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Purification and Characterization of a Primary-Secondary Alcohol Dehydrogenase from Two Strains of *Clostridium beijerinckii*

ADNAN A. ISMAIEL, CHANG-XI ZHU,† GARY D. COLBY, AND JIANN-SHIN CHEN*

Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

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Two primary alcohols (1-butanol and ethanol) are major fermentation products of several clostridial species. In addition to these two alcohols, the secondary alcohol 2-propanol is produced to a concentration of about 100 mM by some strains of *Clostridium beijerinckii*. An alcohol dehydrogenase (ADH) has been purified to homogeneity from two strains (NRRL B593 and NESTE 255) of 2-propanol-producing *C. beijerinckii*. When exposed to air, the purified ADH was stable, whereas the partially purified ADH was inactivated. The ADHs from the two strains had similar structural and kinetic properties. Each had a native M_r of between 90,000 and 100,000 and a subunit M_r of between 38,000 and 40,000. The ADHs were NADP(H) dependent, but a low level of NAD^+-linked activity was detected. They were equally active in reducing aldehydes and 2-ketones, but a much lower oxidizing activity was obtained with primary alcohols than with secondary alcohols. The k_{cat}/K_m value for the alcohol-forming reaction appears to be a function of the size of the larger alkyl substituent on the carbonyl group. ADH activities measured in the presence of both acetone and butyraldehyde did not exceed activities measured with either substrate present alone, indicating a common active site for both substrates. There was no similarity in the N-terminal amino acid sequence between that of the ADH and those of fungi and several other bacteria. However, the N-terminal sequence had 67% identity with those of two other anaerobes, *Thermoanaerobium brockii* and *Methanobacterium palustre*. Furthermore, conserved glycine and tryptophan residues are present in ADHs of these three anaerobic bacteria and ADHs of mammals and green plants.

Alcohol dehydrogenase (ADH) is widespread in nature. The ADHs of human and horse liver and baker's yeast are well characterized (7, 15, 30). Although the metabolism of ethanol is a recognized physiological role of ADH, isozymes of ADH often show higher levels of activity toward other substrates. ADHs react with normal and branched-chain aliphatic and aromatic alcohols, both primary and secondary, as well as the corresponding aldehydes and ketones. The broad substrate range has led to suggestions for other roles for ADH (42), and it remains difficult to determine the physiological reaction and significance of an ADH when multiple ADHs are present in an organism. In anaerobic bacteria that produce alcohols as major end products (27) or that grow on alcohols (e.g., references 5 and 16), the physiological role of the predominant ADH is the formation or oxidation of the pertinent alcohols.

Although ADHs generally have a broad substrate range, few are equally reactive toward both primary and secondary alcohols. For example, the liver ADHs react with both primary and secondary alcohols (12). Among the liver ADHs, the human αα isozyme (class I) is far more efficient than the others in oxidizing secondary alcohols (49). Nevertheless, the human αα ADH is still considered a primary ADH because it reacts with primary alcohols more efficiently than with secondary alcohols (49). ADHs that are specific for or show a higher level of activity toward secondary alcohols (or ketones) have been purified from bacteria (5, 8, 10, 21, 37, 55), a yeast (*Candida boidinii* [46]), and a protozoan (35). The physiological significance of the secondary alcohol-ketone-linked activity is not clear for some of the ADHs.

Among alcohol-producing bacteria, most produce ethanol only, but several *Clostridium* species produce 1-butanol and ethanol (plus acetone) as the major products (27). Distinct from species that produce only primary alcohols, there are two species, *Clostridium beijerinckii* (including those organisms formerly known as "*Clostridium butylicum*") and *Clostridium aurantibutylicum*, that produce 2-propanol, in addition to 1-butanol and ethanol, as a major product (9, 18). Both 1-butanol and 2-propanol are important industrial chemicals, and the property of the ADH producing the two alcohols is relevant to practical uses of the fermentation and to research aimed at an understanding of the structure-function relationship in ADHs.

The purpose of this study was to determine whether or not a single ADH can produce both 1-butanol and 2-propanol. Properties of a partially purified ADH from *C. beijerinckii* NRRL B593 suggest that to be the case (19). To establish this point more conclusively, we examined the ADH in four strains of *C. beijerinckii* that produce both primary and secondary alcohols. A primary-secondary ADH has been purified to homogeneity from two of the strains. In this paper, we report the purification and characterization of the two ADHs.

MATERIALS AND METHODS

Materials. Phenazine methosulfate, *p*-iodonitrotetrazolium violet, semicarbazide-HCl, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2-(*N*-morpholino)ethanesulfonic acid (MES), chloride determination kit, lysozyme (chicken egg white), DNase I, RNase A (bovine

* Corresponding author.
† Present address: Department of Biochemistry and Molecular Biology, New York Medical College, Valhalla, NY 10595.

pancreas), bovine serum albumin, chicken egg albumin, catalase (bovine liver), immunoglobulin G (human), myoglobin (whale muscle), thyroglobulin (bovine), α-chymotrypsinogen A, and *Thermoanaerobium brockii* ADH (purified) were from Sigma Chemical Co.; 1-propanol, 1-butanol, 2-butanol, 2-pentanol, propionaldehyde, 2-butanone, and 2-pentanone, all of the gold-label grade, were from Aldrich Chemical Co.; ethanol (95%) was from AAPER Alcohol and Chemical Co.; methanol was from Burdick & Jackson Laboratories; acetaldehyde, acetone, butyraldehyde, and 2-propanol were from Fisher Scientific Co.; α-toluenesulfonyl fluoride (PMSF) was from Eastman Kodak Co.; sodium dodecyl sulfate (SDS), hydroxylapatite (Bio-Gel HTP), dye-binding protein assay kit, and immunoglobulin G (bovine) were from Bio-Rad Laboratories; Matrex Gel Red A and ultrafiltration membranes were from Amicon Corp.; tryptone and yeast extract were from Difco Laboratories.

Bacteria and growth conditions. Four strains (NRRL B593 [= VPI 13437] and NESTE 253, 254, and 255) of 1-butanol- and 2-propanol-producing clostridia were used in this study. (The NESTE strains were from J.-F. Selin of NESTE Oy, Porvoo, Finland.) These four strains were identified as *C. beijerinckii* by DNA sequence similarity measurements (18, 26), but the pattern of the cellular fatty acids of the NESTE strains had a low identification similarity when compared with a *C. beijerinckii* library (Moore Broth Library, version 3.0, Microbial ID, Newark, Del. [38]), and strain NESTE 255 differed from the other *C. beijerinckii* strains in its ability to grow after three serial transfers in a glucose-mineral salts-biotin medium (20, 38). These four strains also differed from one another in rates of growth and solvent production (51).

Spores were produced either in a tryptone-yeast extract-sucrose medium or in a potato extract-glucose medium (17, 18) and were stored in the same medium at −70°C. Culture conditions were as described previously (19), except that tryptone-yeast extract-glucose was used for growth of the NESTE strains. Cells were harvested in early solvent-producing phase (about 15 h after inoculation) when the level of ADH reached the maximum (60). Washed cells were first frozen in liquid nitrogen and then stored at −70°C.

Analytical procedures. Protein was determined by the dye-binding assay (6) with bovine immunoglobulin G as a standard. The chloride concentration was assayed with the Sigma chloride determination kit. ADH activities were routinely measured by following the reduction of butyraldehyde (33 mM) or acetone (6.7 mM) with NADPH (ε_{340} = 6.2 mM^{-1} cm^{-1}) at 25°C under Ar. The assay mixture (1 ml) contained 50 mM Tris-Cl buffer (pH 7.5), 1 mM dithiothreitol (DTT), and 0.2 mM NADPH. Acetone was first diluted 10-fold with water before use. Because butyraldehyde is not readily dissolved in water, it was first diluted 10-fold with methanol before use (19). Methanol did not affect the ADH activity. One unit of activity is the oxidation of 1 μmol of NADPH per min.

Preparation of crude extracts. Cell paste was thawed under N_2 or Ar in 50 mM sodium–HEPES buffer (pH 8.5) containing lysozyme (2 mg/ml), DTT (0.2 mM), DNase I (0.1 mg/ml), and PMSF (0.3 mg/ml). The cell suspension (3 ml of buffer per g of cells) was then incubated at room temperature under N_2 or Ar for 2.5 h. Cell debris was removed by centrifugation at 43,500 × *g* for 30 min at 4°C. The supernatant (crude extract) was collected, and glycerol was immediately added to a final concentration of 20% (vol/vol). Crude extracts prepared with lysozyme had a higher clarity than those prepared with a French pressure cell (19).

Purification of ADH. The previously reported partial purification of ADH from *C. beijerinckii* NRRL B593 (19) gave specific activities of about 25% of those obtained with the current procedure. Major changes in the procedure included the use of lysozyme in cell disruption and the inclusion of glycerol (20%, vol/vol) in the buffer during the first (DE-52) and the last (Matrex Gel Red A) steps of purification. All purification steps were carried out under Ar or N_2 at room temperature (about 23°C). Between purification steps, active fractions were stored anaerobically at 4°C overnight, and the purified ADH was stored as frozen droplets in liquid nitrogen. All buffers contained DTT (2 mM) and PMSF (0.3 mg/ml).

(i) DEAE-cellulose column. Crude extracts (2.1 to 2.5 g of protein) were applied to a DE-52 column (5 by 7 cm) which had been equilibrated with 50 mM Tris-Cl buffer (pH 8.0) containing glycerol. After being washed with 100 ml of the same buffer, the column was washed with a linear gradient of KCl (0 to 0.6 M in the same buffer; total volume, 400 ml). The flow rate was 100 ml/h, and the ADH was collected in 40- to 60-ml fractions during washing.

(ii) Hydroxyapatite column. To lower the glycerol concentration in the sample, the pooled active fractions from the DE-52 column were concentrated 10-fold through an Amicon Diaflo membrane (PM-30) and then diluted 5-fold with Tris-Cl buffer without glycerol. The treated sample was applied to a Bio-Gel HTP column (2.5 by 10 cm) equilibrated with 0.1 M sodium phosphate buffer (pH 7.0) containing 0.1 M KCl. After loading, the column was washed with 1 bed volume of the equilibrating buffer and then with a linear gradient of sodium phosphate buffer (0.1 to 0.5 M; total volume, 300 ml). The flow rate was 80 ml/h, and 8-ml fractions were collected.

(iii) Sephacryl S-300 column. Pooled active fractions from the Bio-Gel HTP column were concentrated 10-fold through an Amicon Diaflo membrane (PM-30). The concentrated sample was applied to a Sephacryl S-300 column (2.6 by 47 cm), and the eluant was 50 mM sodium phosphate buffer (pH 7.0) containing 0.1 M KCl. The flow rate was 50 ml/h, and 4-ml fractions were collected. The active fractions were pooled, and glycerol was added to a 20% (vol/vol) concentration.

(iv) Matrex Gel Red A column. The pooled active fractions from the gel filtration step were applied to a Matrex Gel Red A column (1.5 by 6 cm) which was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 M KCl and 20% (vol/vol) glycerol. The column was washed with 30 ml of the equilibrating buffer and then with a linear gradient of KCl (0.2 to 1 M in phosphate buffer; total volume, 80 ml). The flow rate was 40 ml/h, and 3-ml fractions were collected.

Determination of molecular weight. The molecular weight of the native ADH was estimated by gel filtration on a Sephacryl S-300 column, with the elution position of ADH determined by activity assays. Molecular weight standards used were thyroglobulin (669,000), baker's yeast alcohol dehydrogenase (150,000), bovine serum albumin (66,000), and ribonuclease A (13,700). The K_{av} values were plotted in the estimation of the molecular weight.

The molecular weight of subunits was determined by continuous and discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) (36, 53). Molecular weight standards used were bovine serum albumin (66,000), catalase (57,500), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), α-chymotrypsinogen A (25,700), trypsinogen (24,000), and myoglobin (17,200). The R_f values were plotted in the determination of the molecular weight.

Metal analysis. The purified ADH from strain NRRL B593 was analyzed for 11 elements (Al, B, Ca, Cu, Fe, K, Mg, Mn, Na, P, and Zn) with an inductively coupled plasma spectrometer (Jarrell-Ash ICAP 9000) at the Soil Testing and Plant Analysis Laboratory of Virginia Polytechnic Institute and State University. Active enzyme samples were diluted 10-fold with distilled water before analysis so that the samples contained 5 mM sodium phosphate (pH 7.0), 50 mM KCl, and 2% (vol/vol) glycerol. One sample was also passed through a Sephadex G-25 column (PD-10, Pharmacia) to remove low levels of DTT and PMSF. All buffer components were analyzed for these elements, and Zn standards prepared in sample buffer were also measured.

PAGE and activity stain. Electrophoresis of enzyme samples under nondenaturing conditions was performed in 7% polyacrylamide gels as described previously (19). Protein was stained with Coomassie Brilliant Blue R-250. The ADH activity band was developed by coupling alcohol oxidation to *p*-iodonitrotetrazolium violet reduction in 100 mM Tris-Cl (pH 8.2) buffer. The alcohol concentration used was 400 mM.

Determination of the N-terminal amino acid sequence. The N-terminal amino acid sequence of ADH from strain NRRL B593 was first analyzed with an Applied Biosystems Model 470A sequencer (20 cycles by M. O. Lively of the Protein Analysis Core Laboratory, the Bowman Gray Oncology Research Center, Winston-Salem, N.C.). ADHs from both strains were later analyzed with an Applied Biosystems Model 477A sequencer (20 to 30 cycles in three analyses by R. Peery and C. L. Rutherford of the Protein Sequencing Facility, Virginia Polytechnic Institute and State University). Between 25 and 50 μg of ADH was used in each analysis. ADH samples that were desalted by washing with distilled water in an Amicon Centricon-30 concentrator and without drying gave unambiguous results from the first cycle of sequencing, whereas those prepared by dialysis and drying (22) gave ambiguous results up to the third cycle of sequencing.

Stability measurements. Crude extracts were prepared in different buffers (50 mM) between pHs 7 and 8.5. Glycerol (20%, vol/vol), when being tested as a stabilizing agent, was added to a crude extract immediately after the supernatant was collected. To test the effect of oxygen on the ADH, an anaerobically prepared sample (500 μl of a crude extract or the purified ADH) was gently aerated with 60 bubbles of air (through polyethylene tubing, 0.86 mm inside diameter) during a 1-min period, which was to ensure rapid equilibration of O_2 between the liquid and the gas phases. The aerated sample was kept on ice and assayed for acetone- and butyraldehyde-linked activities at different time intervals. The thermal stability of purified ADHs from *C. beijerinckii* NRRL B593 and from *T. brockii* was tested in 25 mM potassium phosphate buffer at pH 7.1. The samples (100 μl) were incubated for 10 min at the following temperatures: 40, 50, 60, 70, 75, and 80°C for the *Clostridium* ADH (18 μg/ml) and 70, 75, and 80°C for the *Thermoanaerobium* ADH (100 μg/ml). After the heat treatment, the samples were cooled on ice and assayed for acetone-linked activity.

Effect of pH on ADH activities. The effect of pH on ADH activities was measured in both the alcohol-forming (physiological) and the alcohol-oxidizing (nonphysiological) directions. The substrates and buffers and their concentrations used are given in the figure legends.

Determination of Michaelis constants. The K_m value for alternative substrates of the ADH was first estimated with a wide range of substrate concentrations. The final measurement was made in duplicate with at least five substrate concentrations, generally covering the range of 0.5 to 5 times the estimated K_m value. The true kinetic constants (for acetone, butyraldehyde, and NADPH) were determined by using secondary plots of intercepts and slopes from the primary double reciprocal plots.

The determination of K_m values for aldehydes and ketones was carried out in 50 mM Tris-Cl buffer (pH 7.5) containing 1 mM DTT. NADPH was kept at 0.2 mM unless otherwise noted. Propionaldehyde and *n*-butyraldehyde were first diluted 10- to 100-fold with methanol, whereas acetaldehyde and ketones were diluted 10- to 100-fold with ice-cold deoxygenated water. Exposure of acetaldehyde to air was minimized to prevent the formation of NAD(P)H-oxidizing substances (59). The substrates and concentrations used were acetaldehyde (0.27 to 56 mM), propionaldehyde (0.14 to 5.6 mM), *n*-butyraldehyde (4.4 to 90 mM), acetone (0.25 to 3.3 mM), 2-butanone (0.34 to 5 mM), and 2-pentanone (4.6 to 94 mM). In addition, the concentration of NADPH was varied between 14 and 200 μM when the true kinetic constants for acetone and butyraldehyde were determined.

The determination of K_m values for alcohols was carried out in 50 mM Tris-Cl buffer (pH 8.0) containing 6.2 mM of semicarbazide-HCl. $NADP^+$ was kept at 3 mM. The alcohols and concentrations used were ethanol (49 to 1,060 mM), 1-propanol (13 to 300 mM), 1-butanol (0.21 to 218 mM), 2-propanol (4.4 to 325 mM), 2-butanol (2.5 to 210 mM), and 2-pentanol (2.7 to 130 mM).

RESULTS

ADHs of *C. beijerinckii* strains producing 1-butanol and 2-propanol. We previously reported the presence in *C. beijerinckii* NRRL B593 of ADHs with different substrate and coenzyme specificities, and the predominant ADH is an NADP(H)-dependent enzyme with similar activities toward both acetone and butyraldehyde (19). In this study, we first compared the ADHs present in four 1-butanol- and 2-propanol-producing strains of *C. beijerinckii* (NRRL strain B593 and NESTE strains 253, 254, and 255). In the routine assay, the acetone-linked ADH activity in crude extracts from all four strains was NADPH dependent, and no NADH-dependent secondary ADH activity was detected at either pH 7.5 or 6.0. With butyraldehyde as the substrate, an NADH-dependent activity was detected, but it was lower than the NADPH-dependent activity at either pH 7.5 (5 to 15%) or pH 6 (10 to 50%) for all four strains.

In crude extracts, the ratio between the acetone-linked (A) and the butyraldehyde-linked (B) ADH activities (the A/B ratio) for the four strains was between 2.0 and 2.2 with NADPH as the coenzyme. Following fractionation on a Sephacryl S-300 column, extracts from each strain yielded only one symmetric peak of NADPH-dependent ADH activities (data not shown). Acetone-linked and butyraldehyde-linked ADH activities coeluted at a position corresponding to an M_r of between 90,000 and 100,000, and the A/B activity ratio of the isolated ADH ranged between 1.75 and 1.80 for the four strains. The results suggest that either a primary-secondary ADH is present or a primary ADH and a secondary ADH form a tight complex in these strains. To differentiate between the two possibilities, the ADH with acetone- and butyraldehyde-linked activities was purified to homogeneity from two strains (NRRL B593 and NESTE 255) of *C. beijerinckii* for a detailed analysis.

Purification of ADH. Before purification, the stability of the NADPH-linked ADH activities in extracts of *C. beijer-*

TABLE 1. Purification of the primary-secondary ADH from *C. beijerinckii* NRRL B593[a]

Step	Protein (mg)	Acetone-linked activity				Butyraldehyde-linked activity				A/B ratio
		TU (U)	SA (U/mg)	Yield (%)	Fold	TU (U)	SA (U/mg)	Yield (%)	Fold	
Crude extract	2,420	314	0.130	100	1	157	0.065	100	1	2
DEAE-cellulose	136	312	2.30	99	18	155	1.14	99	18	2
Ultrafiltration	105	314	2.98	100	23	154	1.47	98	23	2
Hydroxyapatite	20.1	305	15.2	97	117	152	7.58	97	116	2
Ultrafiltration	17.8	287	16.1	92	124	147	8.25	94	127	2
Sephacryl S-300	7.95	193	24.3	62	187	98	12.3	62	189	2
Matrex Gel Red A	1.37	89.5	62.5	29	481	45.7	31.7	29	486	2

[a] TU, total units; U, unit (μmoles of NADPH oxidized per minute); SA, specific activity (units per milligram of protein); fold, fold of purification.

inckii NRRL B593 was examined at 4°C under anaerobic conditions. In extracts prepared in Tris-Cl buffer (pH 8 or 8.5), ADH was unstable with or without glycerol present, and ADH in extracts prepared with lysozyme was less stable than that in extracts prepared with a French pressure cell (data not shown). However, the ADH was stable for at least 4 days when glycerol was present in crude extracts prepared with lysozyme in sodium phosphate (pH 7.0) or sodium HEPES (pH 8.5) buffer. The latter condition was used for the preparation of crude extracts during this purification.

An ADH with primary and secondary alcohol-forming activities has been purified 480-fold, with a 29% recovery of enzyme activities, from *C. beijerinckii* NRRL B593 (Table 1), and the specific activities were fourfold higher than what was obtained with an earlier scheme (19). The first chromatographic step of the present purification was a significant improvement over the earlier one. With glycerol present in the buffer, ADH was eluted first from the DEAE-cellulose column and was clearly separated from the bulk of proteins (Fig. 1), resulting in an 18-fold purification in one step. A salt gradient is not needed for this step if ADH is the only enzyme to be purified. The eluting buffer for the final step of purification also contained glycerol (20%, vol/vol), and the peak fractions reached a constant specific activity (data not shown). Under routine assay conditions, the purified ADH had a specific activity of 62.5 U/mg of protein with acetone and 31.7 U/mg of protein with butyraldehyde. The ratio of acetone-linked versus butyraldehyde-linked ADH activities remained at 2 throughout the purification.

With this purification scheme, an ADH with a similar substrate specificity (see below) was purified 245-fold to homogeneity, with a 20% recovery of enzyme activities, from *C. beijerinckii* NESTE 255 (data not shown). Under routine assay conditions, the purified ADH had a specific activity of 35.5 U/mg of protein with acetone.

The purity of the ADH from each strain was examined by protein and activity stains after nondenaturing PAGE and by protein stain after SDS-PAGE. These procedures gave a single, intense band when 10 to 40 μg of ADH was used, and the result of SDS-PAGE is shown in Fig. 2. On the basis of the intensity of bands stained by Coomassie Brilliant Blue R-250, the purity of the ADH from both strains was at least 95%. N-terminal amino acid sequence analyses (see below) of purified ADHs gave a single residue in each cycle.

Determination of native and subunit molecular weights and metal content. The ADHs purified from the two strains of *C. beijerinckii* had similar molecular weights: gel filtration gave a native M_r of between 90,000 and 100,000, and SDS-PAGE gave a subunit M_r of between 38,000 and 40,000. The native M_r of the partially purified ADH from strain NRRL B593 was found to be 100,000 (19). Both the ADH from baker's

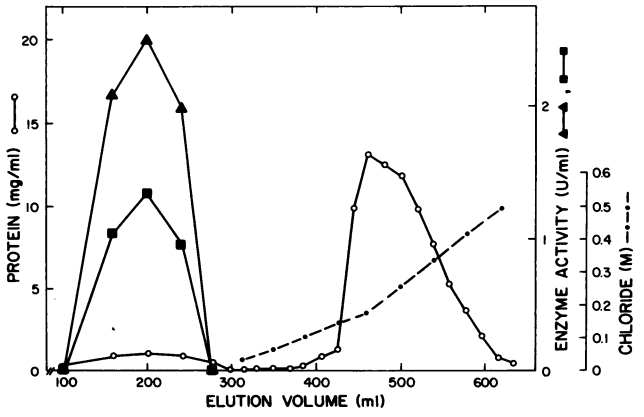

FIG. 1. Elution of the ADH of *C. beijerinckii* NRRL B593 from a DEAE-cellulose column. A crude extract (2.4 g of protein in 160 ml) was applied onto a DE-52 column (5 by 6 cm). Acetone-linked (▲) and butyraldehyde-linked (■) activities were monitored. See Materials and Methods for other conditions.

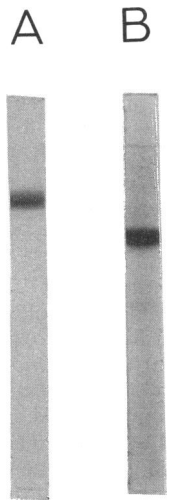

FIG. 2. Examination of the purity of the ADH from *C. beijerinckii* NRRL B593 (A) and NESTE 255 (B) by SDS-PAGE. Approximately 20 μg of ADH was analyzed on a cylindrical gel (5 by 55 mm) by the procedure of Weber and Osborn (53). Protein was stained with Coomassie Brilliant Blue R-250.

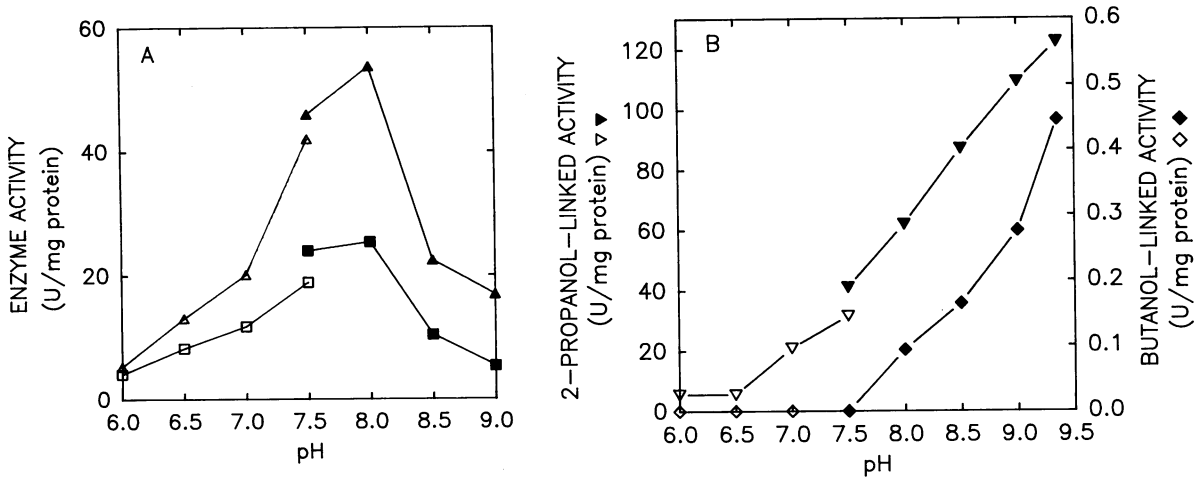

FIG. 3. Effect of pH on activities of the ADH from *C. beijerinckii* NRRL B593 in the physiological (A) and nonphysiological (B) directions. (A) Effect of pH on acetone (△ and ▲)- and butyraldehyde (□ and ■)-linked activities. The reaction mixture (1 ml) contained buffer (50 mM; sodium phosphate between pHs 6 and 7.5 and Tris-Cl between pHs 7.5 and 9), NADPH (0.2 mM), acetone (6.6 mM) or butyraldehyde (33 mM), and 0.29 μg of ADH. (B) Effect of pH on 2-propanol (▽ and ▼)- and 1-butanol (◇ and ◆)-linked activities. The reaction mixture (1 ml) contained buffer (50 mM; sodium phosphate between pHs 6 and 7.5 and Tris-Cl between pHs 7.5 and 9.4), semicarbazide (6.3 mM), and $NADP^+$ (3 mM). Butanol (1 mM)- and 2-propanol (330 mM)-linked activities were measured, respectively, with 2.9 and 0.29 μg of ADH.

yeast (M_r = 150,000) and the ADH from *T. brockii* (M_r = 150,000) were examined along with the ADH from the two strains of *C. beijerinckii* to confirm the apparent native M_r of the clostridial ADH. The quaternary structure of the *C. beijerinckii* ADH is intriguing and remains to be elucidated. Metal analysis detected zinc and iron in the purified ADH from strain NRRL B593, and two preparations gave, respectively, 0.94 and 1.36 g-atoms of zinc per subunit (M_r, 38,000) of the ADH. Further metal analysis will await the preparation of active ADH samples after the removal of any adventitiously bound metals.

N-terminal amino acid sequences. The N-terminal amino acid sequence of the ADH from *C. beijerinckii* NRRL B593 was Met-Lys-Gly-Phe-Ala-Met-Leu-Gly-Ile-Asn-Lys-Leu-Gly-Trp-Ile-Glu-Lys-Glu-Arg-Pro-Val-Ala-Gly-Ser-Tyr-Asp-Ala-Ile-Val-Arg-. The first 21 residues of the ADH from *C. beijerinckii* NESTE 255 were identical to those of the ADH from NRRL B593.

Stability of purified ADH. The purified ADH from *C. beijerinckii* NRRL B593 was fully active after an incubation at 70°C for 10 min, but it lost 70 and 100% of activity, respectively, after 10 min at 75 and 80°C.

The purified ADH from both strains of *C. beijerinckii* was stable in air as no activity was lost after a 1-h exposure. However, the ADH in crude extracts was inactivated by exposure to air. Between 25 and 50% (strain dependent) of acetone-linked activity was lost after a 30-min exposure to air, but the remaining activity was more stable (data not shown). The ADH no longer exhibited oxygen sensitivity after it had passed through the hydroxyapatite column step (no glycerol in the buffer), which was also when the ADH was separated from colored contaminants.

Effect of pH on ADH activities. The acetone-linked (A) and butyraldehyde-linked (B) activities were measured between pHs 6 and 9. The ADH from strain NRRL B593 had an apparent activity peak between pHs 7.5 and 8.0 (Fig. 3A), whereas the ADH from strain NESTE 255 had an activity peak between pHs 7.0 and 7.5 (data not shown). For the ADH from both strains, the activity ratio between A and B rose from about 1 to 3 when the pH was raised from 6 to 9. Thus, a change in intracellular pH could change the product pattern (the ratio between 1-butanol and 2-propanol formed) if the ADH activity is a determining factor.

Figure 3B shows the effect of pH on the alcohol-oxidizing activities (the nonphysiological reactions) of the ADH from strain NRRL B593. The 2-propanol-linked activity remained the same between pHs 6 and 6.5 and then continued to increase when the pH rose from 6.5 to 9.4. The 1-butanol-linked activity was not detected between pHs 6 and 7.5, and it then continued to increase between pHs 7.5 and 9.4. The low alcohol-oxidizing activity below pH 7 is consistent with the physiological role of the ADH.

Coenzyme specificity. The purified ADH from both strains exhibited no NAD(H)-linked activity (less than 0.01 U/mg) in the spectrophotometric assay with acetone, 2-propanol, butyraldehyde, or 1-butanol as the substrate, whereas NADP(H)-linked activities were readily measured with a range of ketones, aldehydes, and alcohols (Table 2). The results are consistent with the finding that in crude extracts the acetone-linked activity was observed with NADPH only. However, the apparent specificity for NADP(H) is not absolute. When activity stain (with 2-propanol) was performed following gel electrophoresis of the purified ADH from both strains, NAD^+-linked activity was observed although it was much lower than $NADP^+$-linked activity (data not shown). The activity stain was performed in the presence of *p*-iodonitrotetrazolium violet, which was shown to facilitate the detection of alcohol-oxidizing activities (19).

Kinetic properties. (i) Alcohol formation. The ADH from both strains was examined for activities with alternative substrates in the physiological (alcohol-forming) direction. The apparent K_m and k_{cat} values are shown in Table 2. For acetone, butyraldehyde, and NADPH, the true K_m value was determined by varying both the substrate and coenzyme concentrations. An apparent relationship existed between the catalytic efficiency and the size of the substituent on the carbonyl group of the substrates (see Discussion).

To characterize the active site(s) of the ADH for reactions toward both ketones and aldehydes, ADH activities were measured in the presence of the two physiological sub-

TABLE 2. Kinetic constants of the primary-secondary ADH of *C. beijerinckii* NRRL B593 and NESTE 255

Substrate	K_m (mM)		k_{cat} (min^{-1})		k_{cat}/K_m (min^{-1} mM^{-1})	
	NRRL B593	NESTE 255	NRRL B593	NESTE 255	NRRL B593	NESTE 255
Aldehydes						
Acetaldehyde	11	13	21,000	14,000	1,900	1,100
Propionaldehyde	8.9	10	29,900	20,000	3,400	2,000
Butyraldehyde	34	36	8,000	5,550	240	150
Ketones						
Acetone	0.98	0.94	8,330	6,250	8,500	6,700
2-Butanone	1.5	1.1	3,850	2,130	2,600	1,900
2-Pentanone	8.5	6.9	1,050	650	120	94
Primary alcohols						
Ethanol	260	260	97	280	0.37	1.1
1-Propanol	57	77	88	140	1.5	1.8
1-Butanol	ND[a]	ND	ND	ND		
Secondary alcohols						
2-Propanol	9.8	7.5	3,310	7,010	340	930
2-Butanol	5.7	6.3	6,450	8,730	1,100	1,400
2-Pentanol	5.2	8.3	400	470	77	57
NADPH (acetone)	0.022	0.016				
NADPH (butyraldehyde)	0.031	0.033				
NADH (acetone or butyraldehyde)	NA[b]	NA				

[a] ND, not determined because activities were at the detection limit (0.01 to 0.05 U/mg); a V_{max} of 0.05 U/mg corresponds to a k_{cat} of 5.
[b] NA, no measurable activity (less than 0.01 U/mg).

strates, acetone and butyraldehyde, singly or combined over a range of concentrations (Table 3). When both substrates were present, the measured activity did not exceed the higher activity obtained with one of the substrates alone. The results suggest that a common (or overlapping) active site is responsible for the aldehyde- and ketone-linked activities of the ADH.

(ii) Alcohol oxidation. In the alcohol-oxidizing (nonphysiological) direction, the ADH had a significant activity only with the secondary alcohols (Table 2). 1-Butanol was inhibitory above 1 mM (data not shown). In addition, 1-butanol was a competitive inhibitor against 2-propanol, with a K_i of 0.21 mM for 1-butanol with the ADH from strain NRRL B593. The competition between 1-butanol and 2-propanol further suggests that the ADH has common or overlapping active sites for both primary and secondary alcohols, but it remains to be elucidated why primary alcohols are oxidized only very slowly.

TABLE 3. Activity of the primary-secondary ADH of *C. beijerinckii* NRRL B593 in the presence of alternative substrates[a]

Substrate (mM)		NADPH-oxidizing activity ($\mu mol \cdot min^{-1} \cdot mg$ of $protein^{-1}$)
Acetone	Butyraldehyde	
13.2	None	38.6
None	33	20.0
13.2	33	32.9
13.2	16.5	32.1
6.6	None	38.7
None	44	21.8
6.6	44	32.9
2.64	44	30.6

[a] Assay mixtures (1 ml) contained 50 mM sodium phosphate (pH 7.0), 1 mM reduced glutathione, and 0.2 mM NADPH.

DISCUSSION

The presence of ADH isozymes in eukaryotic organisms is common, but the physiological role of a specific ADH isozyme is sometimes difficult to establish. ADH isozymes also occur in bacteria. For example, *Bacillus stearothermophilus* (45, 47), *Thermoanaerobacter ethanolicus* (8), and *Zymomonas mobilis* (11, 33, 34, 39, 56) each contain two structurally distinct ADHs. The solvent-producing clostridia also contain multiple ADHs. *Clostridium acetobutylicum* has at least three different primary ADHs (4, 14, 44, 54), and the amino acid sequences encoded by *adh1* (61) and *bdhA* and *bdhB* (52) have been deduced. *C. beijerinckii* has a primary-secondary ADH (reference 19 and this study) and multiple primary ADHs with different coenzyme specificities (24, 57). The physiological significance of the different forms of primary ADHs in solvent-producing clostridia remains to be determined.

The coenzyme specificity of the ADH isozymes of solvent-producing clostridia has been the subject of several studies. The primary-secondary ADH of *C. beijerinckii* is practically an NADP(H)-dependent enzyme, but a low level of NAD^+-dependent activity can be detected (this study). The primary-secondary ADH is the predominant ADH present in 2-propanol-producing strains of *C. beijerinckii*. Butanol-producing clostridia that do not produce 2-propanol contain only primary ADHs. There are contradictory reports concerning the coenzyme specificity of the primary ADHs of *C. acetobutylicum* and *C. beijerinckii*. The primary ADH was earlier reported to be NADH dependent (2, 41) or NADPH dependent (17, 44). These earlier studies used crude extracts, and ADH activities were measured within a narrow pH range. Besides the complication caused by the presence of ADHs with different coenzyme specificities and different pH profiles, the determination of the coenzyme specificity of ADH in crude extracts can be complicated by the presence of other NAD(P)H-dependent dehydrogenases that share a common substrate with ADH (e.g., butyralde-

hyde is a substrate for both aldehyde dehydrogenase and ADH).

The purified aldehyde dehydrogenase from *C. beijerinckii* NRRL B592 can use either NAD(H) or NADP(H) as the coenzyme (58). However, only NAD^+-linked activities were detected in crude extracts when A_{340} changes were monitored. Measurements of the other product (acyl-coenzyme A) of the reaction showed that both NAD^+- and $NADP^+$-linked activities are present in crude extracts (58). The presence in *C. acetobutylicum* and *C. beijerinckii* of primary ADHs reactive toward both NAD(H) and NADP(H) is now established (24, 52, 54, 57, 61). The coenzyme-binding site of the ADH isozymes of clostridia can be examined to elucidate the structural basis for the different coenzyme specificities.

The subunit M_r of 38,000 to 40,000 is comparable between the primary-secondary ADH of *C. beijerinckii* (this study) and that of *T. brockii* (references 1, 37, and 40 and this study). However, the apparent native molecular weight differed significantly between the ADHs from these two species. An M_r of 90,000 to 100,000 was estimated for the primary-secondary ADH from four strains of *C. beijerinckii*. However, the ADH of *T. brockii* has an M_r of about 150,000 (references 1 and 37 and this study). The quaternary structure of the *C. beijerinckii* ADH remains to be determined.

The oxygen sensitivity of the primary secondary ADH in crude extracts was not expected because ADHs generally are stable in air. It has not been determined whether the O_2-dependent inactivation of the ADH involved other cellular components. A possible explanation for the O_2-dependent inactivation of the ADH in impure preparations is that an enzymic or nonenzymic entity (such as a metal-catalyzed oxidation system) that uses NAD(P)H or a mercaptan as an electron donor to convert O_2 into more reactive H_2O_2 or oxygen radicals is present (48).

On the basis of coenzyme and substrate specificities, the *C. beijerinckii* primary-secondary ADH was most similar to two acetone-reducing ADHs purified from two thermophilic anaerobic bacteria, *T. brockii* (37) and *T. ethanolicus* (8), and to a lesser extent to the two acetone-reducing ADHs purified from, respectively, a methanogen, *Methanobacterium palustre* (5), and a parasitic protozoan, *Tritrichomonas foetus* (35). All five of these ADHs are NADP(H) dependent, but low levels of activity with NAD(H) have been detected with the *C. beijerinckii* ADH (this study) and the *T. brockii* ADH (1). The acetone-reducing ADHs purified from *C. beijerinckii* (this study), *T. brockii* (32, 37), and *T. ethanolicus* (8) have comparable activities toward aldehydes, and they are, therefore, primary-secondary ADHs.

All five of these NADP(H)-dependent, acetone-reducing ADHs reacted with 2-pentanone or 2-pentanol. The *T. brockii* ADH is active with 2-octanone (32), and the *T. ethanolicus* ADH is active with 2-heptanol (8). There is a large drop in activity for the ADHs when the size of the substrate is increased from 2-butanone (or 2-butanol) to 2-pentanone (or 2-pentanol), or from 2-propanol to 2-butanol for the *T. ethanolicus* ADH, which gives a general indication of the volume of the substrate-binding pocket for these ADHs.

The activity of the *C. beijerinckii* ADH in the formation of primary and secondary alcohols can be compared in the manner shown in Table 4. Here the catalytic efficiency (based on the k_{cat}/K_m value) of the ADH from both strains is compared between a ketone and an aldehyde that contain the same larger substituent (designated R) on the carbonyl group. An apparent trend between the catalytic efficiency and the size of the larger substituent is seen (Table 4). A comparable catalytic efficiency was observed with 2-butanone and propionaldehyde, both of which have an ethyl group, or with 2-pentanone and butyraldehyde, both of which have a propyl group. However, acetone gave a significantly higher (four- to sixfold) k_{cat}/K_m value than acetaldehyde while the two share a common methyl group. Judging from the trend of the catalytic efficiency, acetaldehyde appears to give an unusually low catalytic efficiency.

TABLE 4. Catalytic efficiency of the primary-secondary ADH of *C. beijerinckii* NRRL B593 and NESTE 255 in relation to the size of the substituent on the carbonyl group

Substrate	R	R′	k_{cat}/K_m (min^{-1} mM^{-1})	
			NRRL B593 ADH	NESTE 255 ADH
Acetone	CH_3-	CH_3-	8,500	6,700
Acetaldehyde	CH_3-	H-	1,900	1,100
2-Butanone	CH_3CH_2-	CH_3-	2,600	1,900
Propionaldehyde	CH_3CH_2-	H-	3,400	2,000
2-Pentanone	$CH_3CH_2CH_2$-	CH_3-	120	94
Butyraldehyde	$CH_3CH_2CH_2$-	H-	240	150

The low level of activity with acetaldehyde may be explained by the formation of a nonproductive but reversible enzyme-substrate complex when acetaldehyde binds to ADH in one of two possible orientations. For ketones and aldehydes, the smaller substituent (designated R′) on the carbonyl group is either a methyl group (for 2-ketones) or a hydrogen atom (for aldehydes). It is postulated that the substrate-binding pocket on the ADH contains two subsites: subsite *R* for the substituent group R and subsite *R′* for the substituent group R′ of a substrate (Table 4). Subsite *R′* is postulated to accommodate equally well either a hydrogen atom or a methyl group but not a larger group. It is proposed that a methyl group (present in acetone and acetaldehyde) best fits subsite *R*, whereas a larger alkyl group (C_2 or C_3) is accommodated but not as well. A hydrogen atom may not bind productively at subsite *R*. Thus, when acetaldehyde binds with the methyl group at subsite *R′* and with the hydrogen atom at subsite *R*, a much less productive complex with the ADH is formed. If the dissociation of this nonproductive complex is relatively slow, the ADH activity with acetaldehyde may be severalfold lower than expected, as the result seems to indicate. The presence of a small alkyl site and a large alkyl site has been proposed for the ADH from *T. brockii* to explain the enzyme's activity pattern toward various ketones (32).

The N-terminal sequences of the primary-secondary or secondary ADHs from *C. beijerinckii*, *T. brockii*, and *M. palustre* had 67% identity (Fig. 4). A comparison of the N-terminal sequences of the three ADHs with those of eukaryotic and other bacterial ADHs revealed an interesting feature. While ADHs from the three anaerobic bacteria did not show any sequence similarity to *Saccharomyces cerevisiae* ADH and other bacterial ADHs (including ADHs of *C. acetobutylicum*), they contained several residues conserved in liver and maize ADHs (Fig. 4). The presence of a conserved tryptophan residue in the ADHs of mammals, higher plants, and these three anaerobic bacteria is significant because tryptophan does not occur frequently in proteins. (See Fig. 1 in reference 31 for the conservation of Gly-3 and Trp-14 in 11 ADHs from four mammals and three

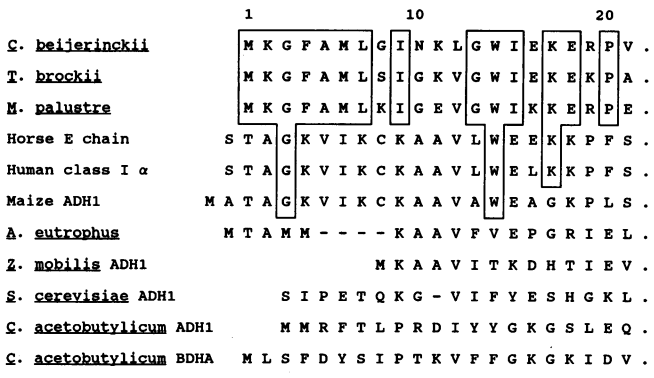

FIG. 4. Comparison of the N-terminal amino acid sequences of ADHs from the following organisms: *C. beijerinckii* NRRL B593 (this study and reference 43), *T. brockii* (40), *M. palustre* (5), horse liver E chain (28), human liver class I α (23, 50), maize ADH1 (13), *Alcaligenes eutrophus* (25), *Z. mobilis* ADH1 (33), *S. cerevisiae* ADH1 (3, 29), *C. acetobutylicum* P262 ADH1 (61), and *C. acetobutylicum* ATCC 824 BDHA (52). Amino acid residues conserved in the ADH of *C. beijerinckii* and at least two other organisms are boxed. The N-terminal sequences of ADHs from *T. brockii*, *M. palustre*, and horse liver were determined with protein. Those from *C. beijerinckii*, *S. cerevisiae*, and humans were both determined with protein and deduced from DNA. The other sequences were deduced from DNA. Dashes were added to align the sequences.

higher plants but not in 5 ADHs from three fungi.) The possible cause for the conservation of these Gly and Trp residues in ADHs of mammals, higher plants, and these three anaerobic bacteria merits further investigations.

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