vol) glycerol [hereafter referred to as the Tris acetate buffer] was used throughout the purification. All operations were performed at 0-4°C under anaerobic conditions using argon (Chen and Blanchard, 1979; Jin et al., 1983). Active fractions were stored at 4°C overnight between purification steps. A typical purification is described below.

**(i) DEAE-cellulose column.** A crude extract (3.2 g protein) was loaded onto a Whatman DE-52 column (2.6 by 8.5 cm) which had been equilibrated with Tris acetate buffer. The column was then washed with one bed volume of the same buffer and eluted with a linear gradient of KCl (0 to 0.2 M in the same buffer; total volume, 300 ml). The flow rate was 50 ml/h, and 10-ml fractions were collected.
(ii) **Matrex Gel Red A column.** Active fractions from the DE-52 column step were pooled and applied to a Matrex Gel Red A column (1.4 by 8.5 cm) which had been equilibrated with Tris acetate buffer. The column was washed with one bed volume of the same buffer and then with a linear gradient of KCl (0 to 1 M in the same buffer; total volume, 90 ml). The flow rate was 25 ml/h, and 4-ml fractions were collected.

(iii) **Cibacron Blue 3GA-Agarose column.** To lower the concentration of KCl in the active fractions from the Matrex Gel Red A column step, the pooled fractions were diluted to 1.5 volume with Tris acetate buffer. The diluted sample was loaded onto a Cibacron Blue 3GA-Agarose column (1.4 by 7.5 cm) which had been equilibrated with Tris acetate buffer containing 0.2 M KCl. The column was washed with one bed volume of the equilibrating buffer and then washed stepwise with one bed volume each of 2, 3, 4, and 5 mM NAD$^+$ in the same buffer. ALDH was eluted during the elution with 3 to 4 mM of NAD$^+$. Purified enzyme was kept as frozen pellets in liquid nitrogen.

**Dialysis of purified ALDH.** To reduce the concentration of DTT in the enzyme sample, the purified ALDH (0.2 ml containing 5 mM DTT) was first diluted 5-fold with Tris acetate buffer containing 0.2 M KCl. It was then dialyzed against one liter of the same buffer at 4°C for 7.5 h under argon. The final DTT level in the sample was estimated at 30 µM, based on the result of dialysis of a one mM solution of bromophenol blue under identical conditions.
In the presence of 1 mM DTT, ALDH in the diluted sample was not inactivated by \( O_2 \).

**Determination of the native \( M_r \).** The native \( M_r \) of ALDH was determined with, respectively, a crude extract and the purified enzyme by gel filtration under anaerobic conditions. The sample was passed through a Sephacryl S-300 column (2.5 by 44 cm) which was equilibrated and eluted with Tris acetate buffer (pH 7) containing 0.1 M KCl. Molecular weight standards used were RNase A (13,700), bovine serum albumin (66,000), human IgG (160,000), and thyroglobulin (669,000).

**SDS-PAGE.** To examine the purity and to determine the subunit molecular weight of ALDH, SDS-PAGE was performed following Weber and Osborn (1969). Protein bands were detected by staining with Coomassie blue. Molecular weight standards used were lysozyme (14,400), soybean trypsin inhibitor (21,500), bovine carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,000), and rabbit muscle phosphorylase b (97,400).

**Determination of N-Terminal Amino Acid Sequence.** Purified ALDH (200 \( \mu \)g of protein) was desalted with 4 changes of distilled water with a Centricon-30 apparatus (Amicon) at a centrifugation speed of 3,000 \( x \) g. The desalted sample was analyzed with an Applied Biosystems Model 477A sequencer by Dr. C. L. Rutherford and R. Peery of the Protein Sequencing Facility, VPI & SU, Blacksburg, VA.
**Kinetic Studies.** To determine the $K_m$ and $V_{max}$ of ALDH for NAD(P)H, acetyl CoA, and butyryl CoA (the physiological direction), enzyme activities were measured in 50 mM potassium MES buffer, pH 6.5, with 21 μg of purified enzyme. NAD(P)H was used as the variable substrate, and acetyl CoA or butyryl CoA was used as the changing fixed substrate. $K_m$ and $V_{max}$ values were determined from a secondary plot using intercepts from the primary double reciprocal plot. To determine the apparent $K_m$ and $V_{max}$ of the ALDH for CoA, NAD(P)⁺, and butyraldehyde, enzyme activities were measured in 50 mM glycylglycine buffer, pH 9, containing 5 mM DTT and 8 μg of purified enzyme. To determine the apparent $K_m$ and $V_{max}$ for CoA, 11 mM butyraldehyde and either 0.32 mM NAD⁺ or 4 mM NADP⁺ were used; for butyraldehyde, either 0.32 mM NAD⁺ and 0.12 mM CoA or 4 mM NADP⁺ and 0.2 mM CoA were used; for NAD⁺, 0.12 mM CoA and 11 mM butyraldehyde were used; for NADP⁺, 0.2 mM CoA and 11 mM butyraldehyde were used.
RESULTS

Stability of ALDH. The stability of ALDH activity was examined first to determine conditions under which the enzyme may be purified. ALDH in crude extracts was sensitive to O₂ (see below). Therefore, anaerobic conditions were used in this study. Crude extracts (prepared in 50 mM Tris acetate, pH 8, with 2 mM DTT) were diluted 20-fold in test buffers (50 mM) at the specified pH and incubated at 4°C under argon. ALDH of C. beijerinckii NRRL B592 was found more stable at pH 7 than at pH 6, 8, or 9 in either Tris acetate or potassium phosphate buffer (data not shown). At the same pH, the enzyme was more stable in Tris acetate buffer than in potassium phosphate buffer. For example, at pH 7, the half-life of the enzyme in a diluted crude extract (1.2 mg protein per ml) was 3 days in Tris acetate buffer and 2 days in potassium phosphate buffer. Also, a 3-fold increase in specific activity (or 3.5-fold increase in total units) was obtained with crude extracts prepared in Tris acetate buffer than in potassium phosphate buffer.

Glycerol at 20% (vol/vol) further stabilized ALDH in 50 mM Tris acetate buffer at pH 7, as no enzyme activity was lost after 3 days at 4°C under argon. With Tris acetate buffer at pH 7, the enzyme became less stable at higher buffer concentrations (within the range of 10 mM to 150 mM). However, addition of 140 mM KCl to 10 mM Tris acetate stabilized the enzyme as no activity was lost after 3 days, whereas 46% of activity was lost during the same period in 10 mM
Tris acetate alone.

**Purification of ALDH.** Buffers containing glycerol and KCl were used throughout the chromatographic steps because ALDH was stabilized by glycerol and KCl in Tris acetate buffer (pH 7). All purification steps were carried out under anaerobic conditions. The result of purification is summarized in Table III-1.

**Table III-1. Purification of aldehyde dehydrogenase from *C. beijerinckii* NRRL B592.**

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>Total unitsa</th>
<th>Specific activity (U/mg)</th>
<th>Fold</th>
<th>Yield (%)</th>
<th>Activity ratiob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>3195</td>
<td>169</td>
<td>0.053</td>
<td>1</td>
<td>100</td>
<td>12</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>186</td>
<td>125</td>
<td>0.672</td>
<td>13</td>
<td>74</td>
<td>11</td>
</tr>
<tr>
<td>Matrex Gel Red A</td>
<td>60</td>
<td>88</td>
<td>1.47</td>
<td>28</td>
<td>52</td>
<td>12</td>
</tr>
<tr>
<td>Cibacron Blue 3GA-Agarose</td>
<td>30</td>
<td>60</td>
<td>2</td>
<td>38</td>
<td>36</td>
<td>11</td>
</tr>
</tbody>
</table>

a One unit (U) is the production of one μmol of NADH per min in the routine assay with butyraldehyde.

b The ratio of activities measured with butyraldehyde and acetaldehyde as alternative substrates.

The scheme gave an overall purification of 38-fold with a 36% yield of activity from crude extracts of *C. beijerinckii* NRRL B592. Crude extracts with a lower
starting specific activity gave a higher fold of purification (up to 150-fold) when a similar final activity was obtained by the same purification scheme (data not shown); variable starting activities may be caused partly by reoxidation of NADH in assays (see Discussion).

Comparable activities were obtained when purified ALDH was monitored for the formation of NAD(P)H or butyryl-CoA. Activities measured with butyraldehyde and acetaldehyde were copurified with a constant ratio (Table III-1), which resembled the \textit{C. acetobutylicum} ALDH (Palosaari and Rogers, 1988). Both NADH- and NADPH-linked alcohol dehydrogenase activities were present in \textit{C. beijerinckii} strain B592 (R.-T. Yan and J.-S. Chen, unpublished results; see Chapter IV), but purified ALDH contained neither NADH- nor NADPH-linked alcohol dehydrogenase activity. SDS-PAGE (Fig. III-1) showed a single, intense band when 30 \( \mu \text{g} \) of ALDH was examined for purity.

In addition to its stabilizing effect, glycerol also changed the elution properties of ALDH from the DE-52 column. In the presence of 20\% (vol/vol) glycerol, ALDH was eluted by a lower concentration of KCl and in a much narrower range, which resulted in the separation of the enzyme from the bulk of eluted proteins. A 13-fold of purification was obtained by this step alone, in contrast to the 3-fold obtained when glycerol was absent (data not shown).

\textbf{Native and subunit molecular weights.} ALDH showed a native \( \text{M}_n \) of 100,000. SDS-PAGE gave a subunit \( \text{M}_r \) of 55,000, suggesting that the native
Figure III-1. Examination of purity by SDS-page of ALDH purified from *C. beijerinckii* NRRL B592. 30 µg of protein was loaded. Protein was detected by Coomassie brilliant blue stain.
enzyme is dimeric.

**pH profile.** For the forward reaction, the NADH-linked activity showed a broad peak between pH 6 and 7, whereas the NADPH-linked activity showed a broad peak between pH 6.5 and 7.5 (Fig. III-2A). For the reverse reaction, both \( \text{NAD}^+ \) - and \( \text{NADP}^+ \)-linked activities increased when pH increased from 6.5 to 9.5 (Fig. III-2B). The ratio of NAD(H)- to NADP(H)-linked activities increased when the pH was lowered from about 8 to 6 (Fig. III-2B and III-3B).

**Kinetic studies.** The CoA-independent oxidation of acetaldehyde to acetate has a free energy change of \(-12.5 \text{ kcal} (-52.3 \text{ kJ})/\text{mol at pH 7} \) (Burton and Stadtman, 1953; Weiner, 1979). The energetics do not favor the reduction of acids to aldehydes, which explains why the CoA-independent ALDH catalyzes only the oxidation of aldehydes under measurable physiological concentration of substrates. The CoA-acylating reaction of ALDH has a free energy change of \(-4.2 \text{ kcal} (-17.6 \text{ kJ})/\text{mol at pH 7} \) (Burton and Stadtman, 1953), which makes the reduction of acyl-CoA to aldehydes energetically less unfavorable than the reduction of acids to aldehydes. Under conditions used in this study, the CoA-acylating ALDH readily catalyzed the forward and reverse reactions. Therefore, initial velocity measurements were conducted with the purified *C. beijerinckii* ALDH to determine the respective \( K_m \) and \( V_{max} \) values for substrates of both reactions.

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Figure III-2. pH profile of ALDH activity of *C. beijerinckii* NRRL B592. (A) Profile in the physiological direction. The reaction mixture (1 ml) contained 0.2 mM butyryl CoA and 21 μg of protein in 50 mM potassium MES (pH 5.5-7) or 50 mM potassium MOPS (pH 6.5-8). The coenzyme was 0.2 mM NADH (○,●) or 0.2 mM NADPH (△,▲). (B) Profile in the nonphysiological direction. The reaction mixture contained 0.5 mM CoA, 5 mM DTT, 11 mM butyraldehyde, and 21 μg of protein in 50 mM potassium MOPS (pH 6.5-7.5) or 50 mM potassium glycyglycine (pH 7.5-9.5). The coenzyme was 2 mM NAD⁺ (○,●) or 2 mM NADP⁺ (△,▲).
In the forward (physiological) direction, the true $K_m$ and $V_{max}$ values were determined for acetyl CoA, butyryl CoA, NADH, and NADPH (Table III-2). A reciprocal plot (Fig. III-3A), in which $1/v$ was plotted against the reciprocal of either NADH or NADPH concentrations and with changing butyryl CoA levels, yielded apparently parallel lines. A secondary plot of intercepts versus the reciprocal of butyryl CoA concentrations are shown in Fig. III-3B. Similar plots were obtained with acetyl CoA or with NAD(P)H as the coenzyme (not shown).

Table III-2. Kinetic constants for the forward reaction of ALDH from *C. beijerinckii* NRRL B592.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (U/mg)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl CoA (with NADH)</td>
<td>0.154</td>
<td>0.034</td>
<td>0.22</td>
</tr>
<tr>
<td>NADH (with acetyl CoA)</td>
<td>0.0082</td>
<td>0.034</td>
<td>4.3</td>
</tr>
<tr>
<td>Acetyl CoA (with NADPH)</td>
<td>0.0196</td>
<td>0.0174</td>
<td>0.89</td>
</tr>
<tr>
<td>NADPH (with acetyl CoA)</td>
<td>0.206</td>
<td>0.0174</td>
<td>0.085</td>
</tr>
<tr>
<td>Butyryl CoA (with NADH)</td>
<td>0.166</td>
<td>0.238</td>
<td>1.4</td>
</tr>
<tr>
<td>NADH (with butyryl CoA)</td>
<td>0.0076</td>
<td>0.238</td>
<td>31</td>
</tr>
<tr>
<td>Butyryl CoA (with NADPH)</td>
<td>0.0715</td>
<td>0.345</td>
<td>4.8</td>
</tr>
<tr>
<td>NADPH (with butyryl CoA)</td>
<td>0.0673</td>
<td>0.345</td>
<td>5.1</td>
</tr>
</tbody>
</table>
Figure III-3. Initial velocity pattern in the physiological direction with NADH as the variable substrate and with butyryl CoA as the changing fixed substrate. (A) Reciprocal plot of initial velocity data. See Materials and Methods for assay conditions. NADH concentrations ranged from 0.001 to 0.04 mM. Butyryl CoA concentrations were 0.065 (▲), 0.1 (△), 0.2 (●), and 1 mM (○). (B) Secondary plot of y intercepts from panel A against reciprocal of butyryl CoA concentrations.
The $V_{\text{max}}$ and $V_{\text{max}}/K_m$ values were much higher with butyryl CoA than with acetyl CoA (Table III-2). This is consistent with the production of less ethanol than butanol by $C$. beijerinckii. With butyryl CoA, the $V_{\text{max}}$ was higher and $K_m$ lower when NADPH, rather than NADH, was the coenzyme. However, the $K_m$ for NADPH was about nine-fold higher than that for NADH, and the $V_{\text{max}}/K_m$ with respect to the coenzyme was about six-fold lower with NADPH than with NADH. Therefore, NADH is a more effective coenzyme than NADPH for ALDH.

The reverse reaction of ALDH is a terreactant reaction, involving an aldehyde, CoA, and NAD$^+$ or NADP$^+$. Pronounced substrate inhibition occurred when any one substrate exceeded a threshold level while the other two were at related threshold levels as given below: with NAD$^+$ as the coenzyme, 0.34 mM for NAD$^+$, 0.12 mM for CoA, and 10 mM for butyraldehyde; with NADP$^+$ as the coenzyme, 4 mM for NADP$^+$, 0.04 mM for CoA, and 15 mM for butyraldehyde, respectively (Fig. III-4A-F). The apparent $K_m$ and $V_{\text{max}}$ values with respect to these substrates were measured with the fixed substrates kept below the inhibitory level (Table III-3). For the reverse reaction, the apparent $K_m$ for NAD$^+$ was about eight-fold lower than that for NADP$^+$. The apparent $K_m$ values for butyraldehyde and CoA were also lower with NAD$^+$ than NADP$^+$ as the coenzyme. The true $K_m$ values and the true $V_{\text{max}}$ for the reaction in the non-physiological direction will await further statistical analysis (Cleland, 1979).
Figure III-4. Initial velocity pattern in the non-physiological direction with CoA, butyraldehyde, NAD\(^+\), or NADP\(^+\) as the variable substrate. NAD\(^+\) was the coenzyme for reciprocal plots (A), (B), and (C). NADP\(^+\) was the coenzyme for reciprocal plots (D), (E), and (F). See Materials and Methods for assay conditions.
Table III-3. Apparent kinetic constants for the reverse reaction of ALDH from \( C. \) beijerinckii NRRL B592.

<table>
<thead>
<tr>
<th>Substrate (U/mg)</th>
<th>Apparent ( K_m ) (mM)</th>
<th>Apparent ( V_{max} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyraldehyde</td>
<td>3.7</td>
<td>6.0</td>
</tr>
<tr>
<td>( 0.32 \text{ mM NAD}^+ , 0.12 \text{ mM CoA} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butyraldehyde</td>
<td>5.0</td>
<td>8.3</td>
</tr>
<tr>
<td>( 4 \text{ mM NADP}^+ , 0.2 \text{ mM CoA} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>0.064</td>
<td>6.4</td>
</tr>
<tr>
<td>( 0.32 \text{ mM NAD}^+ , 11 \text{ mM butyraldehyde} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coenzyme A</td>
<td>0.14</td>
<td>27</td>
</tr>
<tr>
<td>( 4 \text{ mM NADP}^+ , 11 \text{ mM butyraldehyde} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{NAD}^+ )</td>
<td>0.25</td>
<td>8.2</td>
</tr>
<tr>
<td>( 0.12 \text{ mM CoA, 11 mM butyraldehyde} )</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{NADP}^+ )</td>
<td>2.1</td>
<td>9.4</td>
</tr>
<tr>
<td>( 0.2 \text{ mM CoA, 11 mM butyraldehyde} )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Parallel lines in double reciprocal plots of initial velocities versus \( \text{NAD(P)H} \) as the varying substrate and acetyl CoA or butyryl CoA as the changing fixed substrate (Fig. III-3A and data not shown) suggest a ping pong mechanism for the reaction (Cleland, 1963). This is supported by a linear line in plots of (i) \( 1/v \) versus the reciprocal of butyryl CoA concentrations where \( \text{NADH} \) and butyryl CoA were held in a fixed ratio of 1:22 (the forward reaction; butyryl-CoA concentrations were at 0.166, 0.216, 0.310, 0.432, and 0.830 mM [data not shown]) and (ii) \( 1/v \) versus the reciprocal of CoA concentrations, in which \( \text{NAD}^+ \) and CoA were held in a fixed ratio of 1:0.24 (the reverse reaction; CoA...
concentrations were at 0.044, 0.060, 0.12, 0.18, and 0.60 mM [data not shown]) (Dixon and Webb, 1979). A ping pong mechanism has been proposed for the reaction catalyzed by CoA-acylating ALDH of *E. coli* (Rudolph et al., 1968; Shone and Fromm, 1981) and *C. kluveri* (Smith and Kaplan, 1980). The order of addition for the proposed bi-uni-uni-uni ping pong mechanism is:

\[
\begin{array}{cccccc}
NAD(P)^+ & \text{Aldehyde} & NAD(P)H & \text{CoA} & \text{Acyl CoA} & \text{E} \\
\downarrow & \downarrow & \uparrow & \downarrow & \uparrow & \text{E}
\end{array}
\]

For the *C. beijerinckii* ALDH, the order of interaction with NAD(P)^+ and aldehyde remains to be established. However, it is likely that NAD(P)H, CoA, and acyl CoA interact with the enzyme in the same order as proposed for the *E. coli* and *C. kluveri* enzymes because the enzyme is not expected to complete a redox reaction with NAD(P)H before it reacts with acyl CoA (in the forward direction and with a ping pong mechanism).

For the ALDH of *C. beijerinckii* NRRL B592, substrate inhibition was observed only in the direction of butyryl CoA formation from butyraldehyde and CoA (Fig. III-4A-F), which is consistent with the fact that substrate inhibition is much more common in the non-physiological direction (Cleland, 1979). We further measured the ALDH activity at higher than threshold levels of NAD^+ and CoA, but with the ratio of the two substrates held constant. No inhibition was observed with increasing concentrations of NAD^+ (up to 2.5 mM) and CoA (up to 0.6 mM) when the NAD^+/CoA ratio was kept at 1:0.24 (data not shown).
The result suggests that the substrate inhibition is double competitive in nature; competitive substrate inhibition is usually found in ping pong mechanisms (Cleland, 1979). However, CoA-acylating ALDHs isolated from *C. kluveri* and *C. acetobutylicum* were only inhibited by CoA, but not by NAD⁺ or aldehyde (Palosaari and Rogers, 1988; Smith and Kaplan, 1980). No substrate inhibition was reported for ALDH of *E. coli* (Rudolph et al., 1968).

**Inactivation of ALDH by O₂ and reactivation.** ALDH was sensitive to O₂ in either crude extracts or the purified form (Fig. III-5) when DTT was less than 1 mM. DTT at 5 mM protected ALDH in crude extracts from O₂ inactivation for at least 2 h. Activity of the O₂-inactivated enzyme in a crude extract could be partially restored (75%) by deoxygenation and incubation with DTT. The degree of reactivation depended on the conditions used (Fig. III-5), as CoA is expected to be present in crude extracts. Incubation of the inactivated (purified) ALDH with CoA alone resulted in significant reactivation, whereas DTT alone caused much less reactivation. CoA and DTT together restored a higher activity than CoA. The reactivating effect of CoA may explain why addition of DTT alone afforded a greater degree of reactivation in crude extracts (75%) than in the purified sample (Fig. III-5), as CoA is expected to be present in crude extracts. Reactivation of ALDH by CoA had not been reported before, and the synergistic effect of DTT with CoA was also unexpected. The reactivating effect of these compounds was examined further with purified ALDH.
Figure III-5. Inactivation by $O_2$ and reactivation of purified ALDH from _C. beijerinckii_ NRRL B592. Dialyzed ALDH (0.3 ml) was gently bubbled with air for 1 min and then incubated on ice (○, ●). ALDH activity was measured in the reverse reaction at the indicated times. In one set of experiment (●, △), DTT was omitted from the assay mixture. After 20 min (indicated by arrow), the vial containing the aerated sample was evacuated and refilled with argon for 10 cycles, and portions (5 to 10 μl) of the deoxygenated sample were incubated, under argon and at room temperature, in one ml of glycylglycine buffer (50 mM; pH 9) containing the indicated compound(s). Activity was measured after the indicated length of incubation. Incubation was carried out with 5 mM DTT (△, ■), 0.12 mM CoA (□), or 5 mM DTT plus 0.12 mM CoA (■). At the end of incubation, 0.5 mM of NAD$^+$ and a compound(s) not already present in the assay buffer were added to make up the assay mixture. The reaction was initiated by the addition of 11 mM butyraldehyde. The purified and undialyzed ALDH had an activity of 4.9 U/mg (◇).
We first examined the effect of DTT in assay mixtures on ALDH activity. When measured in anaerobic assays containing 5 mM DTT, O₂-treated ALDH seemed to retain part of its activity (Fig. III-5). However, omission of DTT from the assay mixture showed that no activity remained after ALDH was exposed to air for 10 min. After the inactivated ALDH was incubated with DTT for 20 min, a comparable activity was obtained in assays with and without 5 mM DTT (Fig. III-5). The results suggest that, in the routine assay mixture (with substrates and 5 mM of DTT), a very rapid reactivation of ALDH occurred, but ALDH was not reactivated to the more active state obtained with CoA alone or with CoA plus DTT. Also, the DTT-reactivated ALDH was not further activated in the assay mixture containing DTT. Thus, the apparent degree of reactivation by DTT was much less when the assay mixture contained 5 mM DTT; and this assay condition did not allow an accurate measurement of the extent of O₂ inactivation.

We then compared aerobic and anaerobic assay mixtures for any effect of O₂, along with DTT, on the measured activity. The assay mixture contained sodium glycylglycine buffer (50 mM, pH 9), NAD⁺,(0.5 mM), CoA (0.12 mM), and butyraldehyde (11 mM). DTT was added to 0, 0.05, 0.5, 5, and 10 mM in the assay mixture. For undialyzed ALDH, which carried DTT into the assay mixture to give a calculated final concentration of 50 μM, the same activity (5.6 U/mg) was obtained under both aerobic and anaerobic conditions and with or without added DTT. After dialysis and with the amount of DTT in the ALDH sample
lowered to 30 μM or less, an increase in activity (from 0.88 to 0.19 U/mg) was observed when > 50 μM DTT was added to the assay mixture, but no difference was found between aerobic and anaerobic conditions. After dialyzed ALDH was exposed to air for 10 min, it required the presence of >50 μM DTT in the anaerobic assay mixture to show activity (1 U/mg). Thus, ALDH was less sensitive to O₂ in the presence of CoA and NAD⁺ than in their absence (about 80% loss in 2 min; Fig. III-5). It also suggests that a low level of a thiol compound (e.g., about 50 μM of DTT) in the assay mixture is sufficient for maintaining ALDH activity, which may explain why a thiol compound was not required for the assay of the purified enzyme from C. acetobutylicum, as the enzyme sample contained 10 mM of 2-mercaptoethanol (Palosaari and Rogers, 1988). For the O₂-inactivated ALDH, DTT (5 mM) was required in the anaerobic assay to show activity, although CoA (0.5 mM), which is also a thiol compound, was present in the assay.

Dialysis of the purified enzyme caused about a 70% loss in enzyme activity, but the activity was increased about two-fold when 50 μM or more of DTT was present in the assay, which suggests an oxidative inactivation of the enzyme during dialysis. The dialyzed (partially inactivated) ALDH was used in a study of the effect of preincubation with CoA, DTT, or NAD⁺ on enzyme activity. Butyraldehyde was not tested because the reaction was normally initiated by this substrate.
Activity of dialyzed ALDH was increased by incubation (20 min) with CoA alone, with NAD$^+$ alone, with CoA, NAD$^+$, and DTT, or with any two of these compounds (Table III-4). In contrast, there was no change in activity when the undialyzed enzyme (with 5 mM DTT) was incubated with CoA, DTT, and NAD$^+$ for up to 25 min prior to the initiation of the reaction. Four levels of activation may be recognized with the dialyzed ALDH: (i) no activation was seen with either DTT or NAD$^+$ alone; (ii) an activation of about 1.7-fold was obtained with NAD$^+$ plus CoA, NAD$^+$ plus DTT, or a combination of NAD$^+$, CoA, and DTT, respectively; (iii) an activation of about 2.7-fold was obtained with CoA alone; (iv) an activation of about 5.7-fold was obtained with CoA plus DTT. It was not expected that CoA plus DTT activated the enzyme to an activity (9.62 U/mg) which was about two times the activity (4.88 U/mg) originally measured with the anaerobically purified enzyme. Also, preincubation of the dialyzed enzyme with DTT alone prevented it from activation by further incubation with CoA, which may explain the lack of a response of the purified ALDH (with 5 mM DTT always present) toward incubation with CoA, DTT, and NAD$^+$.

**N-terminal amino acid sequence.** The N-terminal 29 aminol acid residues were determined from the purified ALDH. The sequence is as follows: MNKDTLIPPTT KDLKVKT(N)GE NINLK(N)YKD. The amino acid residues are represented by the single letter code. Residues in parentheses are tentative assignments. The information could be used for the cloning of the gene.
encoding ALDH.

Table III-4. Effect of incubation with DTT, NAD+, or CoA on activity of
dialyzed ALDH\(^2\) from *C. beijerinckii* NRRL B592.

<table>
<thead>
<tr>
<th>Incubated with:</th>
<th>Enzyme activity (U/mg)(^b)</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No preincubation</td>
<td>1.68</td>
<td>100</td>
</tr>
<tr>
<td>No addition</td>
<td>1.59</td>
<td>95</td>
</tr>
<tr>
<td>DTT</td>
<td>1.48</td>
<td>88</td>
</tr>
<tr>
<td>NAD(^+)</td>
<td>1.47</td>
<td>88</td>
</tr>
<tr>
<td>DTT, NAD(^+)</td>
<td>2.98</td>
<td>177</td>
</tr>
<tr>
<td>NAD(^+), CoA</td>
<td>2.63</td>
<td>157</td>
</tr>
<tr>
<td>DTT, NAD(^+), CoA</td>
<td>2.86</td>
<td>170</td>
</tr>
<tr>
<td>CoA</td>
<td>4.50</td>
<td>268</td>
</tr>
<tr>
<td>DTT, CoA</td>
<td>9.62</td>
<td>573</td>
</tr>
<tr>
<td>DTT → CoA(^c)</td>
<td>1.40</td>
<td>83</td>
</tr>
</tbody>
</table>

\(^2\) An 8-μg portion of dialyzed ALDH was incubated in 1 ml of glycylglycine buffer (50 mM; pH 9) in a cuvette for 20 min at room temperature with the indicated compound(s) at the following concentrations: DTT, 5 mM; NAD\(^+\), 0.5 mM; CoA, 0.12 mM. At the end of incubation, the compound(s) not already present was added to make up the assay mixture, and 11 mM of butyraldehyde was added last to initiate the reaction.
The undialyzed ALDH had an activity of 4.88 U/mg, which was not affected by preincubation with DTT, CoA, and NAD⁺.

Incubated first with DTT for 20 min and then with CoA for another 10 or 20 min.
DISCUSSION

Few CoA-acylating ALDHs have been completely separated from other enzyme activities and purified to a high degree of purity. ALDHs purified from \textit{C. acetobutylicum} NRRL B643 (Palosaari and Rogers, 1988) and from \textit{C. beijerinckii} NRRL B592 (this work) shared a number of properties which differ from the ALDH purified from \textit{Propionibacterium freudenreichii} (Hosoi et al., 1979). The \textit{C. beijerinckii} ALDH had native and subunit $M_s$ of 100,000 and 55,000, respectively, whereas those of the \textit{C. acetobutylicum} ALDH are 115,000 and 56,000, suggesting a dimeric structure for ALDH of both \textit{Clostridium} species. The ALDH from \textit{P. freudenreichii}, on the other hand, has a native $M$, of 188,000 and a subunit $M$, of 47,500, suggesting a tetrameric structure.

ALDHs from \textit{C. kluyveri} strain K-1 (the soluble enzyme; Burton and Stadtman, 1953), \textit{E. coli} (Rudolph et al., 1968), \textit{L. mesenteroides} (Kazahaya et al., 1972), and \textit{P. freudenreichii} (Hosoi et al., 1979) are specific for NAD$^+$, whereas the ALDH from \textit{C. kluyveri} (the particulate enzyme; Hillmer and Gottschalk, 1974), \textit{Vibrio harveyi} (Byers and Meighen, 1984), \textit{C. acetobutylicum} NRRL B643 (Palosaari and Rogers, 1988), and \textit{C. beijerinckii} NRRL B592 (this work) can use either NAD(H) or NADP(H) as the coenzyme. For ALDH of \textit{C. beijerinckii} and \textit{C. acetobutylicum}, a much lower $K_m$ was obtained with NAD(H) than with NADP(H), while the $V_{\text{max}}$ values were comparable. Within the pH range of 6 to 8, the ratio of NADH-linked/NADPH-linked activities of the \textit{C.}
beijerinckii ALDH increased with decreasing pH (Fig. III-2A). Because clostridial cells have an acidic intracellular pH when actively producing butanol (see review by Jones and Woods, 1986), the result suggests that NADH is the physiologically more important coenzyme for the ALDH.

When ALDH activity was measured by monitoring the formation of NAD(P)H, only NAD\(^+\)-linked activity was detected in crude extracts of *C. beijerinckii* NRRL B592 and no significant activity was detected with either coenzyme in crude extracts of *C. beijerinckii* NRRL B593 (Yan et al., 1988; this work). However, purified ALDH from *C. beijerinckii* NRRL B592 was active with both NAD\(^+\) and NADP\(^+\). To see whether the discrepancy was caused by reoxidation of NAD(P)H by other enzymes in crude extracts, we further measured ALDH activity by monitoring the formation of butyryl-CoA. Both NAD\(^+\)-and NADP\(^+\)-linked ALDH activities were readily detected in crude extracts of both strains of *C. beijerinckii* when butyryl-CoA formation was measured (data not shown). Although ALDH activity based on NADH formation was measurable in crude extracts of strain NRRL B592, ALDH activity based on thioester formation was still higher than activity based on NADH formation, indicating reoxidation of both coenzymes. Because the assays were done anaerobically, reoxidation of NAD(P)H cannot be attributed to diaphorase activities in crude extracts (Hosoi et al., 1979; Smith and Kaplan, 1980).
It is possible that alcohol dehydrogenases [NAD(P)H + butyraldehyde ⇔ NAD(P)\(^+\) + butanol] are involved in the reoxidation of NAD(P)H, but assays for butanol are not sensitive enough to confirm this. The results indicate that measurements of ALDH activity in crude extracts by NAD(P)H formation may not be reliable even under anaerobic conditions.

The activity of CoA-acylating ALDH was significantly affected by prior exposures of ALDH to thiol compounds and substrates. Reactivation of ALDH by a thiol compound (CoA or DTT) suggests that the inactivation involved the oxidation of an enzymic sulfhydryl group(s), which is consistent with the reported requirement of ALDHs for a thiol compound for activity. This reductive activation of an oxidized ALDH by a thiol compound becomes more effective in the presence of a substrate, such as CoA or NAD\(^+\). CoA alone, but not DTT or NAD\(^+\) alone, caused a rapid and significant activation of the \(\text{C. beijerincki}\) ALDH, and CoA plus DTT caused a greater activation. NAD\(^+\), however, decreased the extent of activation by CoA, although NAD\(^+\) together with DTT showed an activating effect. CoA together with another thiol compound (such as DTT) seems most effective converting ALDH to the active form. It may be postulated that binding of the substrate CoA to ALDH puts ALDH in a conformation which is most conducive to activation by a thiol compound such as DTT. NAD\(^+\) may cause a similar but less effective conformational change in ALDH than CoA. NAD\(^+\) or DTT perhaps also binds to sites that affect the
proper binding of CoA and hence decreases the effectiveness of CoA for activation. These other sites on ALDH may be involved in interactions with a non-protein sulphydryl group (shared by CoA and DTT) and with an adenyl or ADP moiety (a similar structure between CoA and NAD\(^+\)). NAD\(^+\) may compete against CoA. This competition may be related to their inhibitory properties and their structural similarities. Shone and Fromm (1981) showed that NAD\(^+\) was necessary, together with 2-mercaptoethanol, for the activation (and for the maintenance of the active conformation) of the \textit{E. coli} ALDH. These workers did not report whether CoA could replace NAD\(^+\). Because the measured activity of ALDH is easily affected by prior exposure to O\(_2\) (in the presence of a low level of thiol compounds) and by the assay conditions, a comparison of ALDH activity among different sources must be approached carefully.

During butanol formation, the cell must direct butyryl CoA away from the competing enzyme phosphotransbutyrylase [PTB; butyryl CoA + phosphate \(\rightarrow\) butyryl phosphate + CoA (EC 2.3.1.19)], which leads to the formation of butyric acid (Fig. I-1). For \textit{C. beijerinckii} NRRL B592, the \(K_m\) value for butyryl CoA was 0.033 mM (at pH 7.5) for PTB (J.-S. Chen & M. Walker, unpublished) and 0.166 mM (with NADH at pH 6.5; Table III-2) for ALDH. The \textit{in vitro} activity level of PTB was about 3 orders higher than that of ALDH in crude extracts of butanol-producing cells (R.-T. Yan & J.-S. Chen, unpublished data). PTB activity was lower at lower pH, and it had a relatively high \(K_m\) for phosphate
(Thompson and Chen, 1990). However, it remains to be shown whether a decrease in intracellular pH and phosphate concentration is sufficient to make butyryl-CoA more available to ALDH than to PTB during butanol production.
SUMMARY

ALDH was purified to near homogeneity by three chromatographic steps under anaerobic conditions at 4°C. Activities measured with butyraldehyde and acetaldehyde as alternative substrates were copurified. The ALDH showed a native $M_r$ of 100,000. SDS-PAGE gave a subunit $M_r$ of 55,000, suggesting that the native enzyme is dimeric. The ALDH was more effective for the production of butyraldehyde than for acetaldehyde. ALDH could use either NAD(H) or NADP(H) as the coenzyme, but the $K_m$ value for NAD(H) was much lower than that for NADP(H). Kinetic data suggest a ping pong mechanism for the reaction.

ALDH was sensitive to $O_2$. DTT could protect ALDH from $O_2$ inactivation. The $O_2$-inactivated enzyme could be reactivated by incubating the enzyme with CoA in the presence or absence of DTT prior to assay. This appears to be the first observation that CoA alone caused a rapid and significant activation of ALDH. NAD$^+$, on the other hand, decreased the extent of activation by CoA, although NAD$^+$ together with DTT showed an activating effect. This diminished activation in the presence of NAD$^+$ may be related to its structural similarity to CoA. It suggests that the concentration and the ratio of CoA and NAD$^+$ in vivo could affect the ALDH activity, and it is possible that solvent production is regulated at the enzyme level by these parameters.
REFERENCES


inducible coenzyme A-linked butyraldehyde dehydrogenase from


properties of a Heme-containing aldehyde dehydrogenase from


24. Peterson, J. B., and T. A. LaRue. 1982. Soluble aldehyde dehydrogenase and

aldehyde dehydrogenase from *Pseudomonas testosteroni*: a novel

aldehyde dehydrogenase from *Escherichia coli*. J. Biol. Chem. 243:5539-5545.

kinetics of coenzyme A linked aldehyde dehydrogenase from


mechanism of coenzyme A-linked aldehyde dehydrogenase from


Chapter IV.

Distinct Properties of NADPH-dependent and NADH/NADPH-dependent Alcohol Dehydrogenases and Preliminary Purification of NADH/NADPH-dependent Alcohol Dehydrogenase from Clostridium beijerinckii NRRL B592

INTRODUCTION

A few species of saccharolytic clostridia are the only bacteria known to produce 1-butanol as a major fermentation product (Gottschalk, 1986). The production of 1-butanol from butyraldehyde is catalyzed by alcohol dehydrogenase. A NADPH-dependent primary/secondary alcohol dehydrogenase which catalyzes the formation of 1-butanol, ethanol and 2-propanol has been purified and the gene encoding this enzyme has been cloned from Clostridium beijerinckii NRRL B593 (Zhu et al., 1991). A gene encoding a primary alcohol dehydrogenase has been cloned and sequenced from Clostridium acetobutylicum P262 (Youngleson et al., 1989). This C. acetobutylicum enzyme is also NADPH-dependent based on the assay of crude extracts of E. coli in which the gene is expressed. However, the NADPH-dependent primary alcohol dehydrogenase has not been purified from C. acetobutylicum. A second primary alcohol dehydrogenase which is NADH/NADPH-dependent has been purified from C. acetobutylicum ATCC 824 (Welch et al., 1989) and its gene has been cloned (Petersen et al., 1991). Therefore, at least two primary alcohol dehydrogenases are present in C. acetobutylicum: one is NADPH-dependent and the other is NADH/NADPH-dependent (dual specificity for coenzymes). The coenzyme specificity of ADH in crude extracts of C. acetobutylicum has been contradictory in the literature (Petitdemange et al., 1968; Andersch et al., 1983; Rogers, 1986; Durre et al, 1987). Thus, the ratio of NADPH-dependent activity to NADH-
dependent activity (the P/D ratio) in crude extracts of *C. acetobutylicum* vary among different reports. In this chapter, we report the factors affecting the P/D ratio, the separation and properties of NADPH-dependent and NADH/NADPH-dependent ADHs and the preliminary purification of the ADH with dual coenzyme specificities from *C. beijerinckii* NRRL B592.
MATERIALS AND METHODS

Materials. All reagents used in this study were as described in Chapter III except that Reactive Red 120 and HEPES were obtained from Sigma Chemical Co. (St. Louis, MO); Q-Sepharose (fast flow) was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ).

Organisms and growth conditions. C. beijerinckii NRRL B592 was used in this study. Growth conditions were as described in Chapter III.

Preparation of crude extracts. Cell paste was thawed under argon in 50 mM potassium HEPES, pH 8 (1 g of cells per 3 ml of buffer). DNase I (0.1 mg/ml), lysozyme (2 mg/ml) were added to the cell suspension. The cell suspension was incubated at room temperature for 2 h. Cell debris was removed by centrifugation at 37,000 x g for 0.5 h at 4°C. The supernatant (crude extract) was stored as frozen droplets in liquid nitrogen.

Protein determination. Protein was determined by the dye-binding assay (Bradford, 1976), with bovine plasma gamma globulin as a standard.

Enzyme assay. In routine assays, both NADPH-dependent and NADH-dependent activities were assayed in physiological direction under anaerobic conditions (Chen and Blanchard, 1979; Jin et al., 1983). The assay procedures were the same as described in Chapter III.

Purification of NADH/NADPH-dependent ADH. 50 mM potassium HEPES buffer was used throughout the purification. All operations were performed anaerobically at 4°C under argon. Enzyme activity assay was performed immediately after each column chromatographic step, and active fractions were concentrated by ultrafiltration (PM 30 membrane, Amicon) and were stored as frozen droplets in liquid nitrogen.

(i) DEAE-cellulose column. A crude extract (2.9 g of protein) was loaded onto a
Whatman DE-52 column (5 by 5 cm) which had been equilibrated with 50 mM potassium HEPES buffer (pH 8). The column was then eluted with a linear gradient of KCl (0 to 0.4 M in the same buffer; total volume, 600 ml). The flow rate was 150 ml/h, and 20-ml fractions were collected.

(ii) Cibacron Blue 3GA-agarose column. To lower the concentration of KCl in the active fractions from DE-52 column, the pooled fractions containing NADH-dependent activities were concentrated by ultrafiltration (PM 30 membrane) and diluted with 50 mM potassium HEPES buffer (pH 7.2) to facilitate the binding of the NADH/NADPH-dependent activity to Cibacron Blue 3GA-agarose. The KCl concentration was lowered to 10-fold. The sample was applied to a Cibacron Blue 3GA-agarose column (2.6 by 5 cm) which had been washed with 8 M urea and then equilibrated with 50 mM potassium HEPES buffer (pH 7.2). The column was washed with 1 bed volume of equilibrating buffer and eluted with a linear gradient of KCl (0 to 0.5 M in the same buffer; total volume, 160 ml). The flow rate was 50 ml/h, and 10-ml fractions were collected.

(iii) Reactive Red 120 column. To lower the KCl concentration in fractions containing NADH/NADPH-dependent activity from the Cibacron Blue 3GA-agarose column, the pooled fractions were concentrated by ultrafiltration and diluted with 50 mM potassium HEPES buffer (pH 7.2) so that the KCl concentration was lowered 3-fold. The sample was applied onto a Reactive Red 120 column (1.5 by 5.5 cm) which had been washed with 8 M urea and equilibrated with 50 mM potassium HEPES (pH 7.2). The column was washed with 1 bed volume of the same buffer and eluted with a linear gradient of KCl (0 to 0.5 M; total volume, 60 ml). The flow rate was 30 ml/h, and 4-ml fractions were collected.

(iv) Q-Sepharose column. To lower the KCl concentration in active fractions of
Reactive Red 120 column, the pooled fractions were concentrated by ultrafiltration and
diluted with 50 mM potassium HEPES buffer (pH 8) so that the KCl concentration was
lowered 3-fold. The sample was applied onto a Q-Sepharose column (1 by 6 cm) which was
eluted with a linear gradient of KCl (0 to 0.5 M; total volume, 30 ml). The flow rate was 30
ml/h, and 1-ml fractions were collected. Active fractions were stored as frozen droplets in
liquid nitrogen.

**Determination of native M₉.** The native M₉ of the NADH/NADPH-dependent ADH
was determined with the partially purified enzyme by gel filtration on a Sephacryl S-300
column (1 by 44.5 cm) under anaerobic conditions. The column was equilibrated and eluted
with 50 mM potassium HEPES buffer (pH 7) containing 0.1 mM KCl, 1 mM DTT.
Standards used were RNase (13,700), chymotrypsinogen A (25,000), conalbumin (77,000),
and yeast alcohol dehydrogenase (150,000).

**SDS-PAGE.** To examine the purity and determine the subunit M, of the
NADH/NADPH-dependent ADH, SDS-PAGE was performed by the method of Weber and
Osborn (1969). Protein bands were detected by staining with Coomassie blue. Standards
used were lysozyme (14,000), soybean trypsin inhibitor (21,500), bovine carbonic anhydrase
(31,000), ovalbumin (45,000), bovine serum albumin (66,000), and rabbit muscle
phosphorylase b (97,400).
RESULTS

A. Observation with crude extracts.

1. NAD(P)⁺ inhibition and enzyme assay. In routine assay, NADH- and NADPH-oxidizing activities do not give linear reaction progress curve. The NADH-dependent activity is linear with time longer than the NADPH-dependent activity. Two possible factors could cause the non-linearity: the enzyme was destabilized during the assay or was inhibited by product(s) of the reaction. The enzyme activity was not lost in one min in the presence or absence of butyraldehyde in assay buffers without NAD(P)H. However, NADP⁺ strongly inhibited the NADH- and NADPH-dependent activities; NAD⁺ also inhibited the NADH- and NADPH-dependent activities, but to a lesser extent than NADP⁺ (Fig. IV-1). The non-linearity of the reaction progress curve was caused mainly by the formation of NAD(P)⁺ in the reaction but not caused by NAD(P)H based on the evidence that the calculated concentration of NAD(P)⁺ in the reaction mixture and the observed activity at each time point corresponded very closely to the concentration of NAD(P)⁺ required in a reaction mixture to give a comparable initial activity (Fig. IV-1). The reaction can go to near completion although NADP⁺ inhibition was present, as the A₅₄⁶ decreased to near zero from 1.2.

2. Factors affecting the P/D ratio in crude extracts. Both NADH- and NADPH-dependent alcohol dehydrogenase activities were present in crude extracts. However, the P/D ratio varied in crude extracts prepared from either different batches of cells or the same batch of cells but on different dates. In order to understand the nature of the variation in the P/D ratio, several factors have been investigated.
Figure IV-1. NAD(P)⁺ inhibition of the NAD(P)H-dependent ADH activity of crude extracts from *C. beijerinckii* NRRL B592. (A). Different amounts of NAD⁺ (△) were added to the assay mixture (potassium MES, 50 mM, pH 6; 0.2 mM NADH; 350 μg of protein). The reaction was initiated by the addition of 5.5 mM butyraldehyde. No NAD⁺ was added to the assay mixture (▲), and the instantaneous activity was measured at different time points in a reaction and the amount of NAD⁺ present was calculated based on $A_{340}$ at each point. (B). Different amounts of NADP⁺ (□) were added to the assay mixture (Tris chloride, 50 mM, pH 7.5; 0.2 mM NADPH; 175 μg of protein). The reaction was initiated by the addition of 5.5 mM butyraldehyde. No NADP⁺ was added to the assay mixture (■), and the instantaneous activity was measured at different time points in a reaction and the amount of NADP⁺ was calculated based on $A_{340}$ at each point.

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Figure IV-2. O$_2$ inactivation of NADH- and NADPH-dependent ADH activities in crude extracts from *C. beijerinckii* NRRL B592. 500 µl of dialyzed crude extracts (against 50 mM potassium HEPES buffer (pH 8) overnight under anaerobic conditions at 4°C) was bubbled with air in a 1-ml vial for 1 min on ice. A. NADH-dependent (Δ) and NADPH-dependent (□) activities in a sample containing 1 mM DTT; NADH-dependent (▲) and NADPH-dependent (■) activities in a sample without DTT. B. Semi-log plot. Symbols are the same as in A.
(1) O$_2$. Both NADH- and NADPH-dependent ADH activities in crude extracts were sensitive to O$_2$ (Fig. IV-2A). However, the NADH-dependent activity was more sensitive to O$_2$ than the NADPH-dependent activity. The initial half-life for NADH-dependent activity and NADPH-dependent activity, respectively, was 1.7-2.4 and 3.6-5.2 min after exposure of crude extracts to air and kept on ice. Similar results were obtained with dialyzed crude extract either with 1 mM DTT [2.7 min (NADH-dependent) and 5.3 min (NADPH-dependent)], or without DTT [2.9 min (NADH-dependent) and 4.9 min (NADPH-dependent) min]. It showed that DTT and other small molecules in crude extracts had no effect on O$_2$ inactivation. The NADH- and NADPH-dependent activities curved noticeably on the semi-log plot with different patterns (Fig. IV-2B). Therefore, the P/D ratio was increased with O$_2$ inactivation. Since the rate of O$_2$ inactivation was not first-order and trace amounts of O$_2$ would be present in crude extracts before O$_2$ treatment, the real initial half-life of NADH- and NADPH-dependent activities in air should be shorter than those values shown above.

(2) pH. The NADH-dependent activity decreased with increasing pH from 6 to 9, whereas the NADPH-dependent activity increased with increasing pH (Fig. IV-3). Therefore, the P/D ratio was affected by pH.

(3) Growth stage of cell. The NADH-dependent activity was undetectable in a sample collected at the end of a growth study, whereas the NADPH-dependent activity in the same sample was 20% of the peak level found in cells at an earlier stage of growth (Fig. IV-4). Thus, different P/D ratios could be obtained from cells at different stages of growth (Fig. IV-4).
Figure IV-3. pH effect on NADH-dependent and NADPH-dependent ADH activities in crude extracts of \textit{C. beijerinckii} NRRL B592. The reaction mixture (1 ml) contained 0.2 mM NADH (\textDelta) or NADPH (\textsquare), 5.5 mM butyraldehyde, and 170-350 \( \mu \)g protein in 50 mM potassium MES (pH 6 to 7) or 50 mM Tris chloride (pH 7 to 9).
Figure IV-4. NADH-dependent and NADPH-dependent ADH activities in cells at different stages of growth. A550 (○); NADH-dependent ADH activity (△); NADPH-dependent ADH activity (□).
(4) Fe in culture medium. As shown in Table IV-1, the Fe concentration in culture medium affected the P/D ratio although the amount of butanol produced was comparable. The NADH-dependent activity in crude extracts of cells cultured in the medium in which Fe was not added was 13-fold lower than that from the culture in which 0.1 mM Fe was added (normal culture medium), but the NADPH-dependent activity was only 2.8-fold lower in the absence of added Fe.

Table IV-1. Comparison of ADH activity and the P/D ratio in crude extracts from cells grown with or without the addition of 0.1 mM Fe.

<table>
<thead>
<tr>
<th>Fe (mM)</th>
<th>Culture OD (A550)</th>
<th>Acetone (mM)</th>
<th>Butanol (mM)</th>
<th>Enzyme activity (U/mg)</th>
<th>P/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>0\textsuperscript{a}</td>
<td>6.12</td>
<td>22.1</td>
<td>32.8</td>
<td>0.0157</td>
<td>0.193</td>
</tr>
<tr>
<td>0.1\textsuperscript{b}</td>
<td>6.4</td>
<td>15</td>
<td>32.1</td>
<td>0.21</td>
<td>0.546</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Without the addition of Fe to the TYS medium.

\textsuperscript{b} In normal cultures (volume of cultures and growth conditions are as described in Chapter II), with 0.1 mM Fe (FeCl\textsubscript{3}) added to the TYS medium.

(5) Ionic strength. The NADH-dependent activity in crude extracts increased with decreasing ionic strength in the assay mixture, whereas the NADPH-dependent activity decreased with decreasing ionic strength. Different salts give comparable but not identical effects based on ionic strength (Fig. IV-5).
3. **Enzyme thermostability in crude extracts.** \(O_2\) was a major factor that caused the loss of ADH activities. Therefore, the stringency of anaerobic conditions affects both the P/D ratio and the stability of ADH(s). Under anaerobic conditions and at 55\(^\circ\)C, the NADH-dependent activity was much less stable than the NADPH-dependent activity (Fig. IV-6).

**B. Separation of the NADPH-dependent (P) ADH and the NADH/NADPH-dependent (D/P) ADH from *C. beijerinckii* NRRL B592.**

1. **Separation.** Two alcohol dehydrogenases could be separated on a Reactive Red 120 column or on a Cibacron Blue 3GA-agarose column under certain conditions (e.g., low salt concentrations, gel containing no free dye). One was NADPH-dependent (P-ADH) which did not bind to Reactive Red 120 or Cibacron Blue 3GA-agarose, whereas the other was NADH/NADPH-dependent (D/P-ADH) which bound to Reactive Red 120 (Fig. IV-7) and Cibacron Blue 3GA-agarose (Fig. IV-8).

2. **Properties of the P-ADH and the D/P-ADH.** The P-ADH and the D/P-ADH had distinct properties.

   (1) **Thermostability of the P-ADH and the D/P-ADH.** As shown in Fig. IV-9, the D/P-ADH was unstable at 55\(^\circ\)C, and a parallel loss in NADH-dependent and NADPH-dependent activities was observed.
Figure IV-5. Effect of ionic strength on NADH- and NADPH-dependent ADH activities in dialyzed crude extracts of C. beijerinckii NRRL B592. KCl (○), MgCl₂ (△), or MgSO₄ (□) was added to the reaction mixture for the assay of the NADH-dependent ADH activity. KCl (●), MgCl₂ (▲), or MgSO₄ (■) was added to the reaction mixture for the assay of the NADPH-dependent activity. Other conditions were the same as in Fig. IV-1.
Figure IV-6. Thermostability of NADH- and NADPH-dependent ADH activities of crude extracts from \textit{C. beijerinckii} NRRL B592. 1 ml of crude extracts was incubated at 55°C. Aliquots were taken at different time intervals to assay for ADH activities. A. linear plot. B. Semi-log plot. Symbols: NADH-dependent activity (△), NADPH-dependent activity (□). The assay procedures were as described in Materials and Methods.
Figure IV-7. Separation of the P-ADH and the D/P-ADH by a Reactive Red 120 column. A crude extract (1.17 g of protein) was loaded onto a Whatman DE-52 column (2.6 by 5 cm) which had been equilibrated with 50 mM potassium HEPES buffer (pH 8) under anaerobic conditions under argon at room temperature. The column was eluted with a linear gradient of KCl (0 to 0.4 M; total volume, 160 ml in the same buffer). Active fractions were pooled and concentrated by ultrafiltration (Amicon PM 30 membrane) and diluted 10-fold with 50 mM potassium HEPES (pH 7.2). The sample was loaded onto a Reactive Red 120 column (1.4 by 6.6 cm) which had been equilibrated with 50 mM potassium HEPES buffer (pH 7.2) at 4°C. The column was washed with one bed volume of the equilibrating buffer and eluted with a linear gradient of KCl (0 to 0.6 M; total volume, 70 ml in the same buffer). Symbols: protein (○), NADH-dependent activity (△), NADPH-dependent activity (□), chloride concentration (●).
Figure IV-8. Separation of the P-ADH and the D/P-ADH by a Cibacron Blue 3GA-agarose column. A crude extract (1.1 g of protein) was loaded onto a Cibacron blue 3GA-agarose column (2.6 by 6.5 cm) which had been equilibrated with 50 mM potassium HEPES buffer (pH 8) under anaerobic conditions at 4°C. The column was washed with 1 bed volume of the same buffer and eluted with a linear gradient of KCl (0 to 0.5 M; total volume, 240 ml in the same buffer). Symbols were the same as in Fig. IV-7.
Figure IV-9. Thermostability of P-ADH and D/P-ADH activities from _C. beijerinckii_ NRRL B592. Preparation of the P- and the D/P-ADH samples was as described in Fig. IV-7. The samples were incubated at 55°C. Aliquots were taken at different time intervals to assay for ADH activities. A. linear plot. B. Semi-log plot. Symbols: NADH-dependent activity from the D/P-ADH (∆), NADPH-dependent activity from the D/P-ADH (□), NADPH-dependent activity from the P-ADH (○).
However, the P-ADH was quite stable under the same conditions.

(2) **Effect of ionic strength on the P-ADH and the D/P-ADH.** The NADPH-dependent activity of both the P-ADH and the D/P-ADH increased with increasing ionic strength (Fig. IV-10). The NADPH-dependent activity of the P-ADH increased 4-fold when the ionic strength increased from 0.002 to 0.12, whereas the NADPH-dependent activity of the D/P-ADH increased only 1.7-fold. The NADH-dependent activity of the D/P-ADH decreased with increasing ionic strength, whereas an NADH-dependent activity was undetectable in the P-ADH even at low ionic strength (Fig. IV-10).

(3) **O₂ inactivation of the P- and the D/P-ADHs.** Both the NADH-dependent and the NADPH-dependent activities of the D/P-ADH were O₂ sensitive (Fig. IV-11), whereas the NADPH-dependent activity of the P-ADH was O₂ insensitive.

C. **Preliminary Purification of the D/P-ADH**

The D/P-ADH was purified by four column chromatographic steps [DE-52, Cibacron Blue 3GA-agarose, Reactive Red 120, and Q-Sepharose (fast flow)]. Because the D/P-ADH is sensitive to O₂ and unstable at high temperature, all the purification steps were performed at 4°C under anaerobic conditions. The results of purification are summarized in
Figure IV-10. Effects of ionic strength on the D/P- and the P-ADH activities of C. beijerinckii NRRL B592. Preparation of the P- and the D/P-ADH samples was as described in Fig. IV-7. 5 to 125 mM KCl was added to the assay mixture. Symbols: NADH-dependent ADH activity from the D/P-ADH sample (△), NADPH-dependent ADH activity from the D/P-ADH sample (□) or the P-ADH sample (■).
Figure IV-11. Effects of O₂ on the P- and the D/P-ADH activities from \( \text{C. beijerinckii} \) NRRL B592. Preparation of the P- and the D/P-ADH samples was as described in Fig. IV-7. The procedures of O₂ treatment and enzyme assays were as described in Fig. IV-2. Symbols: NADH-dependent activity from the D/P-ADH sample (\( \triangle \)), NADPH-dependent activity from the D/P-ADH sample (\( \square \)), and NADPH-dependent activity from the P-ADH sample (\( \blacksquare \)).
Table IV-2. The P-ADH was separated from the D/P-ADH on a Cibacron Blue 3GA-agarose column. The D/P-ADH from the Cibacron Blue 3GA-agarose column step was further purified by Reactive Red 120 and Q-Sepharose column chromatography. The NADH-dependent and the NADPH-dependent ADH activities were copurified after the Cibacron Blue 3GA-agarose column step (Fig. IV-12), indicating that the D/P-ADH can use both NADH and NADPH as coenzymes. SDS-PAGE showed a single, intense band when >50 μg of ADH was examined for purity (Fig. IV-13). However, two bands were obtained on SDS-PAGE when 10 μg of ADH was loaded (Fig. IV-14). The ratio of the upper band to the lower band was about 0.67 based on the intensity of Coomassie stain, and a similar ratio was obtained from earlier and later active fractions of the Q-Sepharose column step. It suggests that the D/P-ADH contained two nonidentical subunits.

Native and subunit molecular weights. The D/P-ADH showed a native $M_r$ of 70,000. SDS-PAGE of extensively purified D/P-ADH showed two nonidentical subunits with molecular weights of 45,300 and 40,000.

Inactivation of the D/P-ADH by $\alpha,\alpha'$-dipyridyl and restoration by Fe$^{2+}$. The D/P-ADH activity could be inactivated by $\alpha,\alpha'$-dipyridyl and reactivated by the addition of Fe(NH$_4$)$_2$(SO$_4$)$_2$ (Fig. IV-15), indicating the
<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>ADH activity</th>
<th>NADPH</th>
<th>%</th>
<th>Fold</th>
<th>NADH</th>
<th>%</th>
<th>Fold</th>
<th>P/D&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>2910</td>
<td>0.316</td>
<td>918</td>
<td>100</td>
<td>1</td>
<td>0.12</td>
<td>349</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DE52</td>
<td>210</td>
<td>3.1</td>
<td>655</td>
<td>85</td>
<td>9.8</td>
<td>1.54</td>
<td>324</td>
<td>93</td>
<td>13</td>
</tr>
<tr>
<td>Cibacron Blue 3GA-agarose</td>
<td>28</td>
<td>11.6</td>
<td>324</td>
<td>54</td>
<td>36.6</td>
<td>3.33</td>
<td>93.2</td>
<td>27</td>
<td>57</td>
</tr>
<tr>
<td>Reactive Red120</td>
<td>7.6</td>
<td>12.9</td>
<td>96</td>
<td>15</td>
<td>40.8</td>
<td>8.7</td>
<td>65.2</td>
<td>18.7</td>
<td>73</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>2.4</td>
<td>5.6</td>
<td>13.4</td>
<td>2</td>
<td>18</td>
<td>5.4</td>
<td>12.8</td>
<td>3.7</td>
<td>45</td>
</tr>
</tbody>
</table>

<sup>a</sup> The ratio of the NADPH-linked activity to the NADH-linked activity.

<sup>b</sup> One unit (U) is the production of one μmol of NAD(P)H per min.

<sup>c</sup> % of recovery of activity.

<sup>d</sup> Fold of purification.
enzyme required a metal for activity. In the absence of chelating agent, the D/P-ADH was also unstable, and the activities of the partially inactivated D/P-ADH activity could be restored by $\text{Fe}^{2+}$ (Table IV-3).
Figure IV-12. Elution of the NADH- and NADPH-dependent ADH of *C. beijerinckii* NRRL B592 from a Q-Sepharose column. The procedures were as described in Materials and Methods. Symbols: NADH-dependent activity (U/ml) (△), NADPH-dependent activity (U/ml) (□), NADH-dependent activity (U/mg) (▽), NADPH-dependent activity (U/mg) (◇), protein concentration (○), Cl⁻ concentration (●).
Figure IV-13. Examination of purity by SDS-PAGE of the D/P-ADH from \textit{C. beijerinckii} NRRL B592. 50 to 70 \( \mu \text{g} \) of protein was loaded onto each gel. 
Protein was detected by Coomassie blue stain.
Figure IV-14. Determination of subunit molecular weights by SDS-PAGE of the purified D/P-ADH from C. beijerinckii NRRL B592. 10 µg of protein was loaded onto each gel. Protein was detected by Coomassie blue stain.
Figure IV-15. Inactivation of the D/P-ADH by \( \alpha,\alpha' \)-dipyridyl and restoration by \( \text{Fe}^{2+} \). Preparation of the D/P-ADH sample was described in Fig. IV-8. 0.5 mM of \( \alpha,\alpha' \)-dipyridyl was added to the partial purified D/P-ADH sample (1-ml volume) and incubated at room temperature for 2.5 h. 5 mM of \( \text{Fe}((\text{NH}_4)_2\text{SO}_4) \) and 0.05 mM DTT were added to the sample and incubated on ice for 3 days. Symbols: NADH-dependent activity (\( \triangle \)), NADPH-dependent activity (\( \square \)).
Table IV-3. Restoration and stabilization of the D/P-ADH activity by Fe\(^{2+}\) and DTT.

<table>
<thead>
<tr>
<th>Addition</th>
<th>ADH Activity</th>
<th></th>
<th>P/D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NADPH (U/mg)</td>
<td>%</td>
<td>NADH (U/mg)</td>
</tr>
<tr>
<td>Before Addition(^a)</td>
<td>0.194</td>
<td>100</td>
<td>0.293</td>
</tr>
<tr>
<td>1 mM Fe(^{2+})</td>
<td>0.337</td>
<td>174</td>
<td>0.783</td>
</tr>
<tr>
<td>1 mM Fe(^{2+}), 2 mM DTT</td>
<td>0.46</td>
<td>238</td>
<td>1.24</td>
</tr>
<tr>
<td>2 mM DTT</td>
<td>0.087</td>
<td>45</td>
<td>0.13</td>
</tr>
<tr>
<td>No addition</td>
<td>0.098</td>
<td>50</td>
<td>0.185</td>
</tr>
</tbody>
</table>

\(^a\) Partially purified D/P ADH [100 \(\mu_l\) (67 \(\mu_g\) of protein) of sample] was stored in 1 ml Pierce React-vials at 4\(^\circ\)C anaerobically under argon for 3 days. 95\% of NADH and NADPH-dependent activities was lost during this storage. Anaerobically prepared Fe\((NH_4)_2(SO_4)_2\) and/or DTT was added to the sample at the indicated concentration. The samples were then incubated at room temperature for 32 h before assay.
DISCUSSION

Alcohol dehydrogenases from various sources are known to differ in substrate and coenzyme specificities. NADP(H)-dependent [or NADP(H)-preferring] ADH is present in *Clostridium thermohydrosulfuricum* (Lamed and Zeikus, 1981), *Clostridium thermosaccharolyticum* (Hsu and Ordal, 1970), *C. beijerinckii* NRRL B593 (Hiu et al., 1987; Zhu et al., 1990), *C. beijerinckii* NRRL B592 (this work), *C. acetobutylicum* ATCC 824 (Youngleson et al., 1989), *Thermoanaerobium brockii* (Lamed and Zeikus, 1981; Al-Kassim and Tsai, 1989), *Thermoanaerobacter ethanolicus* (Bryant et al., 1988), *Escherichia coli* (Hatanaka et al., 1971), *Saccharomycopsis lipolytica* (Barth and Kunkel, 1979), *Tritrichomonas foetus* (Kleiner and Johnston, 1985), and pig liver (Dutler et al., 1971). NAD(H)-dependent ADHs from liver and the baker’s yeast have been well studied (Branden et al., 1975). The NADH-dependent [or NAD(H)-preferring] ADH is also present in *Clostridium kluyveri* (Hillmer and Gottschalk, 1974), *Zymomonas mobilis* (Scopes, 1983; Conway et al., 1997), *E. coli* (Hatanaka et al., 1971;), *Pseudomonas* sp. (Niehaus et al., 1978; Hou et al., 1983), methanol-oxidizing microbes (Hou et al., 1979), *Comamonas* species (Barrett et al., 1981), *Rhizopus javanicus* (Yonega and Sato, 1979), *Leuconostoc mesenteroides* (Hatanaka et al., 1974), and *Sulfolobus solfataricus* (Rella et al., 1987). An ADH with comparable activities with NAD(H) or NADP(H) (dual coenzyme specificity) is present in *C. acetobutylicum* (Welch et al., 1989) and *C.*
beijerinckii NRRL B592 (this work).

Two primary ADHs have been identified in crude extracts from C. beijerinckii NRRL B592. The P-ADH could be separated from the D/P-ADH (dual coenzyme specific) by dye-ligand chromatography (Reactive Red 120 or Cibacron Blue 3GA-agarose). The purified ADH from C. beijerinckii NRRL B593 is NADPH-dependent but is active toward both primary and secondary alcohols. Thus, the two P-ADHs from the two strains of the same species showed distinct properties. The P-ADH from strain NRRL B592 did not bind to Reactive Red 120, whereas the P-ADH from strain NRRL B593 bound to Matrex gel Red A (a gel similar to Reactive Red 120).

Published data on the ADH from C. acetobutylicum are contradictory with respect to the coenzyme specificity. The P/D ratio varied in crude extracts of C. beijerinckii NRRL B592 prepared from either different batches of cells or the same batch of cells but on different dates (this work). The presence in C. beijerinckii NRRL B592 of two ADHs with different stabilities and coenzyme specificities complicates the situation. Because several factors could affect the P/D ratio, observations made with crude extracts are often inconclusive. The D/P-ADH from C. beijerinckii NRRL B592 showed properties different from the D/P-ADH from C. acetobutylicum. The C. beijerinckii D/P-ADH could be reactivated by Fe$^{2+}$ but not by Zn$^{2+}$, whereas the C. acetobutylicum D/P-ADH required Zn$^{2+}$ for stability (Welch et al., 1989). The C. beijerinckii D/P-ADH
was O$_2$-sensitive, whereas the O$_2$-sensitivity was not noted in the report of the *C. acetobutylicum* D/P-ADH. An NAD-dependent, Fe$^{2+}$-activated ADH was found in *Zymomonas mobilis* (Scopes, 1983) and the ADHs was also O$_2$-sensitive. Since Fe$^{2+}$ can be oxidized to Fe$^{3+}$ and Fe$^{3+}$ could not reactivate the ADH, the O$_2$-inactivation of the ADH may be expected to be caused at least partly by the oxidation of Fe$^{2+}$. That the level of NADH-dependent ADH activity in crude extracts of *C. beijerinckii* NRRL B592 was affected by the iron concentration in culture medium was an evidence supporting that the D/P-ADH required iron for activity.

It was unclear why two ADHs were present in cells of *C. beijerinckii* NRRL B592. An NADH-dependent activity (which should reflect the level of the D/P-ADH) was undetectable in a sample collected at the end of a growth study, whereas the NADPH-dependent activity in the same sample was 20% of the peak sample. This suggests that the two ADHs were regulated separately. Supporting this, the level of iron in culture medium affected the NADH-dependent activity (the D/P-ADH) to a much greater extent than the NADPH-dependent activity (the P-ADH). Because the butanol concentrations in the cultures with or without Fe addition were comparable, a higher level of ADH does not seem required for increased butanol production. The determination of the ADH responsible for butanol production and the mechanism of regulation of the ADHs will require the purification and characterization of the two ADHs and an
analysis of appropriate mutants with defective ADH genes.
SUMMARY

Both NADH- and NADPH-dependent alcohol dehydrogenase activities were present in crude extracts from C. beijerinckii NRRL B592. In routine assays, NADH- and NADPH-oxidizing activities do not give a linear reaction progress curve, which was caused mainly by the formation of NAD(P)⁺ in the reaction. The ratio of the NADPH-dependent activity to the NADH-dependent activity (the P/D ratio) varied in crude extracts. The P/D ratio was affected by O₂, ionic strength, pH, growth stage of cell, Fe in culture medium and temperature. Two ADHs have been identified in crude extracts. The NADPH-dependent ADH (P-ADH) could be separated from the the NADH/NADPH-dependent ADH (D/P-ADH). The D/P-ADH has been extensively purified. The D/P-ADH showed a native $M_r$ of 70,000, and nonidentical subunit $M_r$ of 45,300 and 40,000. The D/P-ADH activity could be inactivated by $\alpha,\alpha'$-dipyridyl and restored by Fe²⁺.
REFERENCES


Chapter V.

Oxidation Product(s) in Acetaldehyde Reacts with NAD(P)H and Interferes with Assay of Alcohol Dehydrogenase

INTRODUCTION

Acetaldehyde is a substrate for alcohol dehydrogenases and aldehyde dehydrogenases. Although ethanol oxidation is the more commonly used reaction for assaying alcohol dehydrogenase (Branden et al., 1975; Bergmeyer and Grassl, 1983), acetaldehyde reduction is the physiological reaction catalyzed by alcohol dehydrogenase that is responsible for ethanol formation (Branden et al., 1975). When studying catalytic and regulatory properties of ethanol-forming alcohol dehydrogenases, it is necessary to measure NAD(P)H-oxidizing (acetaldehyde-reducing) activity. Acetaldehyde up to 75 or 300 mM has been used in such assays (Lamed and Zeikus, 1981; Rudge and Bickerstaff, 1986). During our study of alcohol dehydrogenases from Clostridium beijerinckii, we used acetaldehyde between 1 and 50 mM in the enzymatic assay. We observed that some acetaldehyde samples caused a decrease in absorbance of NAD(P)H at 340 nm in the absence of any enzyme, and the reaction could be attributed to oxidation product(s) present in acetaldehyde. Such acetaldehyde samples gave lower ethanol dehydrogenase activities.

Although redistillation of acetaldehyde is generally recommended (e.g., Black, 1955; Seegmiller, 1955; Greenberg, 1962; Beutler, 1984), the proper procedure for redistillation is not readily available. In this chapter, we report the formation of NAD(P)H-oxidizing substance(s) when acetaldehyde was redistilled in the presence of O₂ and we report a suitable procedure for redistilling acetaldehyde.
MATERIALS AND METHODS

Materials. NAD\textsuperscript{+}, NADH, NADP\textsuperscript{+}, NADPH, glucose-6-phosphate dehydrogenase (baker's yeast), and glucose-6-phosphate were from Sigma Chemical Co. (St. Louis, MO); acetaldehyde, reagent grade, was from Fisher Scientific Co. (Pittsburgh, PA). Other chemicals were of reagent grade obtained commercially. Alcohol dehydrogenase from \textit{C. beijerinckii} NRRL B593 was prepared as described (Hsu et al., 1987).

Assays. Samples of acetaldehyde were tested with NAD(P)H in spectrophotometric cells containing the following components: 50 mM Tris chloride or sodium phosphate buffer (pH 7.5), 0.2 mM NADH or NADPH, and 5-100 mM (nominal concentrations) acetaldehyde; total volume, 1 ml. The concentration of reagent-grade acetaldehyde was taken as 17 M. Before use, acetaldehyde samples were diluted 10-fold with degassed (under Ar of N\textsubscript{2}) distilled water kept on ice. [However, dilution of acetaldehyde in water (on ice) under aerobic conditions did not show any significant changes during a 3-h period.]. The reaction was initiated by the addition of acetaldehyde, and absorbance changes were monitored at 340 nm. An extinction coefficient (340 nm) of 6.2 mM\textsuperscript{-1}cm\textsuperscript{-1} was used for NADH and NADPH.

When ethanol dehydrogenase activity was measured, 150-fold purified alcohol dehydrogenase from \textit{C. beijerinckii} NRRL B593 was added to the above reaction mixture, with acetaldehyde at 5 mM (nominal concentration).

To test whether NADP\textsuperscript{+} was formed when A\textsubscript{340nm} disappeared during the nonenzymatic reaction between NADPH and acetaldehyde redistilled in air, the absorption spectrum of the reaction mixture was taken first. The reaction mixture was then lyophilized. The dried material was redissolved in distilled water, and the pH was adjusted to 7.2 with 1 M Tris.
chloride, pH 8. The sample was then assayed with yeast glucose-6-phosphate dehydrogenase (0.46 unit) in the presence of glucose 6-phosphate (4 mM) and MgCl₂ (10 mM).

**Redistillation.** Acetaldehyde was redistilled in a fume hood using a simple distilling assembly (Adams et al., 1979), except that (i) the 3-way connecting tube at the top of the distilling bottle was closed with a stopper, (ii) the distilling bottle was immersed in a 35°C water bath, (iii) the West condenser (190 mm) was cooled by ice water, and (iv) the vacuum connecting tube had the inner tube extended to reach near the bottom of the receiving bottle and the vent was connected to a N₂-sparge line as in Shriver (1969), and (v) the receiving bottle was immersed in an ice-NaCl mixture (crushed ice:NaCl = 3:1; -20°C). The distilling assembly consisted of Pyrex brand components similar to those found in the Corning Organic Chemistry Kit (19/22 joints). Acetaldehyde (10-15 ml) was easily distilled by such an assembly using 25-ml bottles, and redistilled acetaldehyde was kept under N₂ or Ar at 4°C. To obtain strictly O₂-free conditions, the assembly was thoroughly purged with N₂ or Ar before acetaldehyde was added to the distilling bottle, and sparging through the vent continued during distillation. To test the effect of faulty anaerobic conditions, the assembly was purged with Ar before the addition of acetaldehyde, but it was not sparged during distillation. Aerobic distillation was performed in air without any attempt to exclude O₂ from the assembly. Acetaldehyde from a newly opened bottle was used in redistillation experiments unless otherwise specified.
RESULTS AND DISCUSSION

Apparent oxidation of NAD(P)H by acetaldehyde. When assaying ethanol dehydrogenase activity in samples containing a high level of NAD(P)H-oxidizing diaphorase activity, we routinely added acetaldehyde (5 or 50 mM) last to allow the recording of diaphorase activity for baseline corrections. A necessary control for this assay was to omit enzymes from the reaction mixture, and under such conditions a reaction seemingly between NAD(P)H and acetaldehyde was noticed. The reaction was first seen with acetaldehyde from a bottle that had first been opened over 20 months ago (Fig. V-1 and 2), and the rate of NAD(P)H disappearance (loss of $A_{340 \text{ nm}}$) was within the range of alcohol dehydrogenase activities measured in routine assays. The reaction was readily observed with either NADH (Fig. V-2) or NADPH (Fig. V-1) when acetaldehyde was above 25 mM (nominal concentration).

To study the nature of this reaction, four additional samples were examined: (a) acetaldehyde redistilled under strictly O$_2$-free conditions (Sample A), (b) reagent-grade acetaldehyde from a newly opened bottle (Sample B), (c) acetaldehyde redistilled under faulty anaerobic conditions (Sample C), and (d) acetaldehyde redistilled in air (Sample D). The results are shown in Fig. V-1 and 2. It is apparent that the reaction was not with acetaldehyde itself but with something that was formed after acetaldehyde was exposed to air. Redistillation of acetaldehyde in the presence of O$_2$ significantly increased the amount of NAD(P)H-reacting substance(s), whereas redistillation under strictly O$_2$-free conditions removed the interfering substance(s). NAD(P)H-reacting substance(s) in Sample D (redistilled in air) was effectively removed by further distillation under anaerobic conditions (data not shown). If not subjected to further anaerobic distillation, the level of NAD(P)H-
Figure V-1. Reaction between NADPH and interfering substance(s) present in different acetaldehyde samples. The reaction mixture (1 ml) contained 50 mM Tris-Cl (pH 7.5), 0.2 mM NADPH, and the indicated amount of acetaldehyde. Acetaldehyde samples were (a) redistilled under strictly O₂-free conditions (○); (b) from a newly opened bottle (●); (c) redistilled under faulty anaerobic conditions (○); (d) redistilled in air (■); (e) from a bottle first opened over 20 months ago (▲).
Figure V-2. Reaction between NADH and interfering substance(s) present in different acetaldehyde samples. Experimental conditions were identical to those described in Figure 1, except NADH at 0.2 mM was used in place of NADPH. See Figure 1 for symbols.
reacting substance(s) in Sample D continued to increase rapidly when it was stored at 4°C. This behavior was not seen in samples not exposed to air in the vapor state.

When Sample D was tested (within 24 h of distillation) at a nominal concentration of 30 mM with NADPH (Fig. V-1), the rate of absorbance decrease at 340 nm was 0.0136/min (2.2 uM of NADPH reacted/min) in Tris buffer. With NADH (Fig. V-2), the rate of absorbance decrease was 0.008/min (1.3 uM of NADH reacted/min). The reaction rate in phosphate buffer was about 15% of that in Tris buffer for both NADH and NADPH.

Abelson et al. (1965) reported that 1% (ca. 170 mM) acetaldehyde markedly accelerated the light (253.7 am)-caused conversion of NAD(P)H into NAD(P)⁺, which further breaks down to nucleotide and/or nucleoside. We studied the reaction between NADPH (44 uM) and several acetaldehyde samples (at 1%) in 50 mM Tris chloride (pH 7.5) with and without irradiation at 254 nm. Irradiation was carried out by placing the Teflon-stoppered quartz cuvette horizontally at 1 cm below a Mineralight ultraviolet lamp (Model UVG-54, UVP, Inc., San Gabriel, CA). The dark control was wrapped with aluminum foil and similarly placed under the UV lamp. It was found that at 1%, all acetaldehyde samples caused the reaction mixture to lose A₃₆₀ nm to some extent (up to 40% in 2 min) without irradiation at 254 nm. However the reaction was accelerated under irradiation. Because of the instability of acetaldehyde in air and a potential pH effect from high concentrations (e.g., 170 mM) of air-exposed acetaldehyde, we have not determined whether acetaldehyde or the interfering substance(s) or both played a role in the light reaction.

**Effect on alcohol dehydrogenase activity.** The observed generation of NAD(P)H-reacting substance(s) from acetaldehyde in air is expected to affect ethanol dehydrogenase assay because of the lowering of true acetaldehyde concentration and the formation of
potentially inhibitory substance(s). NADPH-linked ethanol dehydrogenase activity of highly purified alcohol dehydrogenase from *C. beijerinckii* was thus measured with six acetaldehyde samples containing different levels of interfering substance(s) (Table V-1). The results show that the measured ethanol dehydrogenase activity was always lower with acetaldehyde samples containing measurable level of NAD(P)H-reacting substance(s). However, the lowering of enzymic activity was not proportional to the level of NAD(P)H-reacting substance(s) in acetaldehyde samples, especially with aged samples. This is perhaps because the level of NAD(P)H-reacting substance does not reflect the total decrease in acetaldehyde concentration and the level of other inhibitory substances.

Table V-1. Alcohol dehydrogenase activity measured with different acetaldehyde samples.

<table>
<thead>
<tr>
<th>Acetaldehyde*</th>
<th>Enzyme Activity (unit per mg protein)*b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample A</td>
<td>11.3 (100%)</td>
</tr>
<tr>
<td>Sample B</td>
<td>11.6 (103%)</td>
</tr>
<tr>
<td>Sample C</td>
<td>7.68 (68%)</td>
</tr>
<tr>
<td>Sample D</td>
<td>5.47 (48%)</td>
</tr>
<tr>
<td>Sample E</td>
<td>1.72 (15%)</td>
</tr>
<tr>
<td>Sample F</td>
<td>9.55 (85%)</td>
</tr>
</tbody>
</table>

* Sample designations are as follows: (A) redistilled under strictly O₂-free conditions, (B) from a newly opened bottle, (C) redistilled under faulty anaerobic conditions, (D) redistilled in air, (E) from a bottle first opened over 20 months ago, and (F) sample D distilled again under strictly O₂-free conditions. Acetaldehyde was at 5 mM (nominal concentration).

b One unit is defined as the oxidation of one μmol NADPH per min.
Identification of NADP$^+$ as a reaction product. Since the highest amount of NAD(P)H-reacting substance(s) was present in acetaldehyde samples that either had been exposed in the liquid state (at 4°C) to air for many months or had been exposed in the vapor state (above 20°C) to air for a few minutes, it seems that the NAD(P)H-reacting substance(s) is the autoxidation product(s) of acetaldehyde, most likely peroxides and peracetic acid (Hayes, 1963). Therefore, the reaction between NAD(P)H and the interfering substance(s) is likely redox in nature, as suggested by the disappearance of $A_{340\text{ nm}}$. The UV-visible absorption spectra of the reaction mixture were consistent with the conversion of NAD(P)H to NAD(P)$^+$, as the absorption maximum at 259 nm remained when $A_{340\text{ nm}}$ was abolished. However, the spectral data did not rule out the possibility that NAD(P)$^+$ further broke down to mononucleotides. Yeast glucose-6-phosphate dehydrogenase (specific for NADP$^+$) was thus used to examine the reaction product and found that about 70% of the starting $A_{340\text{ nm}}$ from NADPH was recovered by this assay, indicating that NADPH was oxidized to NADP$^+$ by interfering substance(s) present in acetaldehyde.
SUMMARY

A decrease in absorbance at 340 nm, at rates similar to those obtained with alcohol dehydrogenases in routine assays, occurred when NADH or NADPH was mixed with acetaldehyde that had been exposed to air for various durations. NAD(P)H was apparently oxidized to NAD(P)⁺ by interfering substance(s) present in acetaldehyde. Reagent-grade acetaldehyde from newly opened bottles as well as acetaldehyde redistilled under strictly O₂-free conditions contained minimal amounts of NAD(P)H-reacting substance(s). Redistillation under poor anaerobic conditions or in air increased the amount of NAD(P)H-reacting substance(s) in redistilled acetaldehyde. NAD(P)H reacted at a higher rate than NADH with the interfering substance(s) in Tris chloride buffer at pH 7.5. Also, the reaction was faster in Tris buffer than in phosphate buffer at pH 7.5. The NAD(P)H-oxidizing reaction may not be apparent when the nominal concentration of acetaldehyde used was below 5 mM, but the measured ethanol dehydrogenase activity could be significantly lower with acetaldehyde containing a measurable level of interfering substance(s). This study suggests that acetaldehyde is most easily tested with NADPH for the presence of a significant level of interfering substance(s). The assay containing NADPH (0.2 mM) and acetaldehyde (30 mM, nominal concentration) in 50 mM Tris chloride buffer (pH 7.5). At about 20°C, an absorbance decrease (ΔA₃₄₀ nm/min) faster than 0.005 in the test would indicate that the acetaldehyde samples may not be suitable for alcohol dehydrogenase assay. If redistillation of acetaldehyde is necessary, it must be performed under strictly O₂-free conditions.
REFERENCES


Chapter VI.

CONCLUSIONS AND SUGGESTIONS

Few species of saccharolytic clostridia are the only bacteria known to produce 1-butanol as a major fermentation product. Clostridium beijerinckii NRRL B592, which produces acetone, 1-butanol and ethanol, was used in this study. This research included a study of the expression of solvent-forming enzymes and the onset of solvent formation, the purification and characterization of butanol-forming enzymes, and identification of substance(s) present in acetaldehyde samples interfering with the assay of alcohol dehydrogenase. Conclusions from this study and suggestions for future research are described below.

The beginning of solvent production in a batch culture is about 2 h earlier than the previously assigned onset time. The previously recognized low pH (<5) for rapid solvent production is apparently not required for the onset of solvent production in C. beijerinckii. The total acid concentration in cultures at the onset of solvent production was lower than the previously reported value. Expression of solvent forming enzymes started at least 40 min earlier than the apparent onset of solvent production. Activities of all solvent-forming enzymes did not rise simultaneously. The degree of increase in these enzyme activities also varied, from 2- to at least 165-fold. The information is useful for enzyme purification since we can harvest the culture when the enzyme level is high and the cell is still sensitive to lysozyme.

Since the onset of expression of solvent-forming enzymes and the onset of solvent production were much earlier than the previously recognized time, investigations of extra- and intracellular conditions as possible triggering signals for the metabolic transition can
now be carried out with cultures or cells at a more pertinent stage of growth. It may be worthwhile to determine whether a differentiation can be made between conditions that are most effective for triggering the onset of solvent production (enzyme expression) and conditions that are optimal for the ensuing solvent production.

Butanol-forming enzymes (CoA-acylating ALDH and the NADH/NADPH-dependent ADH) with a dual specificity for coenzymes were purified from C. beijerinckii NRRL B592. The ALDH can use butyraldehyde and acetaldehyde as substrates, and it has a native Mₐ of 100,000 and a subunit Mₐ of 55,000, suggesting that the native enzyme is dimeric. The ALDH is sensitive to O₂. DTT can protect the ALDH from O₂ inactivation. The O₂-inactivated enzyme can be reactivated by incubating the enzyme with CoA in the presence or absence of DTT prior to assay. It is the first report that CoA alone, but not DTT or NAD⁺ alone, causes a rapid and significant activation of an ALDH. NAD⁺, on the other hand, decreased the extent of activation by CoA. This competition may be related to their structural similarities. It suggests that the concentration and the ratio of CoA and NAD⁺ in vivo can affect the ALDH activity. Therefore, it is possible that solvent production is also regulated at the enzyme level by these parameters. It may be worthwhile to determine the cysteine residues that are involved in the inactivation/reactivation so as to gain insights into the structure/function and physiological regulation of the ALDH. To do this, cloning and sequencing of the gene and a functional analysis of selected amino acid residues (by site directed mutagenesis) will need to be performed.

Two ADHs are present in C. beijerinckii NRRL B592: one is NADPH-dependent (the P-ADH) and the other is NADH/NADPH-dependent (the D/P-ADH). Distinct properties were observed for the two ADHs. The D/P-ADH is unstable at 55°C, whereas the P-ADH
is quite stable under the same conditions. Both the NADH-dependent and the NADPH-dependent activities of the D/P-ADH were $O_2$ sensitive, whereas the NADPH-dependent activity of the P-ADH was $O_2$ insensitive. Preliminary results with the highly purified D/P-ADH suggest the presence of two nonidentical subunits, which is a property distinct from all other bacterial ADHs.

Dehydrogenases generally contain two major domains with one domain responsible for coenzyme specificity and the binding of the coenzyme and the other domain responsible for substrate specificity and the binding of the substrate (Rossmann et al., 1975; Jornvall, 1977; Taylor, 1977; Wierenga et al., 1985; Wierenga et al., 1986; Pai et al., 1988; Scrutton et al., 1990). Distinct amino acid sequences have been identified as the determinant for the coenzyme specificity: with GXGXXGXXXGXXXXXG specifying NAD(H) and GXGXXAXXAXXXAXXXAXXXAXXXG specifying NADP(H) (Hanukoglu and Gutfinger, 1989; Scrutton et al., 1990). For dehydrogenases exhibiting a dual specificity for coenzymes, it is not clear whether both types of sequences are present or a yet to be identified sequence is involved. Thus, it will be worthwhile to identify the coenzyme-binding sequence(s) in the D/P-ALDH and the D/P-ADH of *C. beijerinckii* NRRL B592. The information is important to the understanding of the nature of the dual specificity for coenzymes, and the possible regulation of the product pattern via differential involvement of the two coenzymes. A structure-function investigation of the genes encoding the two dehydrogenases should be a logical step to follow.
REFERENCES


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and butanol-ethanol-isopropanol dehydrogenase: different alcohol
dehydrogenases in two strains of Clostridium beijerinckii (Clostridium
reacts with NAD(P)H and interferes with assay of alcohol dehydrogenase.
forming enzymes and onset of solvent production during growth in batch
Clostridium beijerinckii NRRL B592. Appl. Environ. Microbiol. 56:2591-
2599.
Plant physiology related:
wheat. Cereals. 5:21-25.
(mini review). 3:14-17.
of phases of growth and development in wheat. Agricultural Science of
China. 2:27-35.

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