ISOLATION AND CHARACTERIZATION OF THE [NAD(P)-INDEPENDENT] POLYOL DEHYDROGENASE FROM THE PLASMA-MEMBRANES OF GLUCONOBACTER OXYDANS ATCC STRAIN 621.

by

Ian Judd VanLare

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fulfillment of requirements for the degree of

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in

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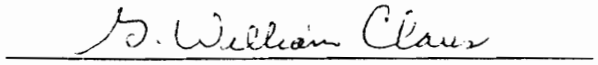

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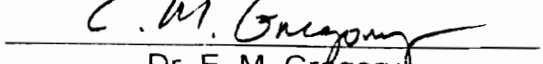

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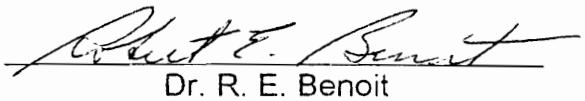

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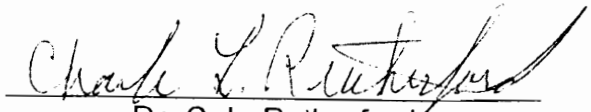

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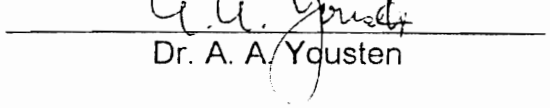

Dr. A. A. Yousten

September 1, 1997

Blacksburg, Virginia

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by

Ian J. VanLare

Dr. G. W. Claus

Department of Biology

(ABSTRACT)

Gluconobacter species rapidly perform limited oxidations of a large number of different polyhydroxy alcohols (polyols). These oxidations are catalyzed by constituitively synthesized plasma-membrane bound dehydrogenases. These bacteria would have to expend much energy to constituitively synthesize a separate substrate-specific dehydrogenase for each polyol substrate oxidized. Therefore, it is my hypothesis that *Gluconobacter* possess a single polyol dehydrogenase that oxidizes many different polyols. To test this hypothesis, a membrane-bound sorbitol dehydrogenase was isolated and tested for its ability to oxidize a wide range of substrates. This enzyme was removed from the membrane fractions with Triton X-100 and fractionated from other membrane proteins by anion and cation-exchange and hydrophobic-interaction chromatographies. This procedure resulted in a 36-fold enrichment of the enzyme and a 31% recovery. The isolated enzyme showed one protein band after non-denaturing polyacrylamide electrophoresis (PAGE) and three polypeptides after SDS-PAGE. Only the 67 kDa subunit had catalytic activity. The 46 kDa subunit was a C-type cytochrome. The isolated enzyme oxidized all

8 polyols tested, but did not oxidize mono-, di-, and cyclic-alcohols, aldehydes, carboxylic acids, or mono-, di-, and oligo-saccharides. Therefore, I propose that this enzyme is a polyol dehydrogenase (PDH). The isolated PDH complex showed optimal sorbitol oxidation from pH 5 to 6 at 40°C, and contained pyrroloquinoline quinone (PQQ) as its prosthetic group. Apo-PDH could be created by salt treatment and the holoenzyme reconstituted with authentic PQQ in the presence of two species of divalent cations. The *c*-type cytochrome of the PDH complex was not reduced by the substrate alone, but it was reduced by substrate if either CoQ_1 or the artificial electron acceptor methylphenazonium methosulfate (MPMS) were present. It is my hypothesis that, in vivo, the electrons removed from the substrate are passed from the PQQ prosthetic group of the catalytic subunit to CoQ_{10} in the plasma membrane, and then to the electron transport chain via the cytochrome *c* subunit of the PDH complex. When the PDH complex is removed from the membranes with detergent, the CoQ_{10} is likely disassociated from the enzyme, but can be replaced with MPMS when assayed with artificial electron acceptors.

DEDICATION

The author dedicates this work to all the special people in his life, including Phyllis, Barry, Susan, Sherryl and Roland VanLare, Evelyn and Clare Judd, Chelle and MaryMegan Vincent, the good Greg (McConnell) and the evil Greg (Church), his students, associates, friends and to himself, for finally finishing what he started.

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Finally, the author wishes to express his deepest gratitude to his Mother and Father and the rest of his family for their unselfish support and understanding throughout this project.

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LIST OF ABBREVIATIONS

BSA = bovine serum albumen

CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CM = carboxy methyl

Co = coenzyme

dddH_2O = double-distilled deionized water

DEAE = diethylaminoethylene

DSMP = detergent solubilized membrane protein

EDTA = ethylenediaminetetraacetic acid

FAD = flavine-adenine dinucleotide

FMN = flavine-mononucleotide

HIC = hydrophobic interaction chromatography

MPMS = methylphenazonium methosulfate

OTG = octyl thioglucoside

PAGE = polyacrylamide gel electrophoresis

PEG = polyethylene glycol

PDH = polyol dehydrogenase

PQQ = pyroloquinoline quinone

polyol = polyhydroxyl alcohol

SDS = sodium dodecyl sulfate

Triton = Triton X-100

CHAPTER 1

Isolation and Initial Characterization of the Plasma Membrane-Bound [NAD(P)-Independent] Polyol Dehydrogenase from *Gluconobacter oxydans.*

ABSTRACT

Gluconobacter oxydans rapidly oxidizes many different polyhydroxy alcohols (polyols). Polyol oxidations are catalyzed by constituitively synthesized membrane-bound dehydrogenases. A polyol-oxidizing enzyme was isolated from the membranes of these bacteria and tested for its ability to oxidize various substrates. The enzyme was solubilized from the membranes with Triton X-100. The detergent solubilized membrane proteins were then fractionated by cation and anion-exchange, and hydrophobic interaction chromatographies. The fraction eluted from the hydrophobic interaction column showed one protein band after non-denaturing polyacrylamide gel electrophoresis (PAGE), and three polypeptides (M_r 67,000 46,000 and 15,000) after SDS-PAGE. The 67,000 molecular weight polypeptide was shown to contain catalytic activity and the 46,000 molecular weight polypeptide was a *c*-type cytochrome. This isolation procedure resulted in a 36-fold enrichment with a 31% yield when compared to the cell extract. The isolated enzyme optimally oxidized sorbitol at 40°C between pH 5 and 6, and had a specific activity of about 3,560 kat kg protein^{-1}. It oxidized compounds containing three or more hydroxyl groups, but not mono-, di-, and cyclic-alcohols, aldehydes, carboxylic acids, or mono-, di-, and oligo-saccharides. Therefore, I propose this enzyme be called a polyol dehydrogenase.

INTRODUCTION

The genus *Gluconobacter* is composed of gram negative, rod-shaped chemoorganotrophs that are strict aerobes but lack a complete tricarboxylic acid cycle (12). These bacteria rapidly oxidize over 100-different hydroxyl containing compounds (14). The oxidation products, which are often industrially important (31), accumulate in stoichiometric amounts in the spent growth medium (7). These industrially important oxidations are catalyzed by NAD(P)-independent dehydrogenases located in the cell's plasma membrane and linked to the electron transport chain (22).

Recent evidence suggests that the enzymes that catalyze many of these oxidations are synthesized constituitively (14). In that study (14), membrane fractions isolated from glycerol-grown *Gluconobacter oxydans* were tested for their ability to oxidize 40 different substrates. The isolated membranes oxidized all 40 compounds tested. It would take considerable biosynthetic energy expenditure for these bacteria to constituitively synthesize a unique substrate-specific enzyme for each of the 40 substrates oxidized. Also, it would seem to be wasted energy, since some of these compounds were xenobiotics and unlikely to be encountered. It is my hypothesis that *Gluconobacter* can constituitively oxidize such a wide variety of substrates, not by constituitively synthesizing unique enzymes for each substrate oxidized, but rather by constituitively synthesizing only a few types of chemical-class specific enzymes

with broad-substrate specificities. Each of these dehydrogenases would then oxidize only one class of chemical compounds, such as alcohols, aldehydes, or aldoses.

This hypothesis is at least partially supported by published reports. An alcohol dehydrogenase was isolated that oxidized aliphatic alcohols of 2-carbons or more in length, to their corresponding aldehydes (1). An aldehyde dehydrogenase was isolated that oxidized aliphatic aldehydes of 2-carbons or more in length to their corresponding carboxylic acids (2). An aldose dehydrogenase was isolated that oxidized several aldoses to their corresponding ketoses (9). However, enzymes responsible for the oxidization of polyhydroxy alcohols (polyols) are reportedly substrate specific (11,27).

Several polyol oxidizing enzymes have previously been isolated from *Gluconobacter*. Sorbitol (27) and mannitol (11) dehydrogenases were each shown to have limited substrate specificities, both oxidizing only sorbitol and mannitol. A glycerol dehydrogenase was enriched that appeared to have a broad substrate specificity towards polyols, however, the investigators failed to show homogeneity of the enzyme (18).

Several polyol oxidations are industrially important. Many polyols, such as sorbitol, mannitol, and glycerol, are efficiently oxidized to their industrially important oxidation products: sorbose (24,31); fructose (31); and dihydroxyacetone (15,17,19,24,30), respectively.

This study was undertaken to isolate a polyol oxidizing enzyme and test it's ability to oxidize a wide range of hydroxyl-containing compounds. In this communication, I describe the isolation and initial characterization of a polyol dehydrogenase from *Gluconobacter oxydans* ATCC strain 621.

MATERIALS AND METHODS

Organism and growth. *Gluconobacter oxydans* ATCC strain 621 was maintained at -11°C as unfrozen sorbitol-stock cultures (32). Three milliliters of sorbitol stock culture was inoculated into a 3 liter BioFlow II fermentor (New Brunswick Scientific Co., Edison, NJ) containing 2.5 liters of the media composed of (wt/vol) 5.0% sorbitol, 1.0% yeast extract, and 1.0% peptone and three milliliters of AF60 anti-foaming agent (General Electric, Waterford, NY) in double-distilled deionized water ($dddH_2O$). Cultures were incubated at 28°C with agitation at 400 rpm and an air flow rate of 3 liters min^{-1}.

Spectrophotometric enzyme assay. Polyol dehydrogenase (PDH) activity was assayed by following the reduction of potassium ferricyanide spectrophotometrically using a modification of the procedure described by Arcus and Edson (6). The assay solution contained 10 μmol of $MgCl_2$, 5 μmol of $CaCl_2$, 250 μmol of sorbitol, 0.4 μmol of potassium ferricyanide (PFC), 0.6 μmol of methylphenazonium methosulfate (MPMS), 200μl of 10 mM Na-acetate buffer (pH 5.0) and sufficient $dddH_2O$ to obtain a total volume of 900 μl. The reaction was started by adding 100 μl of enzyme sample. All reagents except the enzyme samples were brought to the reaction temperature of 38°C before use. Enzyme samples were kept on crushed ice. Change in absorbance of the reaction mixture was monitored at 303.4 nm using a Bausch and Lomb Spectronic 2000 spectrophotometer.

Microtiter-plate enzyme assay. The microtiter-plate assay solution (29), used for detecting MBDH activity of the DSMP fractions, contained the following: 0.6 mM MPMS; 0.4 mM PFC; 250 mM sorbitol; 5 mM $CaCl_2$; and 10 mM $MgCl_2$; all dissolved in 200 mM succinate-NaOH buffer at pH 5.2. For detection of MBDH activity of the membrane fraction, the microtiter-plate assay solution was similar to above, except that MPMS was excluded and the PFC concentration was raised to 1 mM.

Microtiter plate assays were started with the addition of 7µl of enzyme fraction and 35 µl of the appropriate microtiter-plate assay solution to the wells of a microtiter-plate. These were then incubated at 28°C for 10 min. After incubation, each well received 63 µl of a ferric sulfate-Dupanol reagent (26), composed of 5 g of ferric sulfate, 3 g of SDS, and 95 ml of 85% phosphoric acid brought to one liter with $dddH_2O$. Twenty minutes after addition of the ferric sulfate-Dupanol reagent, 252 µl of $dddH_2O$ was added to each well. The color intensity was then measured at 600 nm with a Dynatechs MR 350 microtiter-plate reader. The absorbance of the microtiter-plate reader was set to zero with a well that contained the assay solution plus 7 µl of water instead of enzyme sample, and otherwise treated as described above.

Protein determination. Samples were digested with 2 N NaOH and 10% SDS in a boiling water bath for 30 min. Protein concentrations of these samples

were then determined by a modification (13) of the method of Lowry et al. (20). Bovine serum albumen fraction V was used as the protein standard.

Absorbance spectra. Absorbance of the isolated PDH complex was measured between 400 and 600 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer using a quartz cuvette. Measurements below 400 nm were not possible due to the high absorbance of Triton X-100 (Triton) in the buffer. The absorbance of the spectrophotometer was set to zero using cuvettes filled with 0.6 mM MPMS dissolved in the buffer in which enzyme samples were suspended (10 mM Na-acetate buffer, pH 5.0, containing 0.1% (vol/vol) Triton). The isolated PDH complex was oxidized by agitation for 1 min in 100 μM H_2O_2 and 0.6 mM MPMS. The isolated PDH complex was reduced by incubation in a solution containing 250 mM sorbitol and 0.6 mM MPMS at 38°C for 10 min.

Isolation of PDH. (*i*) Membrane fraction preparation. Cell fractionation procedures were carried out at 4°C or on crushed ice as shown in Figure 1.1. Two liters of early stationary phase cultures, 9.0 OD_{620} (Appendix 1), were harvested by centrifugation for 20 min at 13,500 x *g*, then washed twice by suspending the pellets in 200 mM succinate-NaOH buffer (pH 5.0) and centrifuging as before. The washed cell pellets were suspended in a total of 120 to 200 ml of the same buffer for subsequent breakage.

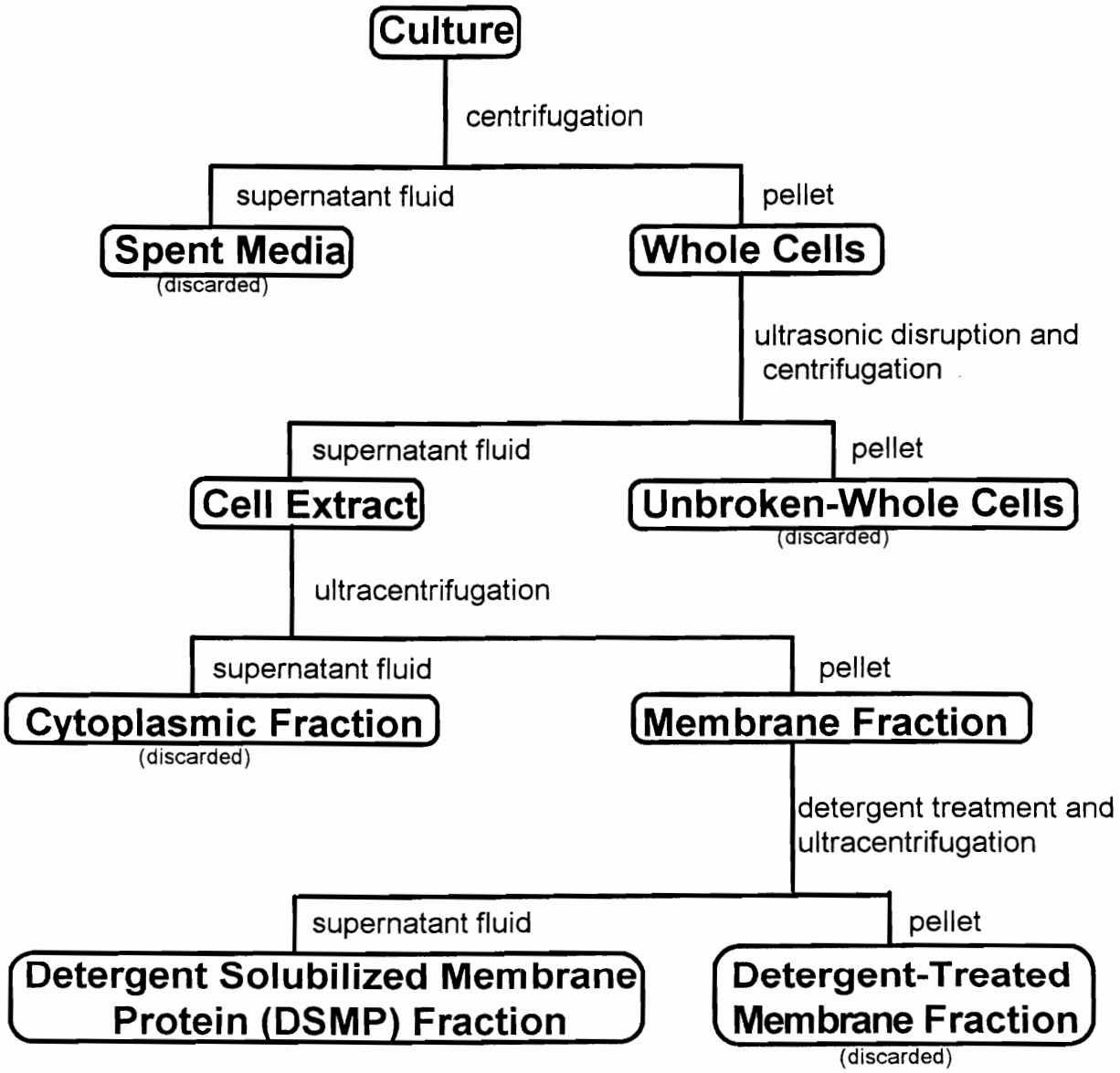

Figure 1.1. Scheme for fractionation of *Gluconobacter oxydans* ATCC strain 621.

Cells were broken by subjecting 40 ml of cell suspension to four-2 minute treatments with a Fisher Sonic Dismembrator Model 300 (Artek, Chantilly, VA) set at full power using the standard one-half inch tip. Samples were cooled to 4°C between each treatment. Treated samples were centrifuged at 4°C for 30 min at 40,000 x *g* to remove unbroken cells and large debris. Whole cells were not detected in the supernatant fluid by microscopic examination (data not shown). This supernatant fluid was called the *cell extract* (Fig 1.1).

The membrane fraction was prepared by ultracentrifugation of the cell extract at 4°C for 90 min at 120,000 x *g* in a swinging bucket rotor (SW41). The resulting supernatant fluid, called the *cytoplasmic fraction* (Fig 1.1), contained no NAD(P)-independent PDH activity (data not shown), and this fraction was discarded. The resulting pellet was washed once, by suspending it in 10 mM Na-acetate buffer (pH 5.0) using a tissue homogenizer, and then ultracentrifuging as before. The supernatant fluid from this ultracentrifugation was discarded. The washed pellet was suspended as before in 10 mM Na-acetate buffer, pH 5.0, and stored at -11°C. This washed pellet was called the *membrane fraction* (Fig 1.1).

(*ii*) Membrane protein solubilization. The membrane fraction was mixed for 3 h with 1.0% (vol/vol) Triton in the presence of 1.5 mM sorbitol and 1.5 mM KCl. The resulting preparation was ultracentrifuged at 4°C for 90 min at 120,000 x *g* in a swinging bucket rotor (SW41). The resulting supernatant fluid

was called the *detergent-solubilized membrane protein* (DSMP) *fraction* (Fig 1.1) and the pellet was called the *detergent-treated membrane fraction* (Fig 1.1).

***(iii)* Ion-exchange chromatography.** DEAE-Sephadex (anion-exchanger) and Carboxymethyl (CM)-Sephadex (cation exchanger) were prepared as directed by the manufacturer. The CM-Sephadex was equilibrated with 10 mM Na-acetate buffer, containing 0.1% (vol/vol) Triton, pH 5.0. The DEAE-Sephadex was equilibrated with the same buffer at pH 5.8. These slurries were separately packed into 1.6 X 15 cm columns and equilibrated by allowing the appropriate Triton-containing Na-acetate buffer to flow through the ion-exchange resin overnight using a gravity-feed system.

The DSMP fractions were applied to the DEAE-Sephadex column and flow through fractions were collected. The pooled fractions from the DEAE-Sephadex column were adjusted to pH 5.8 with NaOH and applied to the CM-Sephadex column. The flow through fractions containing PDH activity from the CM-Sephadex fractionation step were pooled for subsequent hydrophobic interaction chromatography.

***(iv)* Hydrophobic interaction chromatography (HIC).** Sodium chloride was added to a final concentration of 4 M to the pooled fractions from the CM-Sephadex column and the fraction was then applied to a ω-aminooctyl-agarose containing column previously equilibrated with 10 mM Na-acetate buffer (pH 5.0) containing 0.1% (vol/vol) Triton and 4 M NaCl. The column was washed with

five-column volumes of the same buffer and then five-volumes of a similar buffer containing 3 M NaCl. The PDH activity was eluted by passing two-column volumes of 10 mM Na-acetate buffer (pH 5.0) containing 0.1% (vol/vol) Triton and 2 M NaCl. Fractions containing PDH activity were pooled for subsequent concentration and desalting.

***(v)* Concentration and desalting.** Samples eluted from the hydrophobic interaction column were concentrated to 3 to 5 ml by ultrafiltration under air pressure using an Amicon 30,000 molecular weight cutoff filter. To recover any PDH absorbed to the filter, the filter was rinsed several times in 10 mM Na-acetate buffer (pH 5.0) containing 0.1% (vol/vol) Triton.

The removal of salts from samples was performed under similar conditions as described for sample concentration, except a total of 50 volumes of fresh salt-free buffer was added periodically to the sample during the ultrafiltration process.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed as described by Hames and Rickwood (16) at 4°C. For molecular weight and subunit determinations, PAGE was performed with 1% SDS in the stacking gel, resolving gel, and running buffer. Proteins were passed through a 3.9% stacking gel (Tris-HCl, pH 6.8) at 35 mA, and separated through a 10% resolving gel (Tris-HCl, pH 8.8) at 75 mA. For determining the molecular weights of polypeptides separated by SDS-PAGE, Dalton Mark VII and High

Molecular Weight Standard Mixture (Sigma Chem. Corp., St. Louis, MO) ranging from 14.4 to 205 kilodalton (kDa) were electrophoresed in a lane adjacent to PDH.

Native PAGE was performed without the addition of SDS. Proteins were passed through a 3.9% stacking gel (Tris-HCl buffer, pH 8.1) at 25 mA, and separated through a 5.0% resolving gel (Tris-HCl buffer, pH 8.8) at 50 mA.

Staining. Gels were stained for proteins by incubation overnight in a mixture containing 0.1% (wt/vol) Commassie Brilliant Blue R250 in (vol/vol) 10% glacial acetic acid, 25% isopropyl alcohol, and 65% $dddH_2O$. Destaining was accomplished by gently agitating gels for 30 min in a similar mixture not containing Commassie Brilliant Blue.

The PDH activity in gels was detected after electrophoresis by first equilibrating gels in 200 mM succinate-NaOH buffer, pH 5.0 (three changes at 20 min each). Gels were then gently agitated for several hours at 30°C in a PDH activity staining solution containing 25 mmol of sorbitol, 0.2 mg of MPMS, and 0.4 mg of tetranitroblue tetrazolium in 100 ml of $dddH_2O$.

For heme staining after SDS-PAGE, 60 ml of a freshly prepared 6.3 mM solution of 3,3',5,5'-tetramethylbenzidine in methanol was mixed immediately before use with 140 ml of 0.25 M Na-acetate buffer (pH 5.0). Gels were gently agitated in this solution for 1 to 2 h. Hydrogen peroxide was then added to this

solution at a final concentration of 30 mM, and agitation was continued for an additional 30 min.

Reagents. The following reagents were purchased from Sigma Chem. Corp. (St. Louis, MO): Na-acetate (trihydrate); adonitol; DL-α-amino-n-butyric acid; D(-)arabinose; L(+)arabinose; L(-)arabitol; bovine serum albumen fraction V; D(+)cellobiose; citric acid; crotonic acid; dipicolinic acid; formaldehyde; D-gluconic acid; L-glutamic acid; glycine; hippuric acid; *myo*-inositol; DL-isocitric acid; itaconic acid; kojic acid; maleic acid; L-malic acid; D-mannitol; D(+)mannose; 1-O-methyl-α-D-glucopyranoside; polyethylene glycol 8000; L(-)sorbose; D(-)sorbitol; sucrose; D(+)trehalose dihydrate; xylitol; and D(+)xylose.

The following reagents were purchased from Fisher Scientific (Pittsburgh, PA): benzyl alcohol; *tert*-butanol; Na-formate; formic acid; fumaric acid; glycerol; glycolic acid; methanol; and 1-propanol.

The following reagents were purchased from Aldrich Chem. Co. (Milwaukee, WI): 1,3-butanediol; (±)-2-butanol; *trans*-cinnamaldehyde; cycloheptanol; cyclohexanol; cyclooctanol; cyclopentanol; dulcitol; glutaric dialdehyde; 1,6-hexanediol; hexylalcohol; isobutrylaldehdye; lactic acid; mellitic acid; 2-methyl-1-propanol; 1,5-pentanediol; propionaldehyde; D-raffinose pentahydrate; and 1,2,6-trihydroxyhexane.

Dextrose and maltose were purchased from DIFCO (Detroit, MI), and D(-) fructose, cyclohexanone, and cyclohexane were purchased from Eastman (Eastman, TN).

RESULTS

Removal of PDH from the membrane fraction. To isolate polyol dehydrogenase (PDH) from *Gluconobacter*, it was first necessary to remove it from the plasma membrane. Various detergents were employed under different conditions to optimize solubilization of PDH from the membrane fraction.

***(i)* Detergent types.** Brij 58, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), deoxycholate, methylglucoside, octylglucoside, 1-S-Octyl-β-D-thioglucopyranoside (OTG), sodium dodecyl sulfate (SDS), Triton X-100 (Triton), and Tween 80 were separately added at 1.0% concentrations to membrane fractions and the detergent-membrane mixtures stirred for 3 h. To determine the effect of detergent on the total PDH activity, samples of these mixtures (membrane-in-detergent fractions) were assayed for enzyme activity. To determine the effectiveness of the detergents on solubilization of active PDH from the membranes, the membranes-in-detergent mixtures were fractionated into the detergent solubilized membrane protein (DSMP) fraction and the detergent-treated membrane fraction by ultracentrifugation (Fig 1.1). The DSMP fractions and the detergent-treated membrane fractions were assayed for PDH activity.

All detergent treatments of the membrane fractions resulted in a reduction of total PDH activity (Fig 1.2, open bars). The SDS and OTG treatments of the

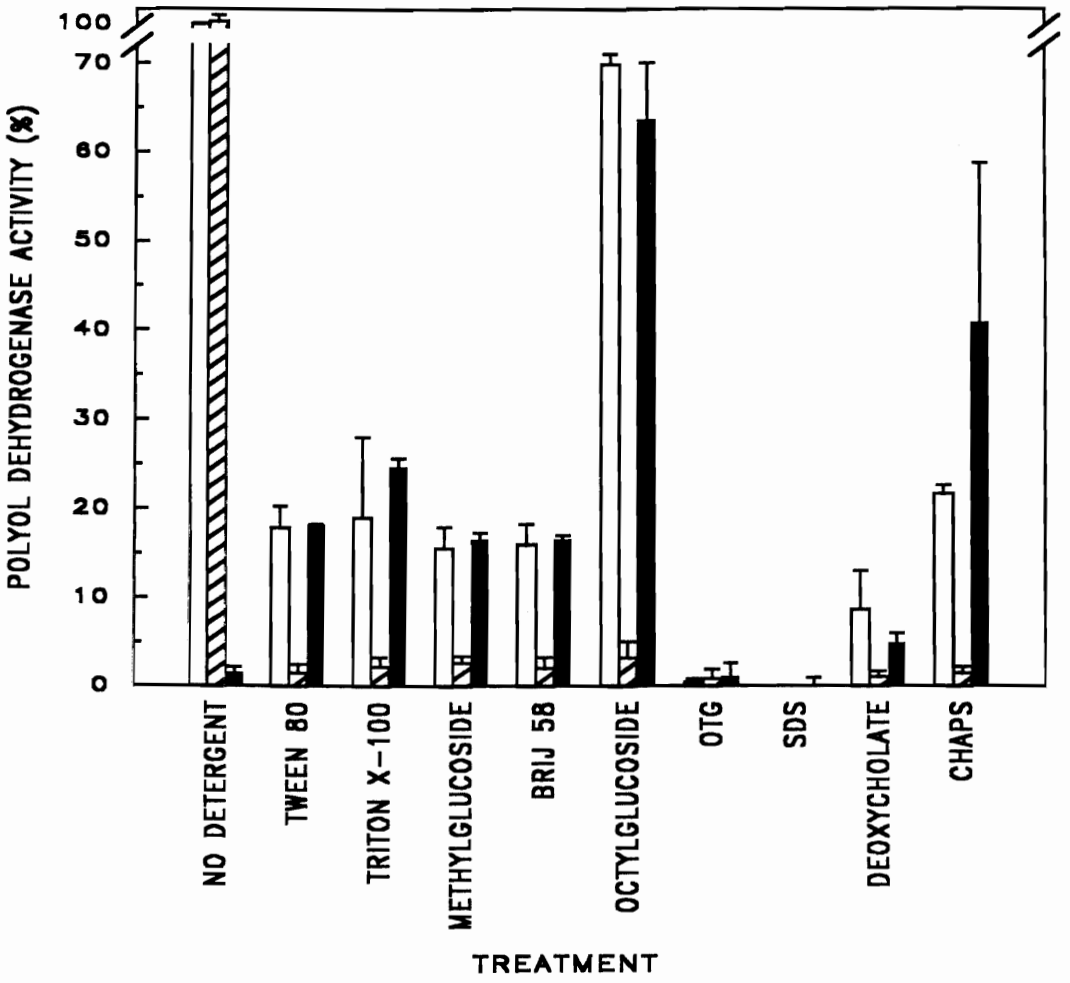

Figure 1.2. Solubilization of PDH activity from the membrane fraction with various detergents. Indicated detergents were separately added to membrane fractions (480, 520, and 730 μg of protein ml^{-1}) at 1.0% concentrations. After stirring for 3 h, the PDH activity of these membrane-in-detergent fractions were determined (□). The detergent-treated membrane protein fractions, or the sham-treated (no detergent) fraction, were then separated from the DSMP fraction by ultracentrifugation. The pellets from these ultracentrifugations were suspended in buffer and assayed for PDH activity (▨). The PDH activity of the DSMP fraction (supernatant fluid) was also determined (■). The PDH activity of the sham-treated membrane fraction (no detergent) was designated as 100% activity. Data represents the average values obtained from three replicates each of three separate cell batches. Standard deviations are shown. Abbreviations are: OTG = 1-S-Octyl-β-D-thioglucopyranoside; SDS = sodium dodecyl sulfate; CHAPS = 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

membranes showed almost complete inactivation of PDH, while the octylglucoside treated fraction retained about 70% activity. Other detergent treatments resulted in a retention of 10 to 25% of the original PDH activity found in the untreated membranes.

At 1.0% concentration, Brij 58, CHAPS, deoxycholate, methylglucoside, octylglucoside, Triton, and Tween 80 removed PDH activity from the membrane fraction, and enzyme activity was found mostly in the DSMP fractions (Fig 1.2, solid bars). Less than 5% of the PDH activity remained in the detergent-treated membrane fraction (Fig 1.2, slashed bars). No PDH activity remained after treatment of membranes with OTG or SDS. Octylglucoside solubilized the greatest amount of PDH activity from the membrane; however, the cost of this detergent was prohibitive considering it would be needed in buffers for all subsequent purification steps. The CHAPS solubilized the second largest amount of PDH activity; however, enzyme activity in the 1% CHAPS-DSMP fraction was unstable and was undetected within 48 h of its solubilization from the membrane fraction. Triton removal is inexpensive and it solubilized the third highest amount of PDH activity (about 24% that of the untreated membranes); therefore, Triton was routinely used for solubilization of PDH from the membrane fraction.

When lower detergent concentrations (0.1 and 0.5%) were tested, only CHAPS released active PDH from the membrane fraction (data not shown).

However, the PDH solubilized with 0.5% CHAPS was also unstable and was undetected within 48 h of its solubilization from the membrane fraction.

***(ii)* Optimal Triton concentration.** Membrane fractions were mixed with Triton so that the final Triton concentrations ranged from 0.2 to 2.0% (vol/vol). After stirring for 3 h, samples were ultracentrifuged, and PDH activity of the Triton-DSMP fractions (supernatant fluid) were assayed (Fig 1.3). Triton-DSMP fractions containing the highest PDH activity were obtained by treatment of membranes with 0.9 to 1.4% (vol/vol) Triton. One percent Triton was chosen for routine solubilization of PDH from the membrane fraction in all subsequent experiments.

***(iii)* Sorbitol and KCl.** To increase the amount of PDH activity released from the membrane fraction, sorbitol and KCl were added to membrane fractions prior to Triton solubilization. When sorbitol and KCl were added together at concentrations of 1.5 mM, the PDH activity in the Triton-DSMP fractions increased over 3-fold (Fig 1.4), and this treatment resulted in recovery of about 80% of the original PDH activity found in the membrane fraction. Therefore, 1.5 mM sorbitol and 1.5 mM KCl were routinely added during all subsequent experiments involving Triton solubilization of PDH from the membrane fraction.

***(iv)* Optimal membrane protein concentration.** Various amounts of membrane fraction were suspended in buffer so that membrane protein

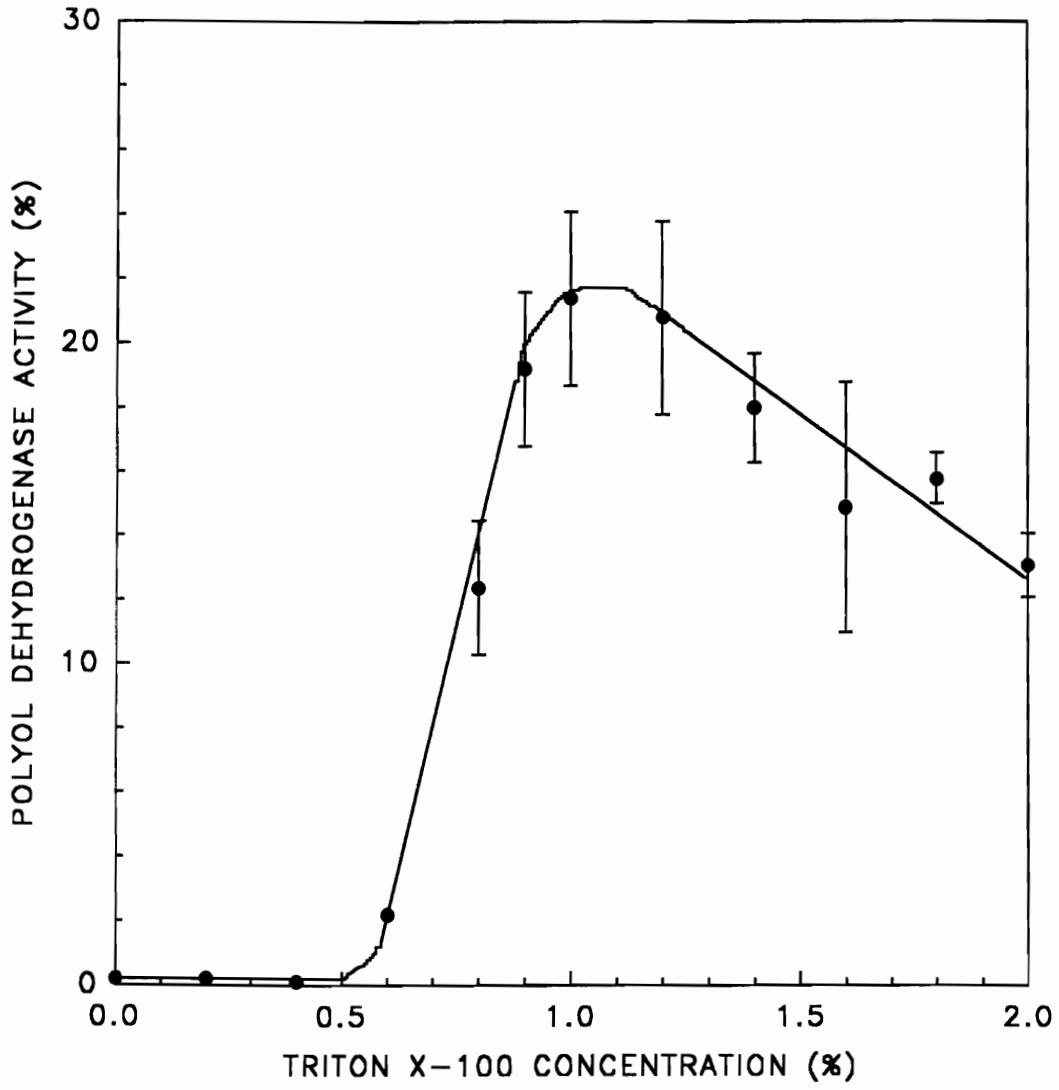

Figure 1.3. Polyol dehydrogenase activity solubilized from membranes treated with various concentrations of Triton. Triton was added to membrane fractions (280, 380, and 620 μg of protein ml^{-1}) in concentrations ranging from 0.2 to 2% (vol/vol). After stirring for 3 h, these membrane-in-detergent fractions were ultracentrifuged, and PDH activity of the Triton-DSMP fractions (supernatant fluid) were assayed. The PDH activity of the membranes fractions prior to treatment were considered to be 100%. Data points represent the average values obtained from three replicates each of three separate cell batches. Standard deviations are shown.

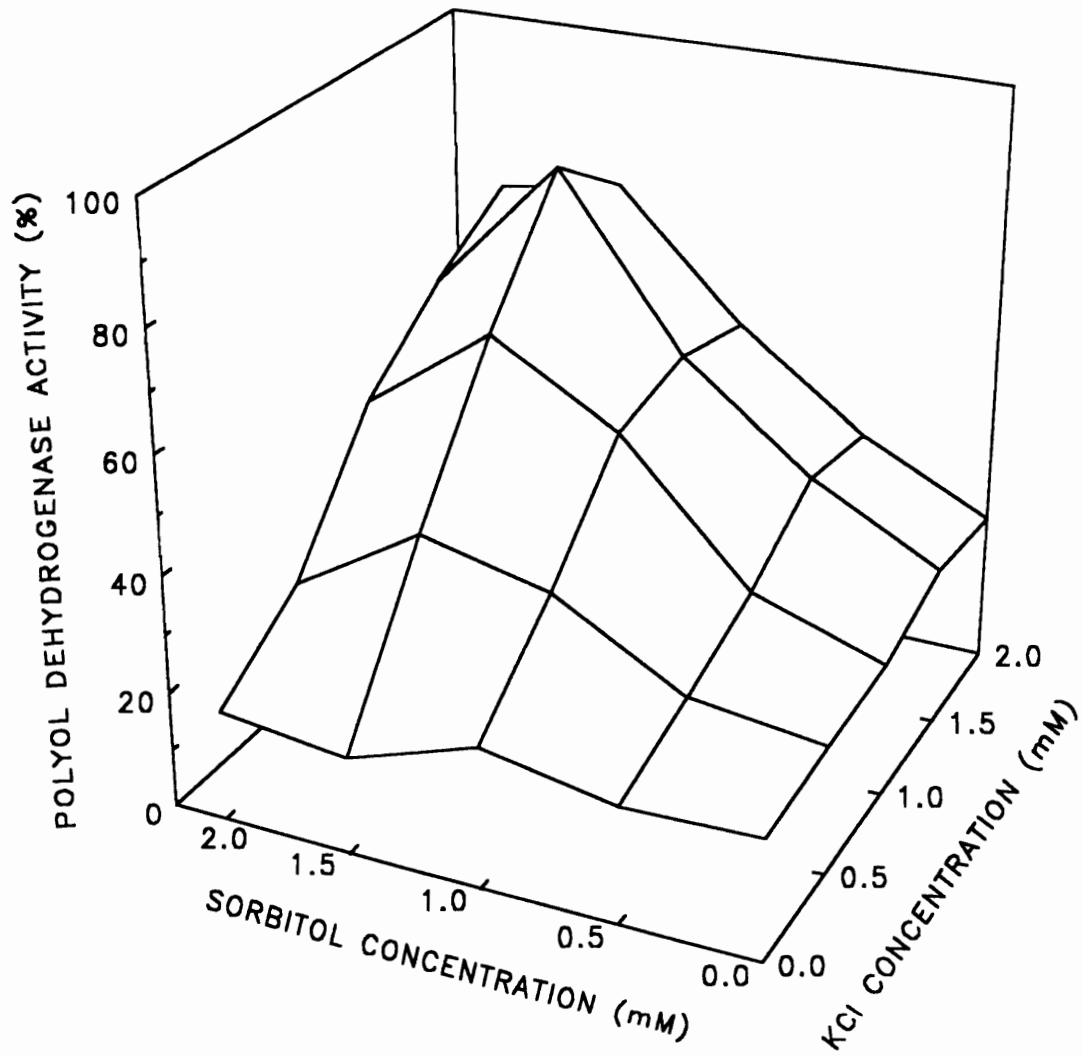

Figure 1.4. Polyol dehydrogenase activity of the DSMP fractions obtained with Triton treatment of membrane fractions in the presence of sorbitol and KCl. Membrane fractions (280, 380 and 620 µg of protein ml^{-1}) were mixed with 1.0% (vol/vol) Triton in the presence of the indicated concentrations of sorbitol and KCl. After stirring for 3 h, the membrane-in-detergent fractions were ultracentrifuged, and the PDH activity of the Triton-DSMP fractions (supernatant fluid) assayed. The membrane fractions prior to treatment were considered to contain 100% PDH activity. Data represents the average values obtained from three replicates each of three separate cell batches.

concentrations ranged from 50 to 600 μg ml^{-1}. Each membrane fraction was then treated with 1.0% (vol/vol) Triton, 1.5 mM sorbitol, and 1.5 mM KCl for 3 h and then ultracentrifuged. Triton solubilization of membrane fractions that contained 500 μg of protein ml^{-1} resulted in DSMP fractions with the highest specific activity (Fig 1.5). When membranes at concentrations above 500 μg of protein ml^{-1} were used, a red precipitate containing large amounts of insoluble PDH activity was deposited on top of the detergent-treated membrane fraction (pellet) during ultracentrifugation (data not shown).

Isolation of PDH by column chromatography. Three types of column chromatography were used to fractionate PDH from other proteins of the DSMP fractions obtained by Triton-treatment of the membrane fractions. These included anion-exchange chromatography using DEAE-Sephadex, cation-exchange chromatography using CM-Sephadex, and hydrophobic interaction chromatography (HIC) using ω-aminooctyl-agarose.

***(i)* DEAE-Sephadex.** The DEAE-Sephadex flow-through fractions obtained had about three-fold higher PDH specific activity than the DSMP fraction applied to the column, and about nine-fold higher specific activity than the cell extract (Table 1.1).

The PDH would bind to DEAE-Sephadex if the column was first pre-equilibrated with 10 mM buffer (pH 6.0) containing 0.1% (vol/vol) Triton. When

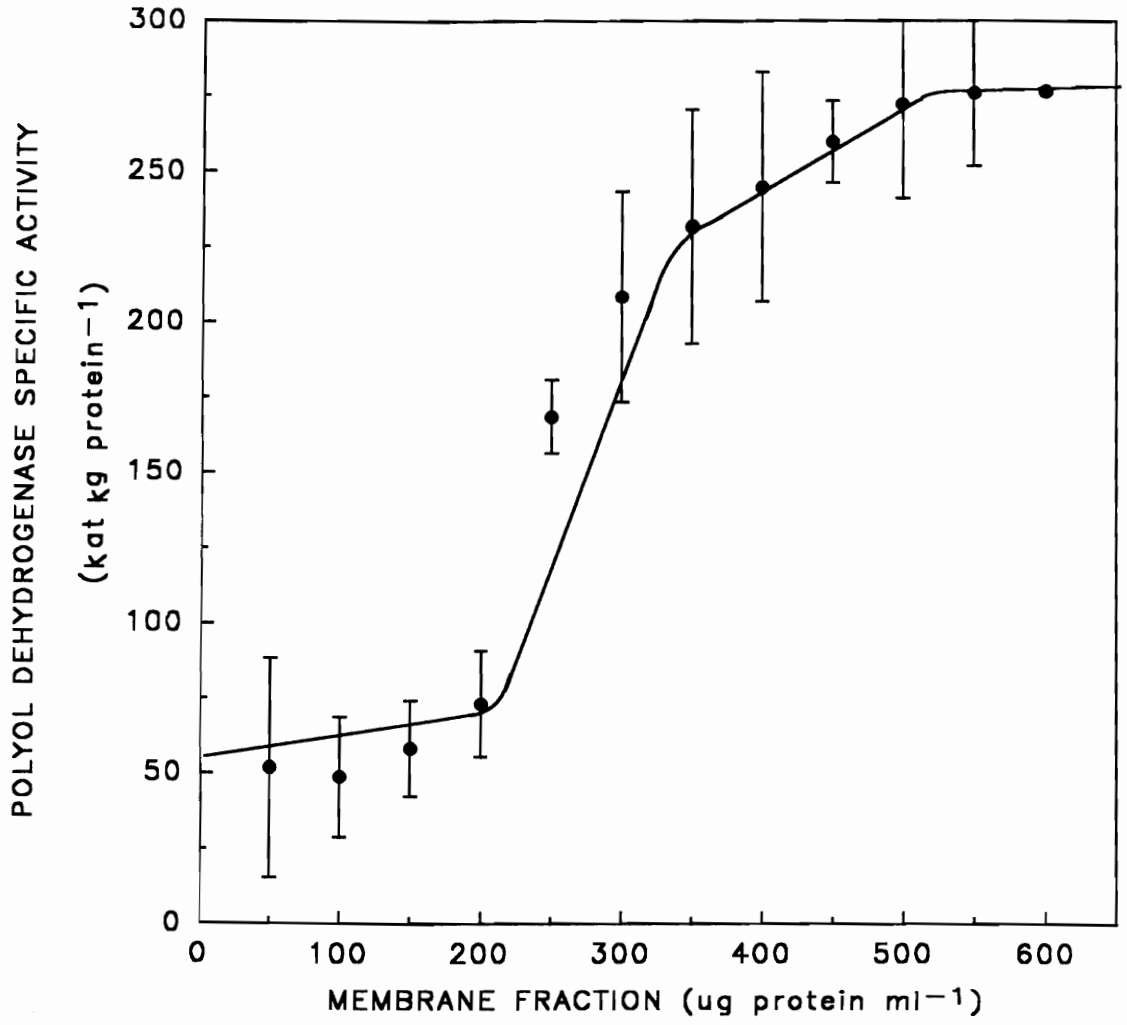

Figure 1.5. Polyol dehydrogenase activity released by Triton from varying quantities of membranes. Membrane fractions, ranging in protein concentrations from 50 to 650 μg ml^{-1}, were separately treated with 1.0% (vol/vol) Triton in the presence of 1.5 mM sorbitol and KCl. After stirring 3 h, these membrane-in-detergent fractions were ultracentrifuged, and the protein content and PDH activity of the DSMP fraction (supernatant fluid) were measured. The specific activities of PDH in the Triton-DSMP fractions were then determined. Data represents average values obtained from three replicates each of three separate cell batches. Standard deviations are shown.

TABLE 1.1. Fractionation and isolation of the membrane-bound [NAD(P)-independent] polyol dehydrogenase complex from *G. oxydans* ATCC strain 621.

Fractionation[a]	Cell batch[b] (replicates)	Total Volume (ml)	Total Protein (mg)	Total Activity (mkat)	Specific Activity (kat kg protein^{-1})	Recovery (%)	Enrichment (fold)
Cell Extract	1	160	233.9	22.9	98	100	1.0
	2	200	238.5	24.3	102	100	1.0
	3	160	347.8	34.9	100	100	1.0
	Av. (n = 15)	141 ± 36	280.5 ± 76.7	30.1 ± 10.1	108.2 ± 11.2	100 ± 0.0	1.0 ± 0.0
Membrane	1	200	117.2	20.6	176	90.1	1.8
	2	180	122.1	22.2	182	91.2	1.8
	3	160	152.3	32.2	211	92.4	2.1
	Av. (n = 15)	142 ± 37	135.1 ± 42.1	28.2 ± 9.8	209.0 ± 29.1	91.4 ± 3.5	1.9 ± 0.2
Triton-Detergent Solubilized Membrane Protein	1	200	31.2	9.7	310	42.2	3.2
	2	180	43.2	11.4	264	47.1	2.6
	3	160	56.4	16.5	292	47.2	2.9
	Av. (n = 13)	145 ± 38	45.6 ± 15.4	12.7 ± 3.1	293.6 ± 55.9	45.6 ± 6.6	2.8 ± 0.6
DEAE-Sephadex	1	81	8.6	9.5	1101	41.3	11.3
	2	210	14.4	10.6	733	45.5	7.2
	3	50	14.1	16.4	1168	47.1	11.7
	Av. (n = 9)	123 ± 55	11.8 ± 4.7	11.1 ± 3.9	981.3 ± 203.2	43.9 ± 6.2	9.4 ± 1.8
CM-Sephadex	1	42	3.1	7.6	2450	32.3	25.0
	2	315	4.4	9.5	2153	39.1	21.4
	3	73	4.6	13.0	2840	37.3	28.3
	Av. (n = 4)	161 ± 127	4.1 ± 0.7	11.1 ±3.1	2674.4 ± 478.4	37.9 ± 3.6	25.0 ± 3.0
Hydrophobic Interaction (Isolated PDH complex)	1	33	1.9	6.4	3405	27.8	34.8
	2	50	2.2	8.0	3614	32.7	35.5
	3	150	2.8	10.3	3668	29.5	36.6
	Av. (n = 3)	78 ± 63	2.3 ± 0.5	8.2 ± 2.0	3562.3 ± 138.9	31.1 ± 2.3	35.6 ± 0.9

[a]Cell breakage, cell-extract, and membrane preparations were accomplished as described in the Materials and Methods.

Triton (1% vol/vol), sorbitol (1.5 mM), and KCl (1.5 mM) were added to membrane fractions. After stirring for 3 h, detergent treated membranes were removed by ultracentrifugation, and the resulting supernatant fluid was called the detergent-solubilized membrane proteins (DSMP) fraction.

Triton-DSMP fractions were passed through a DEAE-Sephadex column pre-equilibrated with pH 5.0 buffer and eluted from the column with the same buffer. Fractions eluted from the column containing PDH activity were pooled and adjusted to pH 5.8 as described in the Materials and Methods. These pooled fractions were called the DEAE-Sephadex fraction.

The pooled DEAE-Sephadex fractions were passed through a CM-Sephadex columns pre-equilibrated with pH 5.8 buffer and eluted with the same buffer as described in the Materials and Methods. Fractions containing PDH activity were pooled and called the CM-Sephadex fraction.

The pooled CM-Sephadex fractions were brought to 4 M with NaCl and passed through a ω-aminooctyl agarose-containing column pre-equilibrated with pH 5.0 buffer containing 4 M NaCl. The columns were washed with buffer containing 3 M NaCl, and the PDH was eluted with buffer containing 2 M NaCl as described in the Materials and Methods. Fractions containing PDH activity were pooled and desalted using an Amicon ultra-filtration device. These fraction were called the HIC fraction or the isolated PDH complex.

[b]Cell batches 1, 2, and 3 represent separately prepared cultures in which the PDH isolation procedures were completed through the final ω-aminooctyl agarose column (HIC) chromatography. The total number (n) of cell batches used to calculate average values and standard deviations are also shown.

PDH was bound to the column, it could not be removed by conventional salt or pH changes (data not shown). Unsuccessful attempts of elution included the use of up to 200 mM buffer (pH 4.0) that contained up to 1.0% (vol/vol) Triton. Polyol dehydrogenase activity was not inactivated, since enzyme activity could be detected after extraction from the DEAE-Sephadex with a tissue homogenizer (data not shown).

***(ii)* CM-Sephadex.** The PDH containing fractions from the DEAE-Sephadex were applied to a CM-Sephadex containing column and the flow-through fractions containing PDH activity were collected and pooled. The pooled fractions from the CM-Sephadex had a PDH specific activity about three-times higher than the fractions applied to the column and about 25-times higher than the cell extract (Table 1.1).

The PDH would bind to CM-Sephadex if the pH of the equilibrated buffer was lowered to pH 4.5. If PDH were bound to CM-Sephadex, it could not be removed by conventional salt or pH changes. Unsuccessful attempts of elution included the use of up to 200 mM succinate buffer (pH 6.0) that contained up to 1.0% (vol/vol) Triton (data not shown). Similarly to when the PDH bound to the DEAE-Sephadex, PDH bound to the CM-Sephadex could be extraction from the CM-Sephadex with a tissue homogenizer.

***(iii)* Hydrophobic interaction chromatography (HIC).** Flow through fractions from the CM-Sephadex columns containing 4 M NaCl were applied to a column containing ω-aminooctyl-agarose. The fractions eluted from the ω-aminooctyl-agarose had about 1.3-times higher specific activity than the CM-fraction applied to the column (Table 1.1). The HIC fraction had a total of a 36-fold increase in specific activity of PDH compared with the cell extract, while retaining over 30% of the original activity of the cell extract (Table 1.1).

Electrophoretic analysis and subunit composition. The HIC fraction (100 μg protein) was subjected to polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions, and gels were subsequently stained for either protein or PDH activity (Fig 1.6). Only one protein band was detected in the HIC fraction, and this protein band contained PDH activity.

Three polypeptides were detected when the HIC-fraction was subjected to SDS- PAGE (Fig 1.7, Lane C). These polypeptides exhibited relative molecular weights of 67,000, 46,000 and 15,000. Since the sample exhibited only one band when separated by native-PAGE (Fig. 1.6) and three by SDS-PAGE (Fig. 1.7) , the fraction from the hydrophobic interaction column was called the isolated PDH complex.

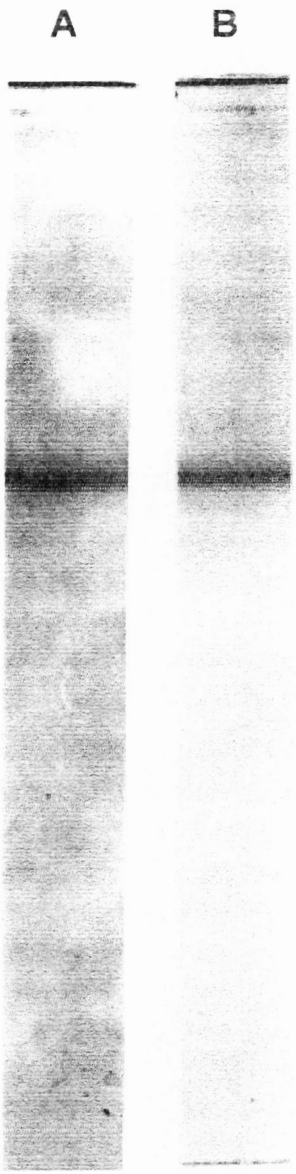

Figure 1.6. Non-denaturing polyacrylamide gel electrophoretic analysis of the isolated PDH complex (HIC fraction). Samples containing 100 μg of the isolated PDH complex from the HIC chromatography fractionation step were applied to each lane, then subjected to electrophoresis, under non-reducing and non-denaturing conditions, through a 5.5% polyacrylamide gel. Lane A was stained for protein while lane B was stained for PDH activity.

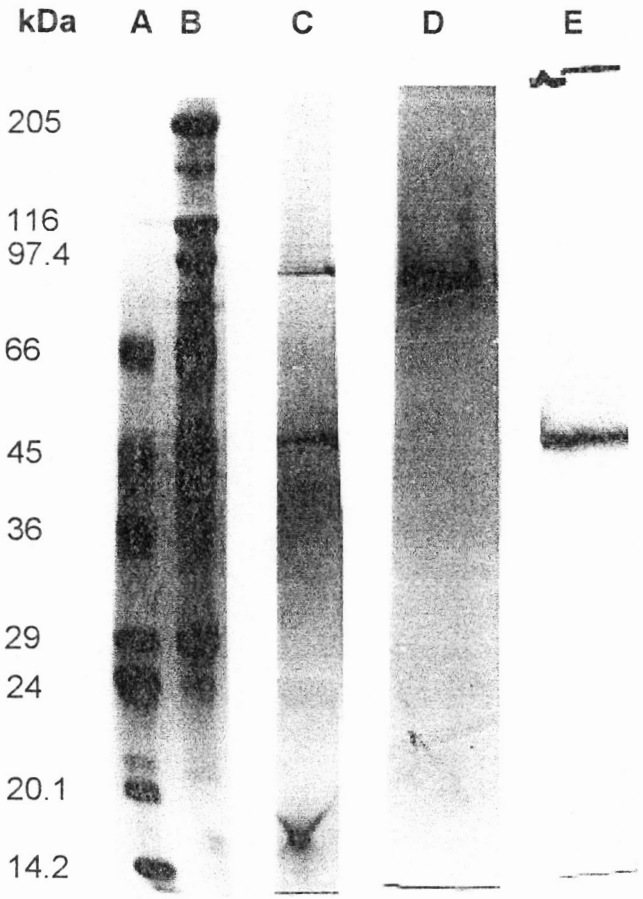

Figure 1.7. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the isolated PDH complex (HIC fraction). Samples were separated through a 10% polyacrylamide gel in the presence of 1% SDS. Lane A and B contained proteins of known molecular weights; Lanes C through E contained 100 μg of the isolated PDH complex (HIC fraction). Lane C was stained for protein, lane D was stained for PDH activity, and lane E was stained for heme.

To identify the subunits of PDH, the isolated PDH complex was separated into its peptide subunits by SDS-PAGE under non-reducing and non-denaturing conditions. After electrophoresis, the gels were stained for either PDH activity or for heme. Only the 67 kDa subunit stained for PDH activity (Fig. 1.7, Lane D), while the 46 kDa subunit stained positive for heme (Fig 1.7, Lane E). The 15 kDa subunit did not stain with either method and was not further identified.

The absorption spectrum of the red-colored, isolated PDH complex was examined to determine if the heme detected in the 46 kDa subunit might be a cytochrome (Fig 1.8). The oxidized form of the isolated PDH complex had absorbance maxima at 417, 523, and 554 nm, while the reduced form had absorbance maxima at 436, 463, 523, and 554 nm.

Catalytic properties. No cofactor requirement was noted for the oxidation of polyols by the isolated PDH complex (data not shown). The rate of sorbitol oxidation was linear for over 13 minutes using the Triton-DSMP fraction when assayed spectrophotometrically using ferricyanide as an indicator (data not shown). However, if $MgCl_2$ and $CaCl_2$ were omitted from the ferricyanide assay solution, oxidation of sorbitol stopped within a few minutes (data not shown).

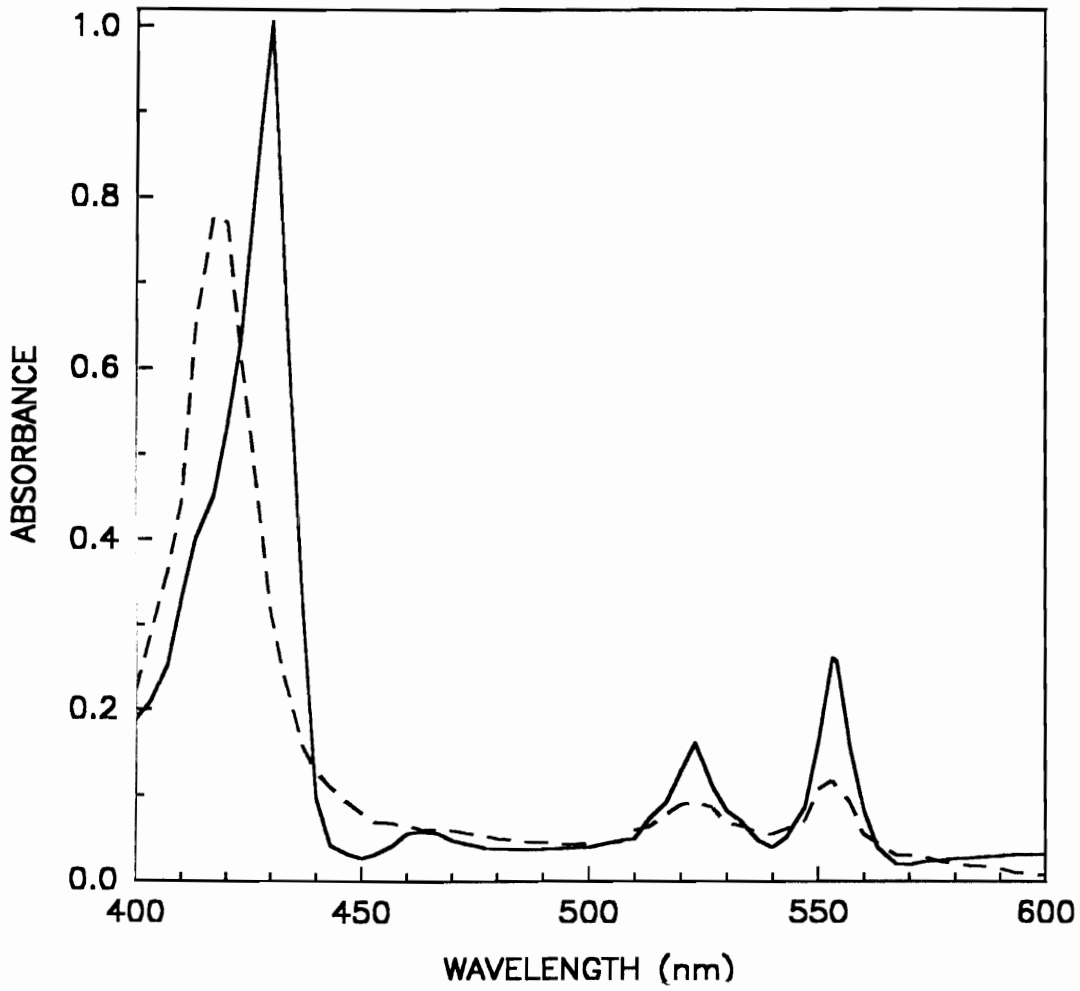

Figure 1.8. Absorption spectra of oxidized and reduced forms of the isolated PDH complex (HIC fraction). The absorbance of the isolated PDH complex (1.065 mg of protein ml^{-1}) was measured between 400 and 600 nm. The oxidized form (- - - - -) was obtained by the addition of hydrogen peroxide and 0.6 mM MPMS. The reduced form (———) was obtained by the addition of 250 mM sorbitol and 0.6 mM MPMS. Data shown is from a typical fraction.

***(i)* Substrate specificity.** From all the substrates reportedly oxidized by *G. oxydans* (5), I wished to determine which ones were oxidized by the isolated PDH complex. Membrane fractions were tested for their ability to oxidize fifty substrates, from 8 chemical classes, using a microtiter plate assay. The membrane fraction oxidized substrates from each chemical class tested, and it oxidized 44 of the 50 substrates tested (Table 1.2). The Triton-DSMP fractions oxidized 38 of 44 substrates tested, including all the tested dialcohols, polyols, cyclic alcohols, and disaccharides, but this fraction failed to oxidize branched-chained aliphatic monoalcohols, L-sorbose, formaldehyde, and D-gluconate. When the ferricyanide-spectrophotometric assay was used to test the isolated PDH complex, all 8 polyols were oxidized, but no other substrate oxidation was detected (Table 1.2).

***(ii)* Hydrogen-ion concentration.** The optimal pH for sorbitol oxidation was investigated using both the cell extract and the isolated PDH complex (Fig 1.9). The optimum hydrogen ion concentration for PDH activity for both fractions was between pH 5 and 6.

***(iii)* Temperature.** Both the cell extract and isolated PDH complex fractions were used to investigate the effect of temperature on the rate of

TABLE 1.2. Substrate oxidations by the membrane fraction, Triton-DSMP fraction, and isolated PDH complex.

Chemical category	Substrate	Fraction assayed			
		Membrane[a]	Triton-DSMP[a]	Hydrophobic Interaction (Isolated PDH complex)[b,c]	
				Cell batches 1 and 2	
Monoalcohols	Methanol	+	0	-	-
	Ethanol	+	+	0	0
	n-Propanol	+	+	0	0
	Isopropanol	+	+	0	0
	n-Butanol	+	+	0	0
	sec-Butanol	+	+	0	0
	tert-Butanol	+	0	-	-
	Isobutanol	+	0	-	-
	n-Pentanol	+	+	0	0
	n-Hexanol	+	+	0	0
Dialcohols	L(+)1,2-Propanediol	+	+	0	0
	1,3-Butanediol	+	+	0	0
	1,5-Pentanediol	+	+	0	0
	1,6-Hexanediol	+	+	0	0
	1,7-Heptanediol	+	+	0	0
Polyalcohols	Glycerol	+	+	92	107
	Erythritol	+	+	30	34
	Adonitol	+	+	91	103
	L-Arabitol	+	+	-	64
	Xylitol	+	+	-	18
	Trihydroxyhexane	+	+	20	32
	D-Sorbitol	+	+	100	100
	D-Mannitol	+	+	103	121
Cyclic alcohols	Cyclopentanol	+	+	0	0
	Cyclohexanol	+	+	0	0
	meso-Inositol	+	+	0	0
	Cycloheptanol	+	+	0	0
	Benzyl alcohol	+	+	0	0

TABLE 1.2. (continued)

Chemical category	Substrate	Fraction assayed			
		Membrane[a]	Triton-DSMP[a]	Hydrophobic Interaction (Isolated PDH complex)[b,c]	
				Cell batches 1 and 2	
Monosaccharides	L-Erythrulose	+	+	0	0
	L(+)Arabinose	+	+	0	0
	D(-)Arabinose	+	+	0	0
	Glucose	+	+	0	0
	Mannose	+	+	0	0
	D-Galactose	+	+	0	0
	D-Fructose	0	-	-	-
	Xylose	+	+	74	0
	D-Ribose	0	-	-	-
	L-Sorbose	+	0	-	-
Disaccharides	Maltose	+	+	0	0
	Sucrose	0	-	-	-
Aldehydes	Formaldehyde	+	0	-	-
	Acetaldehyde	+	+	0	0
	Glyceraldehyde	+	+	0	0
	Propionaldehyde	+	+	0	0
	Glutaraldehyde	+	+	0	0
	Cinnamaldehyde	+	+	0	0
Carboxylic acids	Gluconate	+	0	-	-
	Acetate	0	-	-	-
	Lactate	0	-	-	-
	Pyruvate	0	-	-	-

[a] Oxidations catalyzed by the membrane and Triton-DSMP fractions were determined by a microtiter plate assay. Abbreviations are as follows; "+" represents oxidation of the substrate, "0" indicates that no oxidation was detected, and "-" indicates that the substrate was not tested.

[b] Activity of the isolated PDH complex was determined by the ferricyanide-spectrophotometric assay as described in the Materials and Methods. Numbers represent the relative rate of substrate oxidation compared to sorbitol oxidation (expressed as 100%).

[c] Batch 1 and 2 represent separate experiments from different cell batches in which the isolated PDH complex (same fractions as cell batches 1 and 2 in Table 1) was tested for its ability to oxidize various substrates.

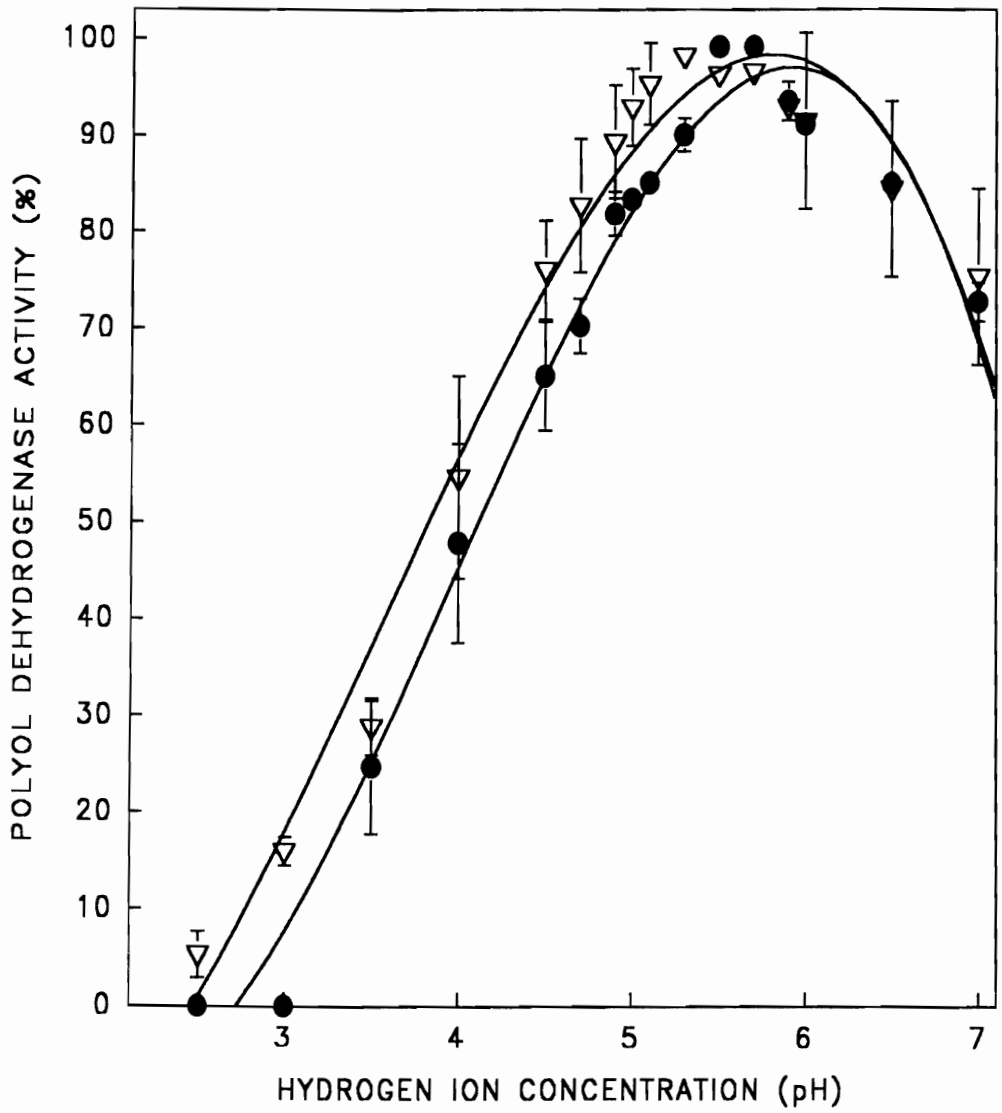

Figure 1.9. Polyol dehydrogenase activity at various hydrogen-ion concentrations. The cell extract (●) and isolated PDH complex (▽) (both in 10 mM Na-acetate buffer, pH 5.0) were diluted 1:10 in buffer containing 66 mM each of Na-acetate, succinate, and Tris. Samples were then adjusted to the indicated hydrogen ion concentration with either NaOH or acetic acid. Polyol dehydrogenase activity was then assayed with the ferricyanide assay. The assay buffer was previously adjusted with NaOH or acetic acid to the pH of the enzyme fractions. Maximum PDH activity detected was considered to be 100%. Each data point represents three replicate experiments using the isolated PDH (842 and 1,023 μg of protein ml^{-1}) fraction or the cell extract (796 and 853 μg of protein ml^{-1}) from two separate cell batches. Standard deviations are shown.

sorbitol oxidation (Fig 1.10). With the cell extract, sorbitol oxidation increased as temperature increased to about 37°C and decreased to about 80% of this activity at 50°C. With the isolated PDH complex, the PDH activity increased to about 40°C and then stayed the same to 50°C. The PDH activity of the cell extract appeared to be more sensitive to higher temperatures than the isolated PDH complex. Little activity was observed below 15°C (data not shown).

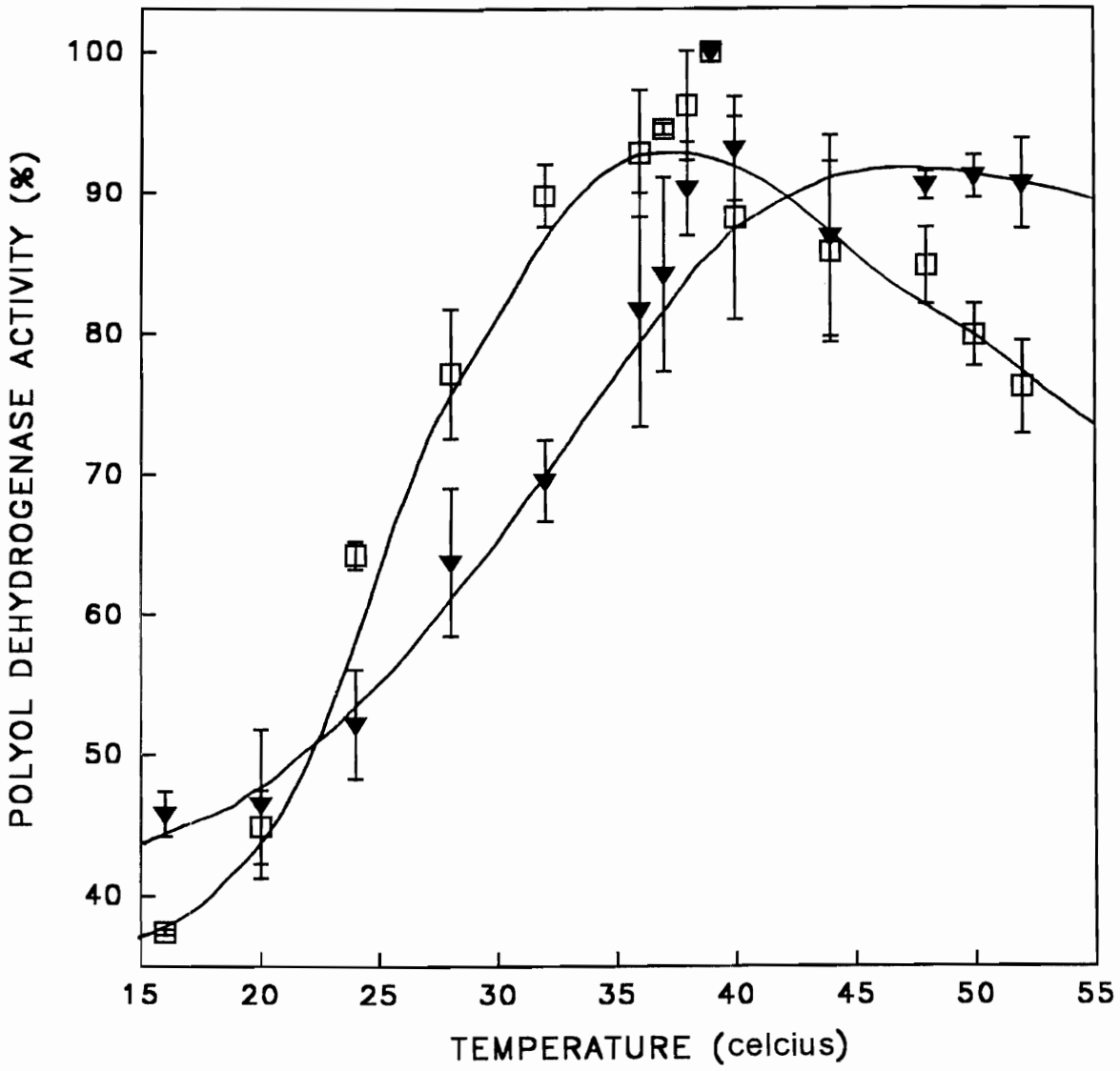

Figure 1.10. Polyol dehydrogenase activity at various temperatures. The cell extract (□) and isolated PDH complex (▼) were assayed for PDH activity using the ferricyanide assay at the indicated temperatures. Maximum PDH activity was considered to be 100%. Each data point represents average of three replicate experiments using the isolated PDH (842 and 1,023 μg of protein ml^{-1}) fraction or the cell extract (796 and 853 μg of protein ml^{-1}) from two separate cell batches. Standard deviations are shown.

Discussion

Substrate specificity. In this study, a membrane-bound [NAD(P)-independent] polyol oxidizing enzyme was isolated from *Gluconobacter oxydans* ATCC strain 621 and tested for its ability to oxidize a wide variety of compounds. The enzyme complex isolated in this study was consistently able to oxidize all the polyols tested, including glycerol, erythritol, adonitol, arabitol, xylitol, 1,2,6-trihydroxyhexane, sorbitol and mannitol. It was generally not able to oxidize compounds from other chemical classes. Therefore, I believe that this enzyme should be called a polyalcohol (polyol) dehydrogenase (PDH). This reasoning is similar to that used to name the alcohol dehydrogenase (1) and the aldehyde dehydrogenase (2), which were also named for the class of substrates they oxidized.

Comparison of PDH to other membrane-bound dehydrogenases.

***(i)* Mass.** The isolated PDH complex from *G. oxydans* ATCC strain 621 shares several properties with membrane-bound dehydrogenases from other oxidative bacteria. First, the relative molecular weight of 128,000 (128K) for the isolated PDH complex from *G. oxydans* (Fig 1.7) is similar to 130K for gluconate dehydrogenase from *G. dioxyacetonicus* (28), 131K for gluconate dehydrogenase from *Pseudomonas aeruginosa* (21), 136K for gluconate dehydrogenase from *Serratia marcescens* (25); 137.5K for fructose dehydrogenase from *G. industrius* (4); 131K for 2-keto-D-gluconate

dehydrogenase from *G. melanogenus* (26); 131K for sorbitol dehydrogenase from *G. suboxydans* var. α IFO strain 3254 (27); 135K for mannitol dehydrogenase from *G. melanogenus* ATCC strain 15163 (11); and 125K and 141K for aldehyde dehydrogenases from *Acetobacter aceti* (23) and *G. oxydans* [17], respectively.

***(ii)* Subunit composition.** Another similarity between other MBDHs and the isolated PDH complex from this study is the subunit composition. The isolated PDH complex from this study was composed of three subunits, like alcohol dehydrogenase from *G. oxydans* (1), gluconate dehydrogenase from *G. dioxyacetonicus* (28), mannitol dehydrogenase *from G. melanogenus* ATCC strain 15163 (11), sorbitol dehydrogenase from *G. suboxydans* var. α IFO strain 3254 (27), fructose dehydrogenase from *G. industrius* (4), and 2-keto-D-gluconate dehydrogenase from *G. melanogenus* (26).

In each case, including this study, the largest of these subunits contained the catalytic activity and the middle-sized subunit was a *c*-type cytochrome. The smallest subunit has not yet been identified in any of these enzymes.

***(ii)* Hydrogen ion concentration.** Like several other dehydrogenases, the isolated PDH complex had its pH optima in the slightly acidic range (1,2,10,27,28,33).

Comparison of PDH to other polyol-oxidizing enzymes. The class-specific, broad-spectrum polyol dehydrogenase isolated in this study is

compared with other polyol-oxidizing enzymes isolated from *Gluconobacter* in Table 1.3. The PDH complex that I isolated from *G. oxydans* ATCC strain 621 has a similar size, subunit composition, and absorption spectra as these other polyol-oxidizing enzymes. The PDH complex isolated in this study may be the same enzyme as other polyol-oxidizing enzymes described elsewhere (3,11,27) and shown in Table 1.3.

(*i*) Comparison to glycerol dehydrogenase. Ameyama et al. (3) enriched a quinoprotein glycerol dehydrogenase which was enriched 100-fold from *G. industrius* IFO strain 3260 by solubilization from the membrane fraction with 0.5% dimethyldodecylamineoxide followed by precipitation with 15 to 25% polyethylene glycol (PEG). The enzyme showed a broad specificity towards polyols, but would not oxidize other compounds. However, the purity of the enzyme was not demonstrated due to its hydrophobicity. There are three noteworthy differences between the glycerol dehydrogenase described by Ameyama et al. (3) and the PDH complex isolated in this study. First is the pH optima (pH 8.0 for solubilized glycerol dehydrogenase compared to pH 5 to 6 for the isolated PDH complex). Second, the isolated PDH from this study oxidized xylitol, but this compound was not oxidized by glycerol dehydrogenase isolated by Ameyama et al. (3). Third, glycerol dehydrogenase was enriched 100-fold by Ameyama et al. (3), compared to the 36-fold enrichment of PDH in this study.

Table 1.3. Comparison of membrane-bound polyol oxidizing enzymes from *Gluconobacter*.

Enzyme	Source	Substrates oxidized[a]	Absorbance maxima	Subunits (kDa)	Temp. maxima	pH optimum	Ref.
polyol dehydrogenase	*G. oxydans* ATCC 621	(see table 2 this study)	554, 523, 417	67 46 15	40°C	5 - 6	this study
sorbitol dehydrogenase	*G. suboxydans* var α IFO 3254	sorbitol (100) mannitol (5)	551, 522, 417	63l 51 17	25°C	4.5	27
mannitol dehydrogenase	*G. melanogenus* ATCC 15163	mannitol (100) sorbitol (50)	552, 522, 518	63l 52 20.5	NG[b]	NG	11
glycerol dehydrongease	*G. industrius* IFO 3260	glycerol (100) sorbitol (45) mannitol (26) D-arabitol (136) dulcitol (29) adonitol (42 meso-erythritol (111) propyleneglycol (54)	NG	NG	NG	7.5 - 8	3

[a]Percent rate of oxidation compared to sorbitol

[b]NG = not given

The first two differences, pH optima and xylitol oxidation, could possibly be explained by the different detergents used to solubilize the enzymes from the membrane fraction. Baker and Claus (8) demonstrated that PDH exhibits different properties when solubilized by different detergents. Ameyama et al. (3) showed that the pH optima of glycerol dehydrogenase changes from the acidic range to pH 8 when solubilized from the membrane fraction.

The difference in enrichment efficiency is probably due to *G. industrius* IFO strain 3260 used by Ameyama et al. (3) having only about one-fourth the total polyol oxidizing activity and one-third the specific activity for PDH than does *G. oxydans* ATCC 621 (= IFO 12528) used in this study (8). Therefore, to achieve homogeneity, it would need to be enriched by a higher degree than the isolated PDH complex from G. oxydans ATCC strain 621.

***(ii)* Comparison to sorbitol dehydrogenase.** An enzyme called sorbitol dehydrogenase was isolated from *G. suboxydans* var. α ATCC strain 3254 by Shinagawa et al. (27). This enzyme was isolated by Triton solubilization from the membrane fraction, PEG precipitation, and DEAE and CM-cellulose ion-exchange chromatography. The enzyme had some similarities and differences from the isolated PDH complex described here (Table 1.3). Similarities include subunit structure and composition of the two enzymes when compared by SDS-PAGE. Also, like the enzyme described in this study, the cytochrome of the sorbitol dehydrogenase could not be reduced directly by the substrate, but

instead was reduced with the substrate in the presence of phenazine methosulfate.

One difference between the enzyme isolated by Shinagawa et al. (27) and the isolated PDH complex from this study were the number of subunits detected by native-PAGE. Shinagawa et al. (27) found two bands when the sorbitol dehydrogenase was separated by native-PAGE, where only one was observed from the PDH complex isolated in this study. It could be that the subunits of the sorbitol dehydrogenase from *G. suboxydans* var. α used by Shinagawa et al. (27) are not held as together as tightly as the subunits of the PDH complex isolated from *G. oxydans* ATCC 621 used this study, which allowed them to dissociate during native PAGE. Another possibility is their sorbitol dehydrogenase isolated by Shinagawa et al. (27) from ATCC strain 3254 is the same enzyme as the PDH complex isolated in this study, but contained a contaminating polypeptide.

Another difference between the enzyme isolated by Shinagawa et al. (3) and the PDH isolated in this study was the range of substrates oxidized. Unlike the enzyme in my study, the sorbitol dehydrogenase was more substrate specific, oxidizing only sorbitol and mannitol (at 5% the rate of sorbitol).

***(iii)* Comparison to mannitol dehydrogenase.** Cho et al. (11) isolated an enzyme that oxidized mannitol and sorbitol from *G. melanogenus* ATCC strain 15163. This enzyme was purified by Triton solubilization, PEG

precipitation, CM-cellulose and DEAE-Sephacyl chromatography, and HPLC. The enzyme had similar subunit size and composition as the PDH complex isolated described in this study (Table 1.3). However, unlike the isolated PDH complex in this study, the enzyme from *G. melanogenus* was eluted from the ion-exchange columns by increased salt concentrations in the buffer.

Also, unlike the isolated PDH complex described here and the sorbitol dehydrogenase described by Shinagawa et al. (27), the enzyme isolated by Cho et al. (11) was reduced by substrate alone and was oxidized by 2,6-dichlorophenolindophenol (DCPIP) alone. The isolated enzymes described here and by Shinagawa et al. (27) were oxidized and reduced only in the presence of the electron mediator phenazine methosulfate.

When Cho et al. (11) subjected their DSMP fraction to CM-cellulose column chromatography, two distinct fractions containing polyol-oxidizing activity were detected. One peak had equal amounts of sorbitol and mannitol oxidizing ability. The other peak contained an enzyme that oxidized mannitol two-times faster than sorbitol. It was this second enzyme that was isolated by these investigators. Therefore, it may be possible that the genus *Gluconobacter* contains two different membrane-bound polyol oxidizing enzymes; one having broad-spectrum activity, needing an electron mediator to become oxidized or reduced. The second enzyme having limited substrate-specificity, and is oxidized by DCPIP and reduced by substrate directly.

Another possibility is that the two peaks represent different forms of the same enzyme. The second peak, isolated by Cho et al. (11) may represent the enzyme in a conformation that allows direct oxidation by DCPIP and direct reduction by the substrate, and is somewhat restricted in its substrate specificity. The first peak, not isolated by these investigators, may represent the enzyme in a conformation that would not allow direct oxidation or reduction of the cytochrome and had a broad substrate specificity.

Summary. *Gluconobacter* constituitively synthesize membrane-bound dehydrogenases for polyol oxidation(14). It was my hypothesis that since it would take considerable biosynthetic energy expenditure for these bacteria to constituitively synthesize a unique substrate-specific enzyme for each polyol oxidized, that *Gluconobacter* might synthesize a single enzyme with broad-substrate specificities towards polyols. This study is significant in that it is the first to show isolation to homogeneity (as indicated by non-denaturing-PAGE) an enzyme from *G. oxydans* with broad-specificity towards polyol, hence named polyol dehydrogenase. These findings are in accord with other broad-spectrum dehydrogenases isolated from *Gluconobacter* species, such as an aldehyde dehydrogenase (2), an aldose dehydrogenase (9), and an alcohol dehydrogenase (1); each with broad-specificity against their respective class of substrates.

It is possible that the isolated PDH complex described in this study is the same enzyme as glycerol dehydrogenase (3), mannitol dehydrogenase (11), and sorbitol dehydrogenase (27), described by others. The differences among these enzymes could be due to the differences in purification and assay procedures. Another possibility is that along with the broad spectrum polyol dehydrogenase isolated in this study, there may be additional substrate-specific dehydrogenases for mannitol and sorbitol.

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CHAPTER 2

The Prosthetic Group of the Membrane-Bound [NAD(P)-Independent] Polyol Dehydrogenase from *Gluconobacter oxydans*.

ABSTRACT

Gluconobacter oxydans rapidly performs limited oxidations of polyhydroxy alcohols (polyols). These oxidations are catalyzed by a constituitively synthesized [NAD(P)-independent] polyol dehydrogenase (PDH) located in the cell's plasma membrane. In this study, PDH from *G. oxydans* ATCC strain 621 was shown to contain pyrroloquinoline quinone (PQQ) as its prosthetic group by three methods. (*i*) The PDH was completely inactivated by CsCl or KI-treatments (and partially inactivated with other salts, EDTA, or acid-treatment), and reconstituted with authentic PQQ but not with flavin mononucleotide or flavin adenine dinucleotide. (*ii*) Salt extracts from the PDH complex isolated from *G. oxydans* restored activity to the apo-form of a known quinoprotein, a membrane-bound glucose dehydrogenase, prepared from *Escherichia coli*. (*iii*) The absorption spectrum of CsCl-extracts of the *G. oxydans* PDH complex were identical to that of authentic PQQ. Various combinations of divalent cations was essential for successful reconstitution of *G. oxydans* apo-PDH with authentic PQQ. The combination of Co^{2+} and Ca^{2+} with PQQ resulted in the immediate restoration of maximum activity to apo-PDH fractions. Considering this evidence, I feel that PDH from *G. oxydans* should be considered a quinoprotein.

INTRODUCTION

The genus *Gluconobacter* is well known for its ability to perform limited single-step oxidations of over 100 different hydroxyl-containing compounds (16,30). The enzymes responsible for these oxidations are NAD(P)-independent, located in the cell's plasma membrane, have their active site facing the periplasmic space, and are linked to the cell's electron-transport chain (45). Substrates are oxidized by these primary dehydrogenases to their corresponding aldehydes or ketoses (45). The oxidation products may then be oxidized by secondary dehydrogenases to their corresponding acids (45).

Polyol dehydrogenase from *Gluconobacter* is used in several industrial processes. This enzyme has been extensively studied because: (*i*) it accomplishes an almost quantitative conversion of sorbitol to sorbose; (*ii*) it is used in the production of vitamin C (12,33,42,51,53,68,69,72,73); (*iii*) it also accomplishes the oxidation of glycerol into dihydroxyacetone (17,36,41,67,71); (*iv*) it may also be used as an antioxidant in foods (62) and beverages (49); (*v*) and this enzyme will also convert a chemically made substrate into a new anti-viral drug (35). The gluconobacters contain at least three sorbitol-oxidizing enzymes. Two of these sorbitol-oxidizing enzymes are cytoplasmic. The first of these cytoplasmic enzymes produces fructose in the presence of NAD (50), and the second cytoplasmic enzyme produces sorbose in the presence of NADP (37,38). Whereas, membrane-bound sorbitol dehydrogenase produces sorbose

and is NAD(P)-independent (18,58). In this chapter, the membrane-bound enzyme that oxidizes sorbitol is exclusively studied.

Many of these membrane-bound dehydrogenases have been purified from the gluconobacters, such as alcohol (1), polyol (18), sorbitol (58), fructose (6,70), sorbose (61), 2-keto-D-gluconate (57), and D-gluconate (59) dehydrogenases. These enzymes were initially characterized as flavoproteins, containing a tightly bound flavin group. However, after the discovery of a novel prosthetic group, 4,5-dihydro-5-hydroxy-4-oxo-5-(2-oxopropyl)-1*H*-pyrrolo [2,3-*f*] quinoline-2,7,9-tricarboxylic acid [trivial name pyrroloquinoline quinone (PQQ)] (23,24,25,26,27,34,55), re-examination of the membrane-bound dehydrogenases from *Gluconobacter* found them to be quinoproteins, containing PQQ as their prosthetic group. This is not surprising, since *Gluconobacter* membranes contain large quantities of PQQ (2). Also, the growth of *Gluconobacter* is stimulated by yeast extract and other complex media components (8), and it is the PQQ in these complex media components that is responsible for the enhanced growth of *Gluconobacter* (8). Also, PQQ reduces the lag phase of growth in a closely related genus, *Acetobacter*, when grown on hydroxyl-containing compounds (7,9).

All except one of the primary dehydrogenases characterized from *Gluconobacter* are quinoproteins [glucose (5), alcohol (60), glycerol (10), and aldehyde (4), polyethylene glycol dehydrogenase (43)]. The exception is polyol

(sorbitol) dehydrogenase (PDH), which is reported to be a flavoprotein (58). The flavin nature of the prosthetic group from PDH was supported by two lines of evidence. First, the purified enzyme, separated by electrophoresis, fluoresced upon exposure to ultraviolet light. Second, proteolytic digestion of partially purified PDH yielded flavin molecules. However, this evidence is not conclusive. After that study (58) was performed, it was shown that PQQ, like flavin, fluoresces when exposed to UV light (21). Also, the enzyme preparation in that study (58) was considered only 90% pure, it was associated with two other proteins, and there was a low recovery of flavin (less then 0.4 moles of flavin mole enzyme^{-1}). Therefore, it is possible that the flavin may have come from contaminating or associated proteins.

This study was performed to determine if the prosthetic group from PDH is PQQ, because all other primary dehydrogenases from *Gluconobacter* are quinoproteins, and the evidence for PDH being a flavoprotein is not conclusive, Specifically, the goals of this study were: (*i*) to remove the prosthetic group from PDH for identification and to form the apo-enzyme; (*ii*) to reconstitute the apo-enzyme with authenticated prosthetic groups, such as PQQ, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD); and (*iii*) to identify the prosthetic group removed from the isolated PDH complex.

MATERIALS AND METHODS

Organism, growth and cell fractionation. (*i*) *G. oxydans.* Organism, growth conditions, cell fractionation, and polyol dehydrogenase (PDH) isolation were performed as previously described (63). Briefly, three milliliters of *Gluconobacter oxydans* ATCC strain 621 sorbitol stock culture was inoculated into 2.5 liters of a complex sorbitol medium and incubated at 28°C in a 3 liter fermentor under heavy aeration and agitation. Cells were harvested by centrifugation about 1 h into maximum stationary phase, washed, and suspended in buffer. Cell extracts were obtained by ultrasonic disruption, and *membrane fractions* were isolated by ultracentrifugation. Polyol dehydrogenase was solubilized from the membrane fraction with 1.0% (vol/vol) Triton X-100 (Triton) in the presence of 1.5 mM KCl and 1.5 mM sorbitol. The Triton-treated membranes were removed by ultracentrifugation, and the supernatant fluid was called the Triton-*detergent-solubilized membrane protein* (Triton-DSMP) fraction. The *isolated PDH complex* was obtained by anion and cation exchange and hydrophobic interaction chromatographies.

(*ii*) *E. coli*. *Escherichia coli* strain K12 (19) was inoculated into a 500 ml Bellco Nephelometer flask containing 50 ml of a minimal medium composed of (wt/vol) 0.1% glucose, 1.64% Na_2HPO_4, 0.15 KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.001% $CaCl_2$, and 0.005% $FeSO_4$. Cultures were incubated at 37°C while shaking at 50 reciprocations min^{-1}. An overnight culture (100 µl) was

inoculated into each two-liter Bellco Nephelometer flasks containing 200 ml of minimal medium and incubated as *G. oxydans* cultures. Cells were harvested by centrifugation at 36 h, and the *membrane fraction* was isolated as described above for *Gluconobacter* (63).

Enzyme assays. Polyol dehydrogenase (PDH) activity was assayed by following the reduction of ferricyanide spectrophotometrically in a modification of the procedure described by Arcus and Edson (15). Each milliliter of assay solution contained 0.4 μmol of potassium ferricyanide, 10 μmol of $MgCl_2$, 5 μmol of $CaCl_2$, 250 μmol of sorbitol, 0.6 μmol of methylphenazonium methosulfate (MPMS) and 200 μl of 200 mM succinate-NaOH buffer, pH 5.0. The bleaching of ferricyanide was monitored at 303.4 nm at 38°C using a Bausch and Lomb Spectronic 2000 spectrophotometer.

A second assay was used when testing the ability of divalent cations to help reconstitute apo-PDH with PQQ, because a precipitate formed when certain divalent cations were added to the ferricyanide assay solution. This assay solution was the same as above, except that ferricyanide was replaced with 0.3 μmol of dichlorophenol indophenol as the artificial electron acceptor, and the change in absorbance was monitored at 600 nm.

Glucose dehydrogenase activity of the membrane fraction of *E. coli* was assayed using the ferricyanide-spectrophotometric assay solution described above, except glucose replaced sorbitol as the substrate in the assay solution.

Protein determination. Cell fractions were first digested for 30 min in a boiling water bath in the presence of 2 N NaOH and 10% sodium dodecyl sulfate. Protein concentrations were then determined by a modification (29) of the method of Lowry et al. (44).

Absorbance spectra of the PDH prosthetic group. The isolated polyol dehydrogenase complex (63) was mixed with 3 M CsCl for 30 min. The mixture was then centrifuged at 5,760 x *g* for 5 min. The supernatant fluid was decanted and incubated overnight at 4°C. This resulted in a red precipitate that was isolated by decanting the supernatant fluid. The precipitate was then suspended in distilled-deionized water and called the *PDH-CsCl-extract*. Its absorption spectra was determined at wavelengths between 300 and 600 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer.

Prosthetic group removal from PDH. (*i*) EDTA treatments. Membrane and Triton-DSMP fractions were prepared as previously described (63) and adjusted to pH 7.5 with NaOH. Crystalline EDTA was slowly added to both these fractions to a final concentration of 100 mM, and the mixtures incubated on ice for 2 h. The treated membranes were isolated from this mixture by ultracentrifugation (120,000 x *g* for 90 min at 4°C). Membrane pellets were suspended with a Teflon tissue homogenizer in EDTA-free 10 mM sodium acetate buffer at pH 5.0 and called *EDTA-treated membrane fractions*.

The Triton-DSMP fraction proteins were removed from this mixture by precipitation with polyethylene glycol 8000 (PEG). To accomplish this, crystalline PEG was slowly added to the supernatant fluid to a final 15% (wt/vol) concentration, mixed, and incubated on ice for 20 min. Samples were then centrifuged (13,500 x *g* for 10 min at 4°C), and the resulting supernatant fluid was removed and discarded. The insides of the centrifuge tubes were wiped to remove any residual supernatant fluid. The precipitated Triton-DSMP fractions were suspended in 10 mM Na-acetate buffer (pH 5.0) containing 0.1% (vol/vol) Triton and called *EDTA-treated Triton-DSMP fractions*.

***(ii)* Acid and base treatments.** Triton-DSMP fractions were prepared as previously described (63) and then mixed for 30 min in buffers ranging from pH 2 to 10. Regardless of the pH, each buffer consisted of 66 mM succinic acid, 66 mM citric acid, and 66 mM dibasic potassium phosphate. The pH was adjusted by adding either NaOH or HCl. After buffer treatment, the Triton-DSMP fractions were precipitated with PEG as described above. Each pellet was dissolved in a volume of buffer [200 mM succinate-NaOH buffer (pH 5.0) containing 0.1% (vol/vol) Triton X-100] equal to that of the starting volume of the original Triton-DSMP fraction sample. These samples were called the *acid-* or *base-treated Triton-DSMP fractions*.

***(iii)* Salt treatments.** Membrane fractions were prepared as previously described (63). Salts were individually added to membrane fractions to a final

concentration of 3 M, except for CsCl and KI which at 2 M. The membrane-salt mixtures were stirred for 2 h at 4°C then ultracentrifuged (120,000 x *g* for 90 min at 4°C). The supernatant fluid was called the *salt-PDH-extract*. The membrane pellet was washed by suspending in 10 mM Na-acetate buffer (pH 5.0) with a Teflon tissue homogenizer. This suspension was ultracentrifuged as before, and the resulting pellet was suspended in 10 mM Na-acetate buffer (pH 5.0) using a volume identical to the original membrane fraction. These resuspended pellets were called the *salt-treated membrane fractions*.

To remove the prosthetic group from PDH, CsCl was added to a 3 M (either to the isolated PDH complex, the Triton-DSMP fraction, or the membrane-bound PDH). All mixtures were stirred for 30 min at 4°C then centrifuged for 30 min at 40,000 x *g* to remove the precipitated enzyme fraction. The pellet was suspended in 10 mM Na-acetate buffer (pH 5.0) (which included 0.1% Triton in experiments that used the Triton-DSMP fraction or the isolated PDH complex) and called the *apo-PDH fractions*. The desalted supernatant fluid obtained from the centrifugation of the isolated PDH complex was called the *PDH-CsCl-extract*.

Apo-enzyme reconstitution. For reconstitution studies, samples containing apo-PDH or apo-glucose dehydrogenase were suspended in 10 mM Na-acetate buffer (pH 5.0) in the presence of 10 mM $MgCl_2$ and 10 mM $CaCl_2$. Either 10 pmol of PQQ, 10 μmol of FMN, or 10 μmol of FAD was added to each

milliliter of enzyme fraction. Samples were then incubated on ice for about 10 min before assaying for enzyme activity using the ferricyanide assay.

RESULTS

Removal of the prosthetic group from PDH. The goals of this study were (*i*) to form apo-PDH for reconstitution studies with authentic prosthetic groups, and (*ii*) to identify the prosthetic group removed from the isolated PDH complex. To accomplish these goals, I needed to remove the prosthetic group from PDH in a way that would not denature the enzyme. The following methods were used in an attempt to remove the prosthetic group from PDH

(*i*) EDTA treatment (3,10,22)**.** The membrane fraction and Triton-DSMP fraction were treated with 100 mM EDTA. The EDTA-treated membrane fractions retained about 95% of their original PDH activity, whereas the EDTA-treated Triton-DSMP fractions lost about 50% of their PDH activity. This suggested that EDTA-treatment did not remove much prosthetic group from the membrane fraction, but EDTA-treatment might be useful for obtaining apo-PDH from Triton-DSMP fractions.

(*ii*) Hydrogen-ion treatment (39,60)**.** The Triton-DSMP fractions were treated with buffers whose pH ranged from pH 3 to 8. The Triton-DSMP fractions incubated at pH values from 3.8 and 6.5 retained over 95% of their PDH activity. Fractions incubated at pH 3 retained 81% activity, and fractions incubated at pH 7, 7.5, and 8 retained 51, 46, and 2% of their PDH activity, respectively. Therefore, it seemed that incubation at alkaline pH values may have been partially successful in apo-PDH formation.

***(iii)* Salt treatment** (28,39,47,52). Membrane fractions were treated with various metal chloride and potassium halide salts in an attempt to form apo-PDH. Membranes separately treated with the metal chloride salts had an approximate 30% reduction in PDH activity (Fig. 2.1A -- top half). The RbCl-treatments resulted in approximately 60% reduction of PDH activity, while CsCl-treatment resulted in almost complete inactivation of PDH. The potassium halide salts also resulted in decreased PDH activity, with KCl-treatment showing the least effect and KI-treatment resulting in almost complete reduction of PDH activity (Fig. 2.1A -- bottom half). Of the three methods used in attempts to form apo-PDH, the salt-treatments, especially CsCl and KI-treatments, seemed to be the most promising for apo-PDH formation.

Reconstitution of apo-PDH. To determine the nature of the prosthetic group of the PDH from *G. oxydans*, various authentic prosthetic groups were added to the apo-PDH in attempts to reconstitute the original enzyme activity of that fraction. Salt, EDTA, and acid-base-treatments of enzyme fractions were used in attempts to remove the prosthetic group from PDH. Each method resulted in some decrease in PDH activity, which could be caused by the removal of the prosthetic group. Alternatively, inactivation of PDH by these treatments could also be due to denaturation of the enzyme. Since the CsCl-treatment of the membrane fraction resulted in the largest decrease in PDH

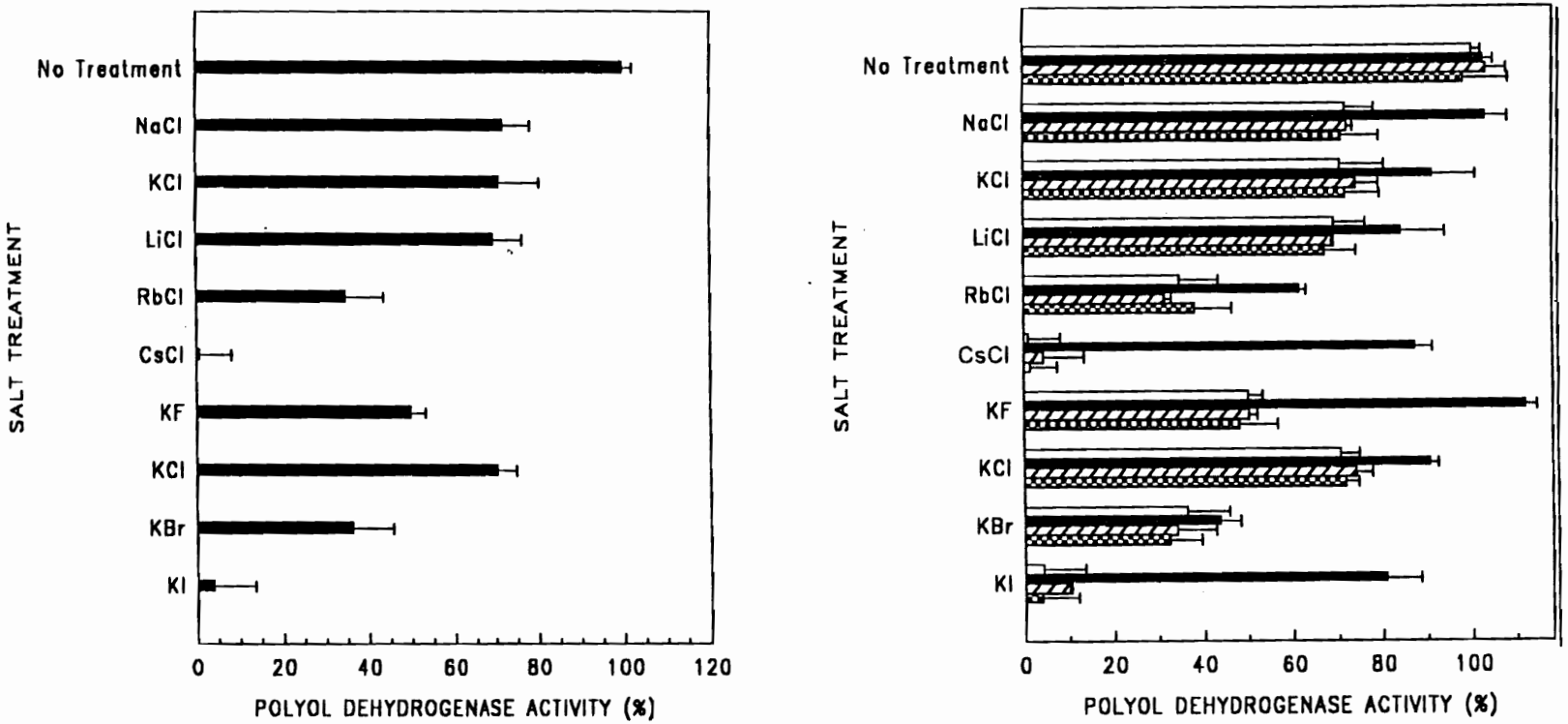

Figure 2.1 (A) Polyol dehydrogenase activity of salt-treated *G. oxydans* membrane fractions and (B) attempts to reconstitute salt-treated fractions with FMN, FAD, and PQQ. (A) Indicated salts were added to the membrane fraction to a final concentration of 3 M (except CsCl and KI, which were used at 2 M concentrations) and stirred for 2 h. The membrane fractions were removed by ultracentrifugation, washed, suspended in salt-free buffer, and PDH activity assayed. (B) Each desalted salt-treated membrane fraction was mixed with either PQQ (■), FMN (▨), or FAD (▩) (in the presence of Ca^{2+} and Mg^{2+}) and assayed for PDH activity. Enzyme activity of the salt-treated membrane fraction with no added prosthetic group is also shown (□). The untreated membrane fraction, assayed in the absence of added prosthetic groups, was considered to contain 100% PDH activity. Data represents the average values obtained from three replicates each of membrane fractions (483, 501, 512 μg of protein ml^{-1}) isolated from three separate cell batches. Standard deviations are shown.

activity, fractions treated with CsCl were chosen for reconstitution studies using either FAD, FMN, or PQQ as the prosthetic group.

When either 10 mM FAD or FMN was added to each type of salt-treated membrane, no restoration of PDH activity was observed (Fig. 2.1B). However, the addition of 10 μM PQQ resulted in (*i*) restoration of over 100% of the activity of the NaCl and KF-treated samples; (*ii*) about 80% activity restoration of the KCl, CsCl, LiCl, and KI-treated fractions; and (*iii*) about 40% of the RbCl and 60% with KBr-treated fractions. When PQQ was added to the untreated membrane fraction (no salt-treatment), there was less than 5% increase in enzyme activity.

The addition of PQQ to the EDTA-treated membrane fraction did not result in a increase in PDH activity (data not shown). However, PQQ addition to the EDTA-treated Triton-DSMP fraction resulted in 85% restoration of activity (data not shown). Addition of PQQ also completely restored PDH activity to the acid-treated Triton-DSMP fractions (data not shown). However, PQQ addition to enzyme fractions subjected to neutral and basic pH did not result in restoration of activity (data not shown).

Identification of PDH's prosthetic group. Reconstitution studies indicated that the prosthetic group of PDH is PQQ. To confirm this, I first used 3 M CsCl to remove the prosthetic group from the PDH complex, which resulted in precipitation and inactivation of PDH. I reasoned that the desalted

supernatant fluid, called the PDH-CsCl-extract, should contain the prosthetic group; therefore it was subjected to the following spectrophotometric and biological analysis.

***(i)* Absorption spectrum of the PDH-CsCl extract.** The absorbance of the PDH-CsCl-extract was examined from 300 to 600 nm. Two absorbance maxima were found (331 and 485 nm) and the absorbance minimum was at 426 nm (Fig. 2.2).

***(ii)* Biological detection of PQQ.** The membrane bound glucose dehydrogenase from *Escherichia coli* is a known quinoprotein, containing PQQ as its prosthetic group, but *E. coli* is incapable of synthesizing PQQ (32,40,64,65,66). Thus, when grown on a minimal medium this bacterium produces only the apo-form of glucose dehydrogenase. Addition of either authentic PQQ or the PDH- CsCl-extract to the apo-glucose dehydrogenase resulted in restoration of enzyme activity (Table 2.1).

Optimum conditions for reconstitution. Optimum conditions for reconstituting apo-PDH from *G. oxydans* with PQQ were investigated using apo-PDH obtained by treating gluconobacter membrane fractions with 3 M CsCl.

***(i)* Divalent cations.** When authentic PQQ was added to the gluconobacter apo-PDH without the addition of divalent cations, or in the presence of only a single type of divalent cation, no reconstitution (enzyme activity) was observed (data not shown). In the presence of certain

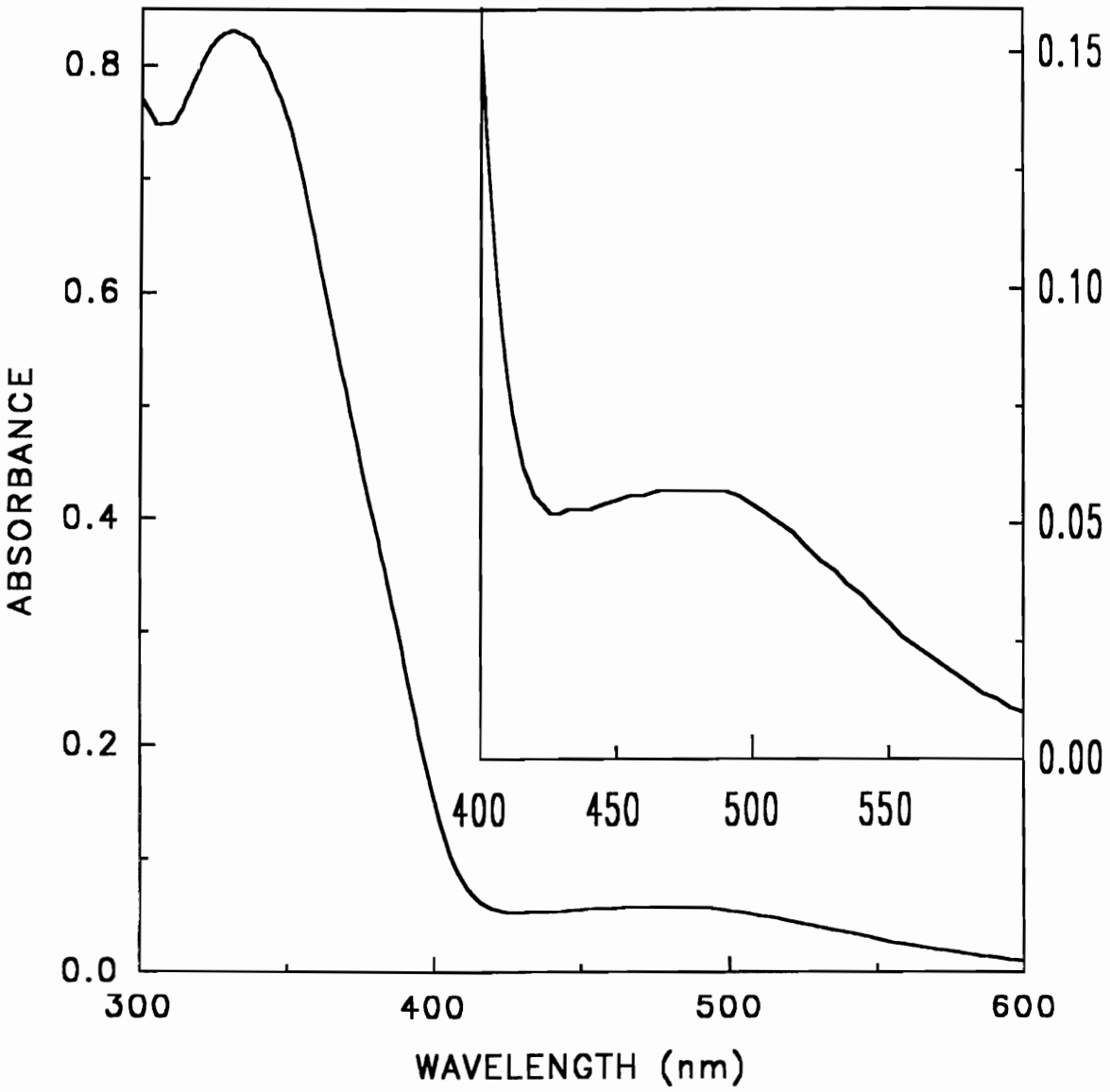

Figure 2.2. Absorption spectrum of the *G. oxydans* PDH-CsCl-extract . The isolated PDH complex was mixed with 3 M CsCl for 30 min and then centrifuged to pellet the enzyme. The supernatant fluid was desalted and its absorption spectra monitored between 300 and 600 nm. Data shown is from a typical experiment.

TABLE 2.1: Reconstitution of apo-glucose dehydrogenase from the membranes of *E. coli* with the PDH-CsCl-extract and authentic PQQ.

Experiment[a]	*E. coli* glucose dehydrogenase activity (change in absorbance at 303.4 nm)		
	No treatment[b]	Authentic PQQ[c]	PDH-CsCl-Extract from *G. oxydans*[d]
1	0.003 ± 0.005	0.132 ± 0.009	0.064 ± 0.004
2	0.007 ± 0.003	0.133 ± 0.010	0.083 ± 0.003
3	0.001 ± 0.002	0.141 ± 0.006	0.059 ± 0.008

[a]Each experiment used PDH-CsCl-extract obtained from isolated PDH complexes from separate cell batches of *G. oxydans*.

[b]Glucose dehydrogenase activity from the membrane fraction of *E. coli* was assayed without the addition of PQQ or the PDH-CsCl-extract.

[c]Glucose dehydrogenase activity from the membrane fraction of *E. coli* was assayed after the addition of authentic PQQ.

[d]Glucose dehydrogenase activity from the membrane fraction of *E. coli* was assayed after the addition of the PDH-CsCl-extract obtained from *G. oxydans*.

combinations of cations, PDH activity could be partially or fully restored (Table 2.2). Various divalent cations in combination with Ca^{2+} restored the greatest amount of activity, with Ca^{2+} plus Co^{2+} being the most effective, closely followed by Ca^{2+} plus Mg^{2+}, and to a lesser extent by Ca^{2+} plus either Ni^{2+}, Mn^{2+}, or Zn^{2+}. The effect of varying the order of addition of PQQ, divalent cation, and apo-PDH addition during reconstitution always demonstrated the same amount of restored activity (data not shown). Since calcium and magnesium were regularly included in the ferricyanide assay, these cations were included in all of the following reconstitution studies.

***(ii)* Incubation time.** To determine the optimum time necessary for reconstitution of PQQ with apo-PDH, the apo-PDH was incubated with PQQ for various time periods (0 to 30 min) prior to measuring the PDH activity. All samples, regardless of time incubated, showed the same amount of PDH activity (data not shown). When PQQ was added after the beginning of the assay, enzyme activity was detected immediately (Fig. 2.3).

***(iii)* PQQ concentration.** Authentic PQQ was added at various concentrations to *G. oxydans* apo-PDH prior to assaying PDH. The PDH activity increased as PQQ concentrations increased from 0 to 200 nM (Fig. 2.4), stayed steady to 500 nM, and above 500 nM PQQ seemed to be slightly inhibitory.

***(iv)* Temperature.** To determine the effect of temperature on reconstitution, authentic PQQ was added to the apo-PDH from *G. oxydans* while

TABLE 2.2: Effect of divalent cations on reconstitution of *G. oxydans* apo-PDH with PQQ.

Cation combination[a]	PDH activity (%)[b]	Cation combination	PDH activity (%)
Cd^{2+} + Co^{2+}	4.1	Ca^{2+} + Co^{2+}	100[b]
Cd^{2+} + Mn^{2+}	1.4	Ca^{2+} + Mg^{2+}	80.7
Cd^{2+} + Ni^{2+}	3.9		
Co^{2+} + Mg^{2+}	5.0	Mg^{2+} + Ni^{2+}	3.5
Co^{2+} + Mn^{2+}	1.5	Mg^{2+} + Sr^{2+}	4.8
Co^{2+} + Sr^{2+}	7.2		
		Mn^{2+} + Ni^{2+}	0.8
Ca^{2+} + Mn^{2+}	57.4	Mn^{2+} + Sr^{2+}	2.7
Ca^{2+} + Ni^{2+}	68.5		
Ca^{2+} + Zn^{2+}	7.4	Ni^{2+} + Sr^{2+}	4.1

[a]All cations were used as chloride salts and added at 10 mM concentrations. Cu^{2+} could not be used because it interfered with the PDH assays.

[b]Enzyme activity was assayed spectrophotometrically with dichlorophenol indophenol and MPMS at 600 nm. Activity detected with Ca^{2+} + Co^{2+} was designated as 100%.

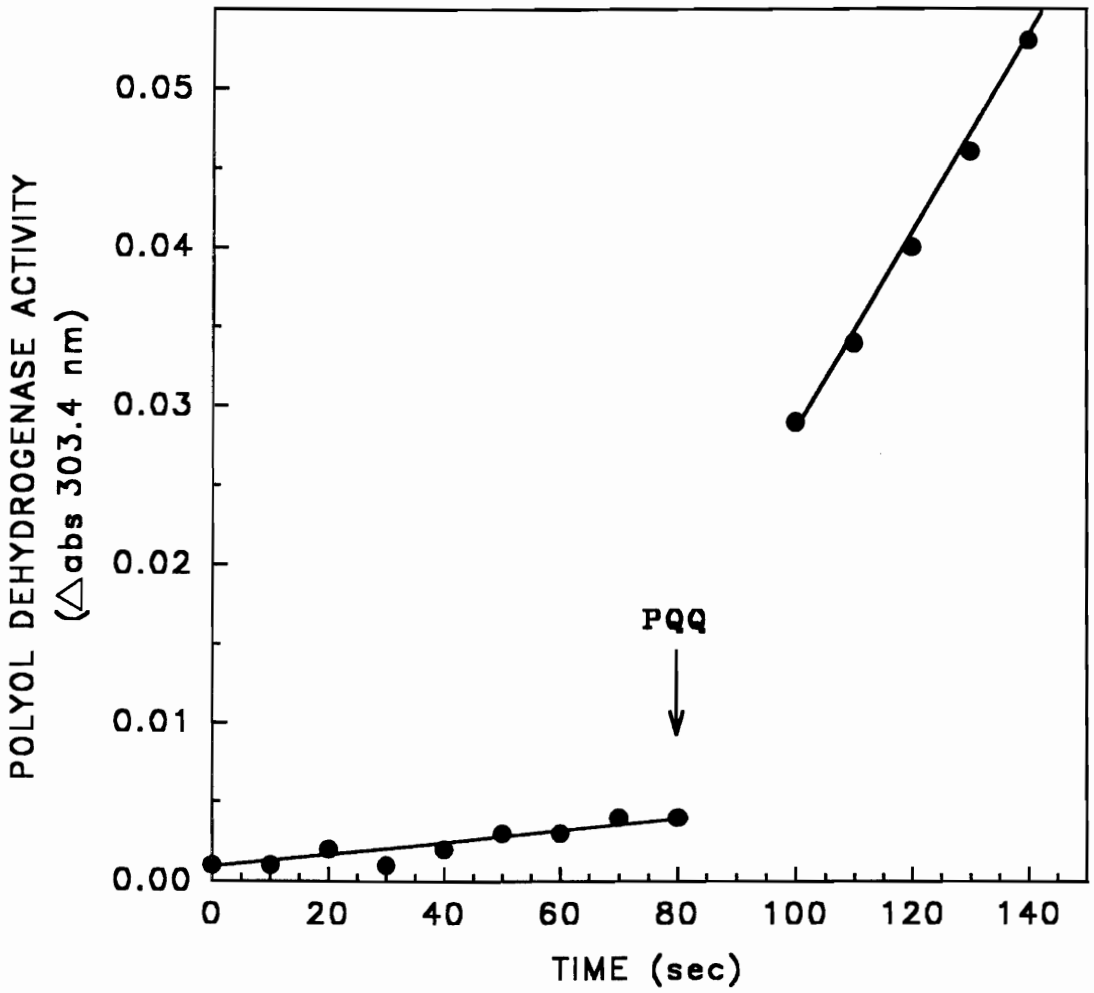

Figure 2.3. Effect of PQQ addition to the CsCl-treated membrane-fraction of *G. oxydans* on PDH activity. Membrane fractions were treated with 2 M CsCl for 2 h. The treated membrane fractions were removed from the salt and suspended in salt-free buffer. Polyol dehydrogenase activity was then assayed. At 80 sec into the assay, 10 μM PQQ was added, and the enzymatic assay continued. Data shown is from a typical experiment.

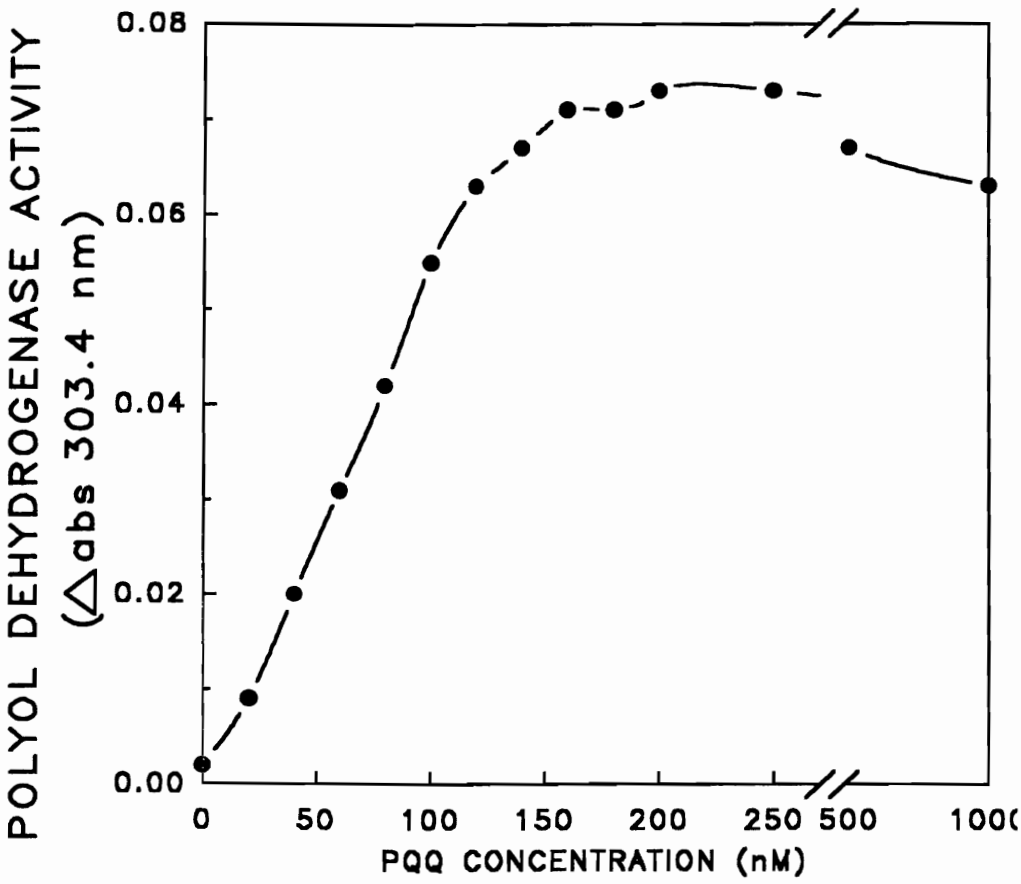

Figure 2.4. Effect of PQQ concentration on reconstitution of *G. oxydans* apo-PDH. The isolated polyol dehydrogenase complex (1.06 μg of protein) was incubated with varying quantities of PQQ for 30 min, and then PDH activity was assayed. Data shown is from a typical experiment.

incubating at 0, 22, and 37°C. Polyol dehydrogenase activity was restored to the same extent, regardless of the temperature used during reconstitution (data not shown).

DISCUSSION

All the primary dehydrogenases isolated from *Gluconobacter* thus far have been shown to be quinoproteins, with the exception of sorbitol dehydrogenase isolated by Shinagawa et al. (58), which was determined to be a flavoprotein. The sorbitol dehydrogenase isolated by Shinagawa et al. (58) has many characteristics of the enzyme I isolated (63) and call polyol dehydrogenase (PDH) and is probably the same enzyme. The major difference in the description is substrate specificity, which could be due to the enzyme assay used (63), or, conversely to a point mutation that can change the substrate specificity of membrane-bound dehydrogenases in *Gluconobacter* (20). Since all other primary dehydrogenases from *Gluconobacter* isolated to date are classified as quinoproteins, except for the polyol oxidizing dehydrogenase, my study was undertaken to determine if PDH might be a quinoprotein, containing PQQ as its prosthetic group.

Prosthetic group removal from PDH. To characterize the prosthetic group of PDH, it was first necessary to remove it from the holoenzyme. Many techniques, such as heat (13,14,54), proteolytic digestion (48,49), organic solvent extraction (24,28,45,54,58), salt-treatment (54), and changes in the hydrogen ion concentration (11,13,14,54) result in irreversible denaturation of the enzyme fractions. However, in this study, I wanted to avoid irreversible denaturation of PDH, so that reconstitution of apo-PDH and authentic prosthetic

groups could be studied. Therefore, I used methods that reportedly allowed reversible separation of the prosthetic group from other membrane-bound dehydrogenases. These methods included (*i*) EDTA-treatment used to reversibly remove the prosthetic group of glycerol dehydrogenase from *Gluconobacter* (10); (*ii*) pH treatment that reversibly removed the prosthetic group of alcohol dehydrogenase from *Gluconobacter* (60); (*iii*) salt-treatment used for reversible removal of the prosthetic group from glucose dehydrogenase from *Acinetobacter calcoaceticus* (28) and *Bacterium anitratum* (29).

Each of these methods, when applied to PDH, resulted in some inactivation of the enzyme, with the 2 M KI or CsCl treatment resulting in the largest loss of activity (Fig. 2.1A and data not shown). However, it was possible that some or all of this loss of enzyme activity was due to enzyme denaturation. To determine if the prosthetic group was being removed reversibly from PDH or if PDH was being denatured, the following reconstitution studies were performed.

Reconstitution of apo-PDH with authentic prosthetic groups. In no case did FMN or FAD restore PDH activity to the inactivated or partially inactivated enzyme samples treated with salt, EDTA, or changes in pH (Fig. 2.1B and data not shown). PQQ did restore some or all the PDH activity to the salt-treated fractions (Fig. 2.1B), as well as the EDTA-treated DSMP and acid treated fractions (data not shown).

This evidence suggests (*i*) that the prosthetic group of PDH is PQQ, not FAD or FMN, as previously reported (58); and (*ii*) apoenzyme is formed by both salt treatment of the membrane fraction or by either acid or EDTA treatments of the DSMP fractions. However, decreases in PDH activity due to base treatment and EDTA treatment of the membrane fraction is apparently due to enzyme denaturation, since reconstitution was not possible.

Identification of the PDH-CsCl-extract. Since PQQ restored PDH activity to several of the treated PDH fractions, this indicates that PDH is a quinoprotein. To further verify this, the extracted prosthetic group from *G. oxydans* was examined (Fig. 2.2) and found to match that published for authentic PQQ (21).

For further verification, a bioassay was used to detect PQQ extracted from the isolated gluconobacter PDH complex. Several bacteria synthesize apo-quinoproteins, but are unable to make PQQ (40,54,56). The resulting apo-quinoproteins become active only when PQQ is present, thus can be used in biological assays for PQQ (64).

I found the prosthetic group from the isolated PDH complex (present in the PDH-CsCl extract) restored glucose dehydrogenase activity to the apo-quinoprotein glucose dehydrogenase from the membranes of an *Escherichia coli* strain incapable of producing PQQ (Table 2.1). This evidence also supports PQQ as the prosthetic group of *Gluconobacter oxydans* PDH.

Divalent cations. To reconstitute PQQ with apo-enzymes, it is often necessary to add divalent cations. Individually, calcium ions (48,60,63) or magnesium ions (3,22) commonly restore the greatest activity. Cobalt and nickel showed some reconstitutive ability in glucose dehydrogenase from *Escherichia coli* and *Pseudomonas fluorescens*, but the most activity was restored with magnesium (3). Ethanol dehydrogenase from *Pseudomonas aeruginosa* was reconstituted with PQQ in the presence of Ca^{2+} or Sr^{2+} ions, however, the Sr^{2+} ions resulted in an enzyme with a different absorption spectrum than the native enzyme (48). Therefore, it is possible that membrane-bound dehydrogenases prefer one cation, but may use others to form a less active holoenzyme.

In this study, no single divalent cation restored gluconobacter PDH activity, when used with authentic PQQ. However, the addition of several combinations of divalent cations restored activity. This is unusual, since reconstitution of membrane-bound apo-quinoproteins is usually accomplished with just a single species of divalent cation (3,22,48,60,63). The combination of Co^{2+} and Ca^{2+} restoring the greatest amount of PDH activity (Table 2.2), and occurred almost immediately (Fig. 2.3), and independent of temperature in the range of 0 to 37°C.

Conclusion. The PQQ has a redox potential which is higher than for flavin-dependent dehydrogenases (25), which would seem to be necessary to reduce its cytochrome subunit (31,58). The prosthetic group, removed with salt-

treatment from PDH isolated from *Gluconobacter* in this study was identified as PQQ by its absorption spectrum and by its ability to activate the apo-quinoprotein glucose dehydrogenase obtained from *E. coli*. Also, apo-PDH from *Gluconobacter* was reconstituted with authentic PQQ and not with FAD or FMN. These data strongly suggests that PDH from *G. oxydans* is a quinoprotein, like the other primary membrane-bound dehydrogenases of *Gluconobacter*. These findings contradict the earlier report of the flavin nature of PDH (58), however, that work was performed before the discovery of PQQ.

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CHAPTER 3

Electron Flow Among the Subunits of the Membrane-Bound Polyol Dehydrogenase of *Gluconobacter oxydans*.

ABSTRACT

Gluconobacter oxydans is a strict aerobe that rapidly performs limited oxidations of polyhydroxyl alcohols (polyols). These oxidations are catalyzed by a constituitively synthesized [NAD(P)-independent] quinoprotein polyol dehydrogenase (PDH) which is located in the cell's plasma membrane and linked to the cells respiratory chain. The PDH complex consists of three subunits: (*i*) a catalytic subunit that oxidizes numerous polyol-compounds; (*ii*) a *c*-type cytochrome; and (*iii*) a protein whose function is not known. This study presents data that suggests that the hydrogens removed from the substrate by the catalytic subunit are passed to Coenzyme Q (CoQ), which subsequently reduces the cytochrome *c* subunit of the PDH complex. This study also demonstrates that CoQ is separated from the PDH complex when membrane fractions are treated with most detergents, resulting in an apparent loss of activity when assayed spectrophotometrically with indicator dyes. To restore enzyme activity, *in vitro*, MPMS was used as a replacement for CoQ, and PDH activity was then detected spectrophotometrically with indicator dyes, such as ferricyanide.

INTRODUCTION

The gluconobacters contain at least three enzymes that oxidize sorbitol. Two of these enzymes are soluble (cytoplasmic) and have pH optima in the neutral range: one soluble enzyme oxidizes sorbitol to sorbose and is NAD-dependent (17); the other soluble enzyme is NADP-dependent and oxidizes sorbitol to fructose (19). The third sorbitol-oxidizing enzyme is a plasma membrane-bound [NAD(P)-independent] quinoprotein that has an acidic pH optimum (12,35,39). It is this membrane-bound enzyme that is being exclusively studied in this investigation.

Polyol oxidation by the gluconobacters is linked to oxygen consumption via the electron-transport chain (9). However, little is known about how this enzyme is linked to the electron-transport system. A polyol dehydrogenase (PDH) was recently isolated in our laboratory (39), and in 1982 and 1990 by Shinagawa et al. (35) and Cho et al. (12), respectively. In all three cases, PDH was shown to contain a cytochrome *c* subunit. The cytochrome *c* subunit of PDH isolated by Cho et al. (12) was reduced by substrate alone. The PDH isolated by Shinagawa *et. al.* (35) was not reduced by the addition of substrate alone, but the cytochrome *c* subunit was reduced in the presence of both substrate and methylphenazonium methosulfate (MPMS).

It is not clear how the PDH complex is linked to the terminal oxidase of the electron transport system in *Gluconobacter.* From studies of redox

potentials, it is expected that the cytochrome *c* subunit of PDH would mediate electron transfer from ubiquinol to the terminal oxidase (14,16). However, the terminal oxidase of the respiratory chain of *Gluconobacter*, an *o*-type cytochrome (10), serves as a ubiquinol oxidase, but not a cytochrome *c* oxidase (6,24). The ubiquinol oxidase activity of the terminal oxidase is inhibited with quinone analogues and cyanide (24,31). Ameyama *et. al.* (6) proposed that the respiratory chain of *Gluconobacter* is branched; containing in, addition to the cyanide sensitive cytochrome *o* terminal oxidase, an unidentified cyanide-insensitive branch, possibly a cytochrome *c* (33) or cytochrome *o* (14). It is possible that the cytochrome *c* subunit of the PDH complex might interact with this unidentified terminal oxidase.

In this communication, evidence is presented which suggests that the catalytic subunit of the PDH complex from *G. oxydans* interacts directly with coenzyme Q (CoQ). The CoQ is then able to reduce the cytochrome *c* subunit of the PDH complex. Also discussed is the need to replace CoQ with the artificial electron mediator MPMS when PDH is removed from the *G. oxydans* plasma membrane fraction with most detergents.

MATERIALS AND METHODS

Organism, growth and cell fractionation. Organism, growth conditions, cell fractionation, and polyol dehydrogenase (PDH) isolation were performed as previously described (39). Briefly, three milliliters of *Gluconobacter oxydans* ATCC strain 621 sorbitol stock culture was inoculated into 2.5 liters of a complex sorbitol medium and incubated at 28°C in a 3 liter fermentor under heavy aeration and agitation. Cells were harvested by centrifugation approximately 1 h into maximum stationary phase, washed, and suspended in buffer. Cell extracts were obtained by ultrasonic disruption, and *membrane fractions* were isolated by ultracentrifugation. Polyol dehydrogenase was solubilized from the membrane fraction with 1.0% (vol/vol) Triton X-100 (Triton) in the presence of 1.5 mM KCl and 1.5 mM sorbitol. The Triton-treated membranes were removed by ultracentrifugation, and the supernatant fluid was called the Triton-*detergent-solubilized membrane protein* (Triton-DSMP) fraction. The *isolated PDH complex* was obtained by anion and cation exchange and hydrophobic interaction chromatographies.

Other detergent treatments. In addition to solubilization of PDH with Triton X-100, other detergents were separately added to membrane fractions at concentrations shown in the Results Section. Each detergent-membrane mixture was stirred for 3 h at 4°C and then ultracentrifuged for 90 min at 120,000 x *g* at 4°C. The resulting supernatant fluids were called the *detergent solubilized*

membrane protein (DSMP) fractions, and the pellets were called the *detergent-treated membrane* fractions.

Assay for PDH activity. Polyol dehydrogenase activity was assayed spectrophotometrically by following the reduction of ferricyanide using a modification of the procedure described by Arcus and Edson (8). The standard assay solution contained 10 mM $MgCl_2$, 5 mM $CaCl_2$, 250 mM sorbitol, 200 µl of buffer (described below), 100 µl of enzyme fraction and artificial electron acceptor(s) as described below for a total volume of 1 ml. When ferricyanide was used alone as an artificial electron acceptor, (*i*) its concentration was 1 mM, (*ii*) 200 mM succinate-NaOH (pH 5.2) was used as the buffer, and (*iii*) and the assay solutions were monitored spectrophotometrically at 420 nm. When ferricyanide and methylphenazonium methosulfate (MPMS) were used together, (*i*) they were used at concentrations of 0.4 mM and 0.6 mM, respectively (*ii*) 10 mM Na-acetate (pH 5.0) was used as buffer, and (*iii*) the assay solution was monitored at 303.4 nm. In each case, the reaction was started by the adding enzyme fraction. All reagents except the enzyme fractions were brought to the assay temperature of 38°C prior to use in the assay. The enzyme fractions were kept on ice. The absorbance change was monitored using a Bausch and Lomb Spectronic 2000 spectrophotometer.

When coenzyme (CoQ) was used, the assay procedure was the same as described for ferricyanide plus MPMS assay, except that the MPMS was replaced with 0.6 mM CoQ.

Protein determination. Samples were digested with 2 N NaOH and 10% sodium dodecyl sulfate (SDS) in a boiling water bath for 30 min and the protein concentrations were determined by a modification (18) of the method of Lowry *et. al.* (23). Bovine serum albumen fraction V was used as the protein standard.

Absorbance spectra. Absorbance spectra of the oxidized and reduced forms of the isolated-PDH complex were measured between 400 and 600 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer using a quartz cuvette. Measurements below 400 nm were not possible due to the high absorbance of the Triton X-100. The spectrophotometer's absorbance was set to zero with the buffer [10 mM Na-acetate buffer, pH 5.0, containing 0.1% (vol/vol) Triton X-100] in which the enzyme samples were suspended and either 0.6 μM MPMS or 0.6 μM CoQ. The isolated PDH complex was oxidized by the addition of H_2O_2 and 0.6 μM MPMS. The isolated PDH complex was reduced by the addition of 250 mM sorbitol and either 0.6 μM MPMS or 0.6 μM CoQ. These samples were then incubated at 38°C for 10 min before absorbencies were measured.

The absorption spectra of MPMS and ferricyanide were determined by separately preparing 0.5 mM solutions in double-distilled deionized water

($dddH_2O$). These solutions were then monitored spectrophotometrically from 250 to 600 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer.

Electrophoresis. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Hames and Rickwood (20) with 1% SDS in the stacking and resolving gel, and in the running buffer. Proteins were passed through a 3.9% stacking gel (pH 6.8) at 35 mA, and separated through a 10% resolving gel (pH 8.8) at 75 mA.

Staining. Gels were stained for proteins by incubation overnight in a solution containing 0.1% (wt/vol) Serva Blue dissolved in (vol/vol) 10% glacial acetic acid, 25% isopropyl alcohol, and 65% $dddH_2O$. Destaining was accomplished by gently agitating gels for 30 min in a solution containing (vol/vol) 10% glacial acetic acid, 25% isopropyl alcohol, and 65% $dddH_2O$.

The catalytic subunit of PDH was visualized after electrophoresis by first equilibrating gels in 200 mM succinate-NaOH buffer, pH 5.0 (three changes of 200 ml buffer at 20 min each), and then immersing the gel in a solution containing 250 μM sorbitol, 0.02% (wt/vol) MPMS, and 0.04% (wt/vol) tetranitroblue tetrazolium in $dddH_2O$. Gels were gently agitated for several hours at 30°C before visualization.

Assay of PDH subunits separated by SDS-PAGE. After electrophoresis of the isolated PDH complex, the areas of the gel containing the subunits of the isolated PDH complex were cut from the gel and assayed for the

ability to reduce CoQ or MPMS by immersion in 3 ml of the ferricyanide assay solution containing either 0.6 mM MPMS or 0.6 mM CoQ. After incubation in a roller drum for 8 h at 28°C, the gel pieces were removed, and the absorbencies of the assay solutions were measured at 303.4 nm. The absorbance of the spectrophotometer was set to zero using an assay solution that was incubated with a gel piece containing no protein.

RESULTS

Detection of PDH activity in the detergent-solubilized membrane protein fractions. A modification of the assay described by Arcus and Edson (8) was employed to monitor polyol dehydrogenase (PDH) activity. In this assay, sorbitol was used as the substrate (electron donor) and ferricyanide was used as an artificial electron acceptor. The PDH activity was detected by spectrophotometrically monitoring the bleaching of ferricyanide at 420 nm. However, attempts to remove (solubilize) PDH from the membrane fraction with most detergents, such as Tween 80, Triton X-100, methylglucoside, Brig 58, or octylthioglucoside, resulted in an apparent loss of >95% of the activity found in the untreated membrane fraction (Fig. 3.1 -- open bars). An exception was the detergent-solubilized membrane protein (DSMP) fraction obtained using the detergent octylglucoside. That fraction retained about 80% of the untreated membrane fraction's PDH activity. No significant enzyme activity was found in the detergent-treated membrane fractions obtained with any of the tested detergent (data not shown).

I considered three possible reasons for the apparent loss of PDH activity following detergent treatment of these membrane fractions. First, the enzyme may be denatured when switched from a lipid environment to an aqueous-detergent environment. Second, the enzyme may undergo a conformational

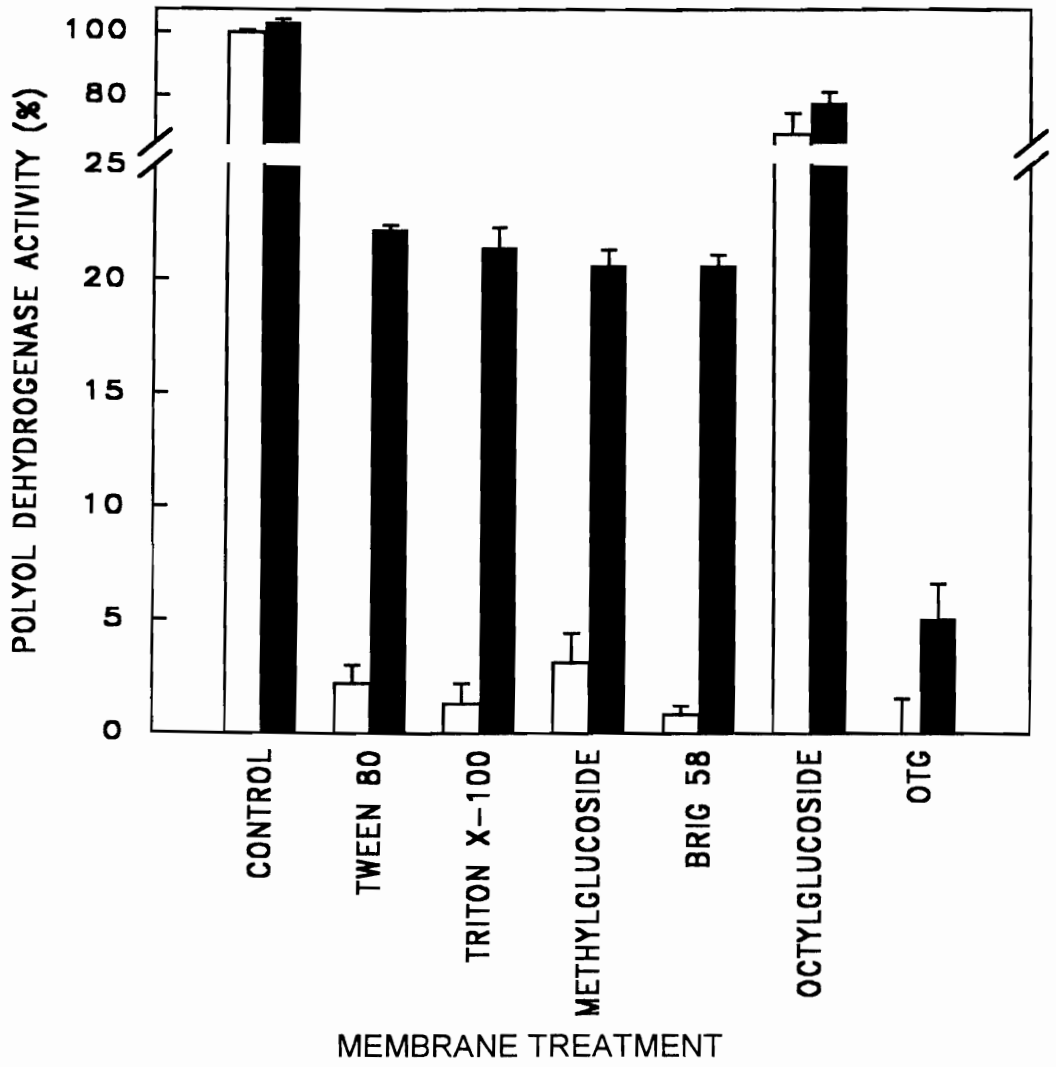

Figure 3.1. Polyol dehydrogenase activity of the DSMP fraction in the presence and absence of MPMS. Membrane fractions were stirred for 3 h in the presence of 1.0% concentrations of the indicated detergents and then ultracentrifuged. The DSMP fractions (supernatant fluid) and the untreated membrane fraction (control) were assayed for PDH activity with either ferricyanide alone (open bars) or with ferricyanide and MPMS (solid bars). The PDH activity of the untreated membrane fraction assayed in the absence of MPMS was considered to have 100% activity. Data represents the average values obtained from three replicates each of membrane fractions (403, 601, 592 μg of protein ml^{-1}) isolated from three separate cell batches. Standard deviations are shown.

change, so that the ferricyanide binding site is no longer available; hence, ferricyanide would not be reduced. Third, ferricyanide may not be reduced directly by PDH, but rather by a subsequent component of the electron transport chain. If this third hypothesis was correct, when the membrane proteins are solubilized with detergent and PDH becomes separated from other electron-transport-chain component, ferricyanide reduction would not occur.

To test the third hypothesis, methylphenazonium methosulfate (MPMS) was added to the assay solution, because MPMS is an artificial electron acceptor that can mediate electron transfer from NAD(P)-dependent enzymes to other artificial electron acceptors (5) and from various reductants to cytochrome *c* (4). Addition of MPMS to the PDH assay solution resulted in an approximate 10-fold increase in PDH activity of most of the DSMP fractions (Fig. 3.1 -- solid bars). The addition of MPMS did not affect PDH activity of the untreated membrane fraction (Fig. 3.1, control), the detergent-treated membrane fractions (data not shown), or the DSMP fraction obtained with the detergent octylglucoside (Fig. 3.1).

MPMS absorbed strongly at 420 nm (see Fig. 3.2), the wavelength conventionally used in ferricyanide assays (8), but I found that ferricyanide reduction could also be monitored at 303.4 nm without significant interference from MPMS (Fig. 3.2). Therefore, 303.4 nm was routinely used in the remainder

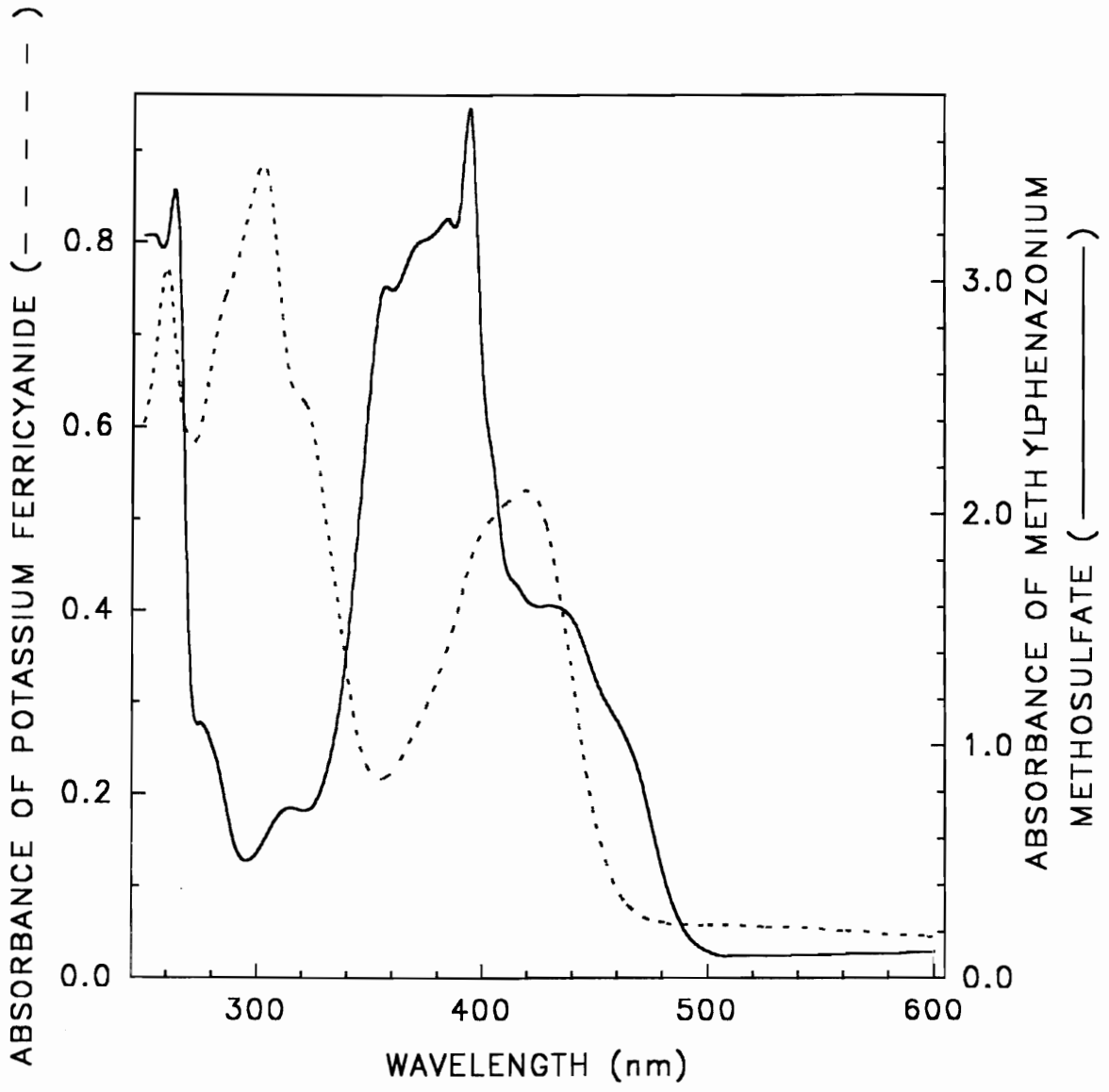

Figure 3.2. Absorbance spectra of ferricyanide and MPMS. Potassium ferricyanide (dashed line -- left axis) and methylphenazonium methosulfate (solid line -- right axis) were dissolved in dddH_2O at 0.5 mM concentrations and their absorbances monitored spectrophotometrically from 250 to 600 nm. Data shown is from a typical experiment.

of this study to monitor ferricyanide reduction (PDH activity) in the presence of MPMS. Ferricyanide has a higher extinction coefficient at 303.4 nm (data not shown), which increased the sensitivity of the ferricyanide-reduction (PDH activity) assay.

To determine which concentrations of ferricyanide and MPMS were needed for optimal detection of PDH activity, the Triton-DSMP fraction was assayed using various concentrations of these two electron acceptors. At all ferricyanide concentrations, the presence of MPMS enhanced detection of PDH activity (Fig. 3.3). From the data shown in Figure 3.3, I selected the concentrations of 0.4 mM ferricyanide and 0.6 mM MPMS for all subsequent assays of PDH activity. At these concentrations, (*i*) high PDH activity was detected, (*ii*) the maximum extent of absorption did not exceed the range of the spectrophotometer, and (*iii*) the enzyme activity could be monitored for several minutes before all the ferricyanide was reduced.

Polyol dehydrogenase-dependent reduction of CoQ. Previous studies demonstrate that glucose dehydrogenase from *Pseudomonas* and *Gluconobacter* interacts with coenzyme Q (CoQ) (26,37) and that CoQ is removed from membranes by treatment with 0.1% Triton (37). To determine if PDH from *Gluconobacter* was using CoQ for electron (hydrogen) transfer, membrane fractions were treated with 0.1% Triton, and the solubilized material was separated from the membrane fraction by ultracentrifugation. The resulting

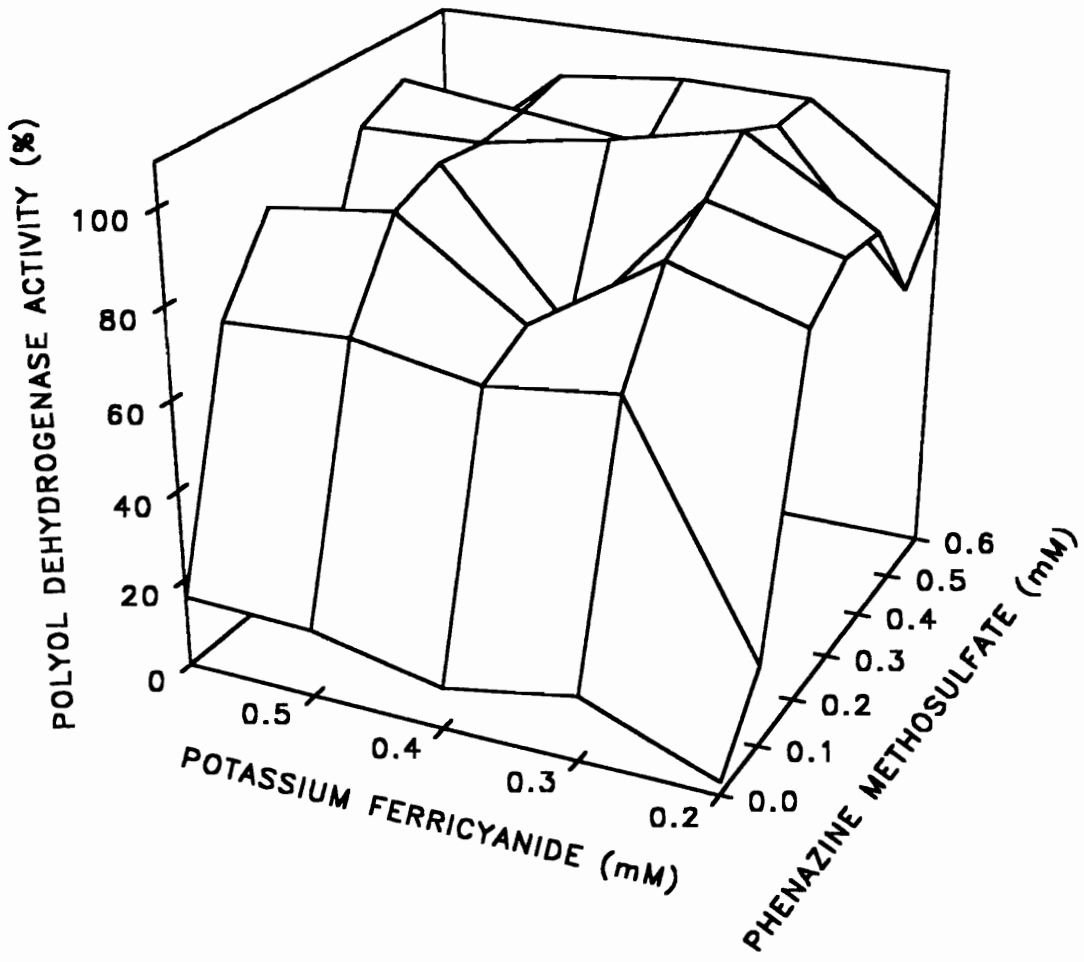

Figure 3.3. Polyol dehydrogenase activity of the Triton-DSMP fraction detected with indicated concentrations of MPMS and ferricyanide. Ferricyanide and MPMS were added to the standard assay solution at the indicated concentrations. The PDH activity of the Triton-DSMP fraction was then assayed for 2 min. Polyol dehydrogenase activity detected with 0.4 mM ferricyanide and 0.6 mM MPMS was considered to be 100% activity. Data represents the average values obtained from three replicates each of Triton-DSMP fractions (381, 232, and 419 μg of protein ml^{-1}) obtained from three separate cell batches.

detergent-treated membrane fractions (resuspended pellets) assayed with ferricyanide alone demonstrated less then 5% of the PDH activity of the untreated membrane fractions for all except the membrane fractions treated with the detergent octylglucoside (Fig. 3.4 -- open bars). When MPMS was added along with ferricyanide, about 55% of the original membrane-fraction activity was demonstrated (Fig. 3.4 -- solid bars). Since PDH activity is greatly reduced when CoQ is removed from the membrane with detergent-treatments, it seemed that CoQ might be involved in the transfer of electrons from PDH to ferricyanide. Perhaps MPMS was substituting for CoQ, *in vitro*, when PDH was solubilized from the membrane.

To test this hypothesis, PDH was removed from the membrane with 1.0% Triton, and this Triton-DSMP fraction was assayed for PDH activity with either ferricyanide alone, ferricyanide and MPMS, or ferricyanide and CoQ. With ferricyanide alone, less than 5% of the untreated membrane fraction activity was observed, but, when assayed with ferricyanide and either MPMS or CoQ, PDH activity was about 20-times higher (Table 3.1 -- row one).

Next, I wanted to determine if CoQ could mediate electron flow directly from the isolated PDH complex (39). When the isolated PDH was used instead of the Triton-DSMP fraction, both MPMS and CoQ independently increased PDH activity about 20-fold over that detected with ferricyanide alone (Table 3.1 -- rows 2 and 3).

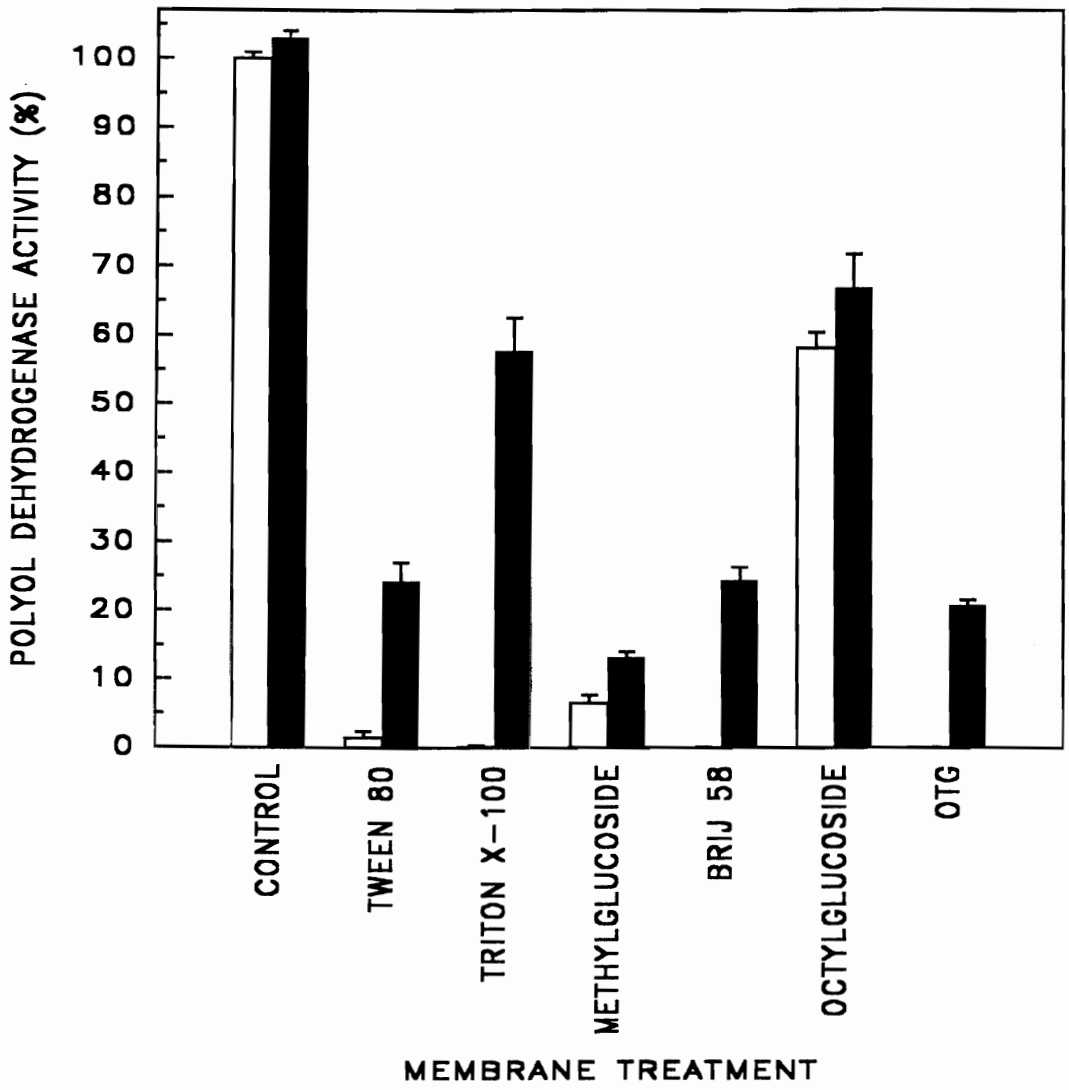

Figure 3.4. Polyol dehydrogenase activity of membranes treated with 0.1% detergent in the presence and absence of MPMS. Membrane fractions were stirred with 0.1% concentrations of indicated detergents for 3 h and then ultracentrifuged. The detergent-treated membrane fractions (pellets resuspended in buffer) and the untreated membrane fraction (control) were assayed for PDH activity with either ferricyanide alone (open bars), or with both ferricyanide and MPMS (solid bars). The PDH activity of the untreated membrane fraction assayed in the absence of MPMS was considered to have 100% activity. Data represents the average values obtained from three replicates each of membrane fractions (403, 601, 592 μg of protein ml^{-1}) isolated from three separate cell batches. Standard deviations are shown.

TABLE 3.1. Detection of polyol dehydrogenase activity with ferricyanide in the presence and absence of MPMS.

Fraction used[c]	Polyol dehydrogenase activity (%)[a]		
	Electron acceptor[b]		
	Ferricyanide	Ferricyanide and MPMS	Ferricyanide and coenzyme Q
Triton-DSMP	4.6 ± 0.4	100 ± 4.7	95.7 ± 2.7
Isolated PDH complex	2.9 ± 0.3	100 ± 2.8	91.0 ± 2.6
Isolated PDH complex	5.5 ± 0.2	100 ± 1.5	93.1 ± 1.5

[a] The PDH activity obtained when assayed with ferricyanide and MPMS was designated as 100%.

[b] Polyol dehydrogenase activity was assayed with 0.4 mM ferricyanide alone, or 0.4 mM ferricyanide and either 0.6 mM MPMS or 0.6 mM CoQ.

[c] Data represents the average values obtained from three experiments, each from a different cell batch. The Triton-DSMP fraction contained 1.456 mg of protein ml^{-1} and the fractions containing the isolated PDH complex contained 44.0 (row two) or 56.6 (row three) ug of protein ml^{-1} . The isolated PDH complex was obtained by ion-exchange and hydrophobic interaction chromatographies of the 1.0% Triton-DSMP fraction (39).

Reduction of CoQ by the catalytic subunit of the PDH complex.

Results shown in Table 3.1 indicated that CoQ and MPMS are directly reduced by the isolated PDH complex. However, PDH is composed of 3 subunits: a catalytic subunit, a cytochrome *c* subunit, and an unidentified subunit (12,35,39). To determine which of these three subunits reduced CoQ and MPMS, the isolated PDH complex was separated into its 3 subunits by SDS-PAGE. Each subunit was then separately tested for their ability to reduce CoQ and MPMS using ferricyanide as an indicator. The cytochrome *c* and the 15 kDa subunits of PDH were not able to reduce CoQ or MPMS (data not shown). However, the catalytic subunit of PDH did reduce CoQ and MPMS, but did not directly reduce ferricyanide (Table 3.2). These experiments support the hypothesis that electrons removed from sorbitol by the catalytic subunit of PDH can be passed directly to either CoQ or MPMS, but the catalytic subunit cannot directly reduce the cytochrome *c* subunit or the 15 kDa subunit of PDH.

Polyol dehydrogenase-dependent reduction of cytochrome *c* via CoQ. Since the catalytic subunit of PDH reduced CoQ, I next wanted to determine if the reduced CoQ would pass its electrons to the cytochrome *c* subunit of the PDH complex. To determine if the cytochrome *c* subunit was being reduced the absorbance of the isolated PDH complex was measured between 400 and 600 nm (Fig. 3.5). The spectrum showing the oxidized form of cytochrome *c* was observed using the isolated PDH complex in the absence of

TABLE 3.2. Reduction of MPMS and CoQ by the catalytic subunit of the isolated polyol dehydrogenase complex.

		Potassium ferricyanide reduction[a] (change in absorbance at 303.4 nm)		
Cell. Batch[b]	Exp. No[a]	Ferricyanide only[c]	Ferricyanide and MPMS[d]	Ferricyanide and coenzyme Q[e]
	1	0.012	0.417	
A	2	0.020	0.623	
	3	0.019	0.312	
	4	0.016		0.289
B	5	0.008		0.196
	6	0.013		0.227

[a] Reduction of CoQ or MPMS was determined by measuring the change in absorbance at 303.4 nm caused by the subsequent reduction of potassium ferricyanide in the assay solution.

[b] The isolated PDH complex (39) was separated into its 3-subunits by electrophoresis. The catalytic subunit was localized using a sorbitol-tetrazolium based activity stain, then cut from the corresponding section of adjacent wells. Experiments 1 through 3 used gel pieces containing isolated PDH complex obtained from one cell batch, while experiments 4 through 6 used gel pieces containing isolated PDH complex from a second cell batch.

[c] Gel pieces containing only the catalytic subunit of the PDH complex were immersed in 3 ml of the assay solution containing the electron acceptor ferricyanide. After incubation in a roller drum for 8 h at 28°C, the gel pieces were removed, and the absorbances of the assay solutions were measured at 303.4 nm. The absorbance of the spectrophotometer was set to zero using an assay solution that was incubated with a gel piece containing no protein.

[d] These experiments were performed as described in footnote c, except that 0.6 mM MPMS was included in the assay solution.

[e] These experiments were performed as described in footnote c, except that 0.6 mM coenzyme Q was included in the assay solution.

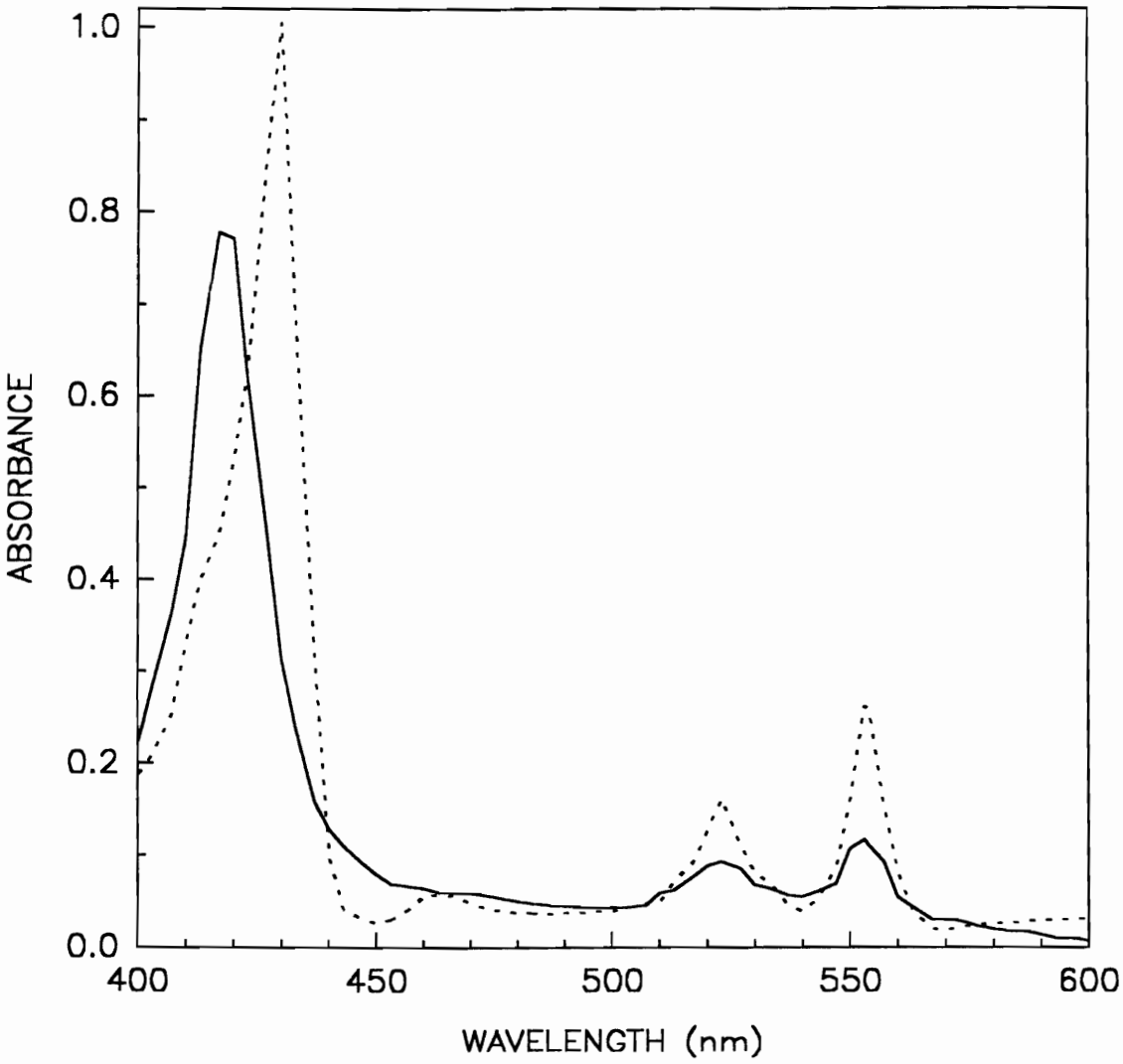

Figure 3.5. Reduction of the cytochrome *c* subunit of the isolated PDH complex with sorbitol in the presence of CoQ. The oxidized absorption spectrum (solid line) was obtained as described in the Materials and Methods. The PDH complex was then treated with 250 mM sorbitol and 0.6 mM CoQ, and its absorption spectrum determined (dashed line). Data shown are from a typical experiment.

sorbitol or other potential electron donors (Fig. 3.5 -- solid line). When sorbitol alone was added to the isolated PDH complex, there was no detectable change in the absorbance spectrum (data not shown). When CoQ and sorbitol were added, the absorption spectrum of the cytochrome *c* subunit shifted to a more reduced form (Fig. 3.5 -- dashed line). This suggests that CoQ passes electrons from the catalytic subunit to the cytochrome *c* subunit of the isolated PDH complex. Similar results were obtained when MPMS was substituted for CoQ (data not shown). Therefore, MPMS seems able to replace CoQ, *in vitro*, as an artificial electron mediator between the catalytic subunit and the cytochrome *c* subunit of the PDH complex.

Once reduced, the cytochrome *c* subunit of the PDH complex was not re-oxidized by air (data not shown). This indicates that the cytochrome *c* subunit of PDH does not serve as a cytochrome oxidase for *G. oxydans*, but instead, the cytochrome *c* subunit of PDH probably passes electrons to one or more additional electron transport chain components that use oxygen as the terminal electron acceptor.

DISCUSSION

The limited oxidation of polyhydroxyl alcohols (polyols) performed by the membrane-bound polyol dehydrogenase (PDH) of *Gluconobacter* has long been known to be linked directly to oxygen consumption via the electron-transport system (9). How polyol oxidations are linked to the electron-transport system was investigated in this study.

Reduction of CoQ by the catalytic subunit of the PDH complex.

Purification of PDH in our laboratory was previously hampered because of an apparent loss of enzyme activity after treating membrane fractions with most detergents and assaying for activity with ferricyanide (Fig. 3.1) (11). Since coenzyme Q (CoQ) is removed from membranes with 0.1% Triton X-100 (37) and PDH was inactivated by such treatment of the membrane fractions (Fig. 3.4), I suspected that PDH may donate the electrons removed from the substrate to CoQ. The data shown in Table 3.1 supports that hypothesis.

Glucose (30,37) and fructose (42) dehydrogenases from *G. oxydans* and glucose dehydrogenase from *Pseudomonas fluorescens* (26) interact directly with CoQ. A proton gradient was formed from *G. oxydans* membrane vesicles containing glucose dehydrogenase, CoQ_{10}, and a terminal oxidase, when glucose was added (29). Several dehydrogenases from *Gluconobacter*, such as fructose dehydrogenase (42), NADH-, glucose-, ethanol-, and glycerol-dehydrogenases (25) have indirectly been shown to interact with CoQ by their

inhibition by HQNO (a CoQ derivative). The interaction of membrane-bound dehydrogenases with CoQ has also been shown for gluconate dehydrogenase from *Pseudomonas aeruginosa* (27).

The interaction of membrane bound dehydrogenases with CoQ seems to be common for membrane-bound dehydrogenases in oxidative bacteria. However, membrane-bound dehydrogenases are usually isolated in complex with cytochrome *c* (1,2,3,12,28,34,35,36,39,41), and it has not been shown whether it is the catalytic subunit or the cytochrome *c* subunit that interacts with CoQ. My study suggests that it is the catalytic subunit of PDH, not the cytochrome *c*, that reduces the CoQ (Table 3.2).

Polyol dehydrogenase-dependent reduction of cytochrome *c* via CoQ. I wanted to determine if the electrons passed from the catalytic subunit to CoQ could subsequently reduce the cytochrome *c* subunit of the PDH complex. Figure 3.5 shows that cytochrome *c* reduction does not occur in the presence of substrate alone, but its reduction is dependent upon addition of CoQ.

Several dehydrogenases from *Gluconobacter* [i.e., gluconate (36), alcohol (2), aldehyde (3), fructose (42) and sorbitol (12,35,39) dehydrogenases] and other bacteria [i.e., gluconate dehydrogenase from *Pseudomonas aeruginosa*, *P. fluorescens* (28), *Serratia marcescens* (34); and alcohol dehydrogenase from *Acetobacter aceti* (1)] have a *c*-type cytochrome subunit. The cytochrome *c* subunit of the above dehydrogenases had similar absorption spectra, with major

absorption peaks at 417-418 nm, 522-523 nm, and 551-554 nm. Cytochrome *c* was purified by Matsushita *et. al.* (32) and cloned by Takeda and Shimizu (38) from *G. oxydans* IFO strain 12528 (= ATCC 621). It was of similar size (48 kDa) and absorption spectra (418, 522, and 553 nm) (24) as the cytochrome *c* subunit of the isolated PDH complex used in this study (39). The cytochrome was reduced with $CoQH_2$ (~50%) and ascorbate-MPMS (~90%) (32). Given the similarities in size, absorption spectrum, and the ability to oxidize reduced forms of CoQ and MPMS, it is likely that the cytochrome studied by Matsushita et. al. (32) is the same type of cytochrome *c* as found in the isolated PDH complex examined in this study.

Detection of PDH activity after its removal from the membrane fraction. After removal from the membrane with most detergents, ferricyanide was no longer reduced by PDH (Fig. 3.1). Table 3.2 shows that ferricyanide does not accept electrons directly from catalytic subunit of the PDH complex, but that electron transfer from the catalytic subunit to ferricyanide can be mediated by MPMS. When Baker and Claus (11) solubilized PDH from the membrane fraction with the detergent octylglucoside, the DSMP fraction retained PDH activity when assayed with ferricyanide alone (also see Fig. 3.1). It may be that octylglucoside more removes the PDH from the plasma membrane in association with CoQ. The CoQ would then be available to reduce the cytochrome *c* subunit of the PDH complex, which could subsequently reduce ferricyanide.

Electron flow through the subunits of *G. oxydans* PDH (Fig. 3.6). The data presented in this study suggests the following model for electron flow from PDH to the electron transport chain during the oxidation of polyols. It appears that electrons (hydrogens) removed from polyols by the catalytic subunit of the PDH complex are passed to CoQ, which subsequently reduces the cytochrome *c* subunit of the PDH complex (Fig. 3.6). The cytochrome *c* subunit presumably donates its electrons to other components of the electron transport chain, which will eventually reduce oxygen to terminate the chain.

It is not clear how the PDH complex is linked to the terminal oxidase of the electron transport system in *Gluconobacter*. The terminal oxidase of the respiratory chain of *Gluconobacter* serves as a ubiquinol oxidase, but not a cytochrome *c* oxidase (6,24). However, Ameyama *et. al.* (6) proposed that the respiratory chain of *Gluconobacter* is branched. It is possible that the cytochrome *c* subunit of the PDH complex might interact with this second terminal oxidase.

Function of Polyol Oxidations in *Gluconobacter*. King and Cheldelin (22) showed that the energy derived by the oxidations catalyzed by these membrane bound dehydrogenases is all that is needed for growth. On the other hand, others demonstrated an increased rate of oxidations at the end of growth (7,13,15,40,41) and thus has lead Heefner (21) to postulate an additional role for the electron transport system in the gluconobacters. Heefner found that

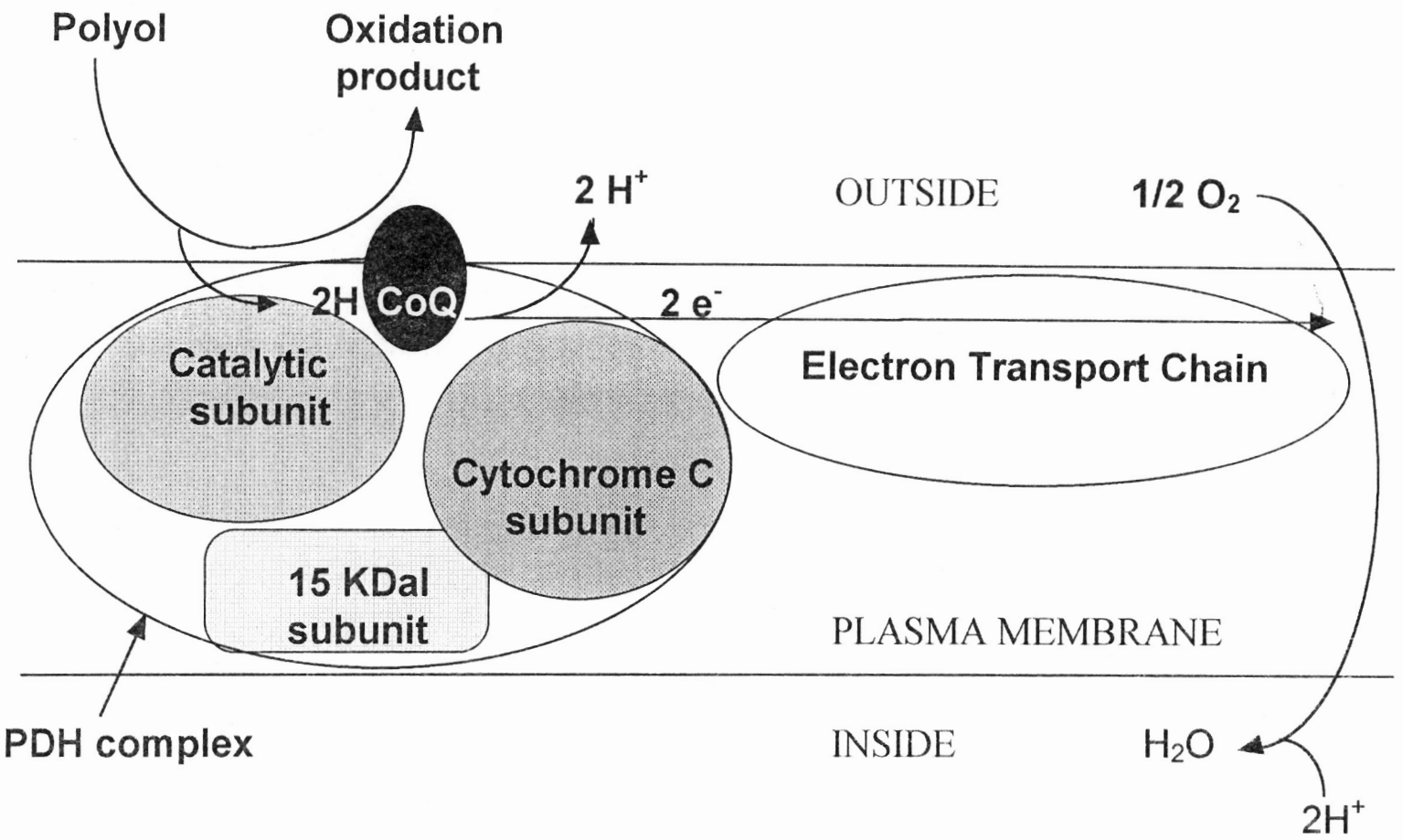

Figure 3.6. Model of electron flow among the subunits of the membrane-bound [NAD(P)-independent] polyol dehydrogenase of *Gluconobacter oxydans*. The PDH complex consists of three subunits (shown shaded in this model): (*i*) the catalytic subunit that oxidizes numerous compounds; (*ii*) a *c*-type cytochrome; and (*iii*) a protein whose function is not known. Hydrogens removed from the substrate by the catalytic subunit are passed to coenzyme Q, which subsequently reduces cytochrome *c*. The electron transport chain from cytochrome *d* has been described elsewhere (33), and the terminal electron acceptor is oxygen.

G. oxydans cells quickly die in pH 3.2 buffer in the absence of polyol, but these cells survive if a polyol is present. He showed that cell death occurred after a rapid influx of hydrogen ions. This influx was accompanied by a denaturation of intracellular proteins, leading to cell death. Heefner, therefore, hypothesized that one function of gluconobacter's PDH is to remove electrons from the polyol and pass them to the electron transport system, which then used the electron's energy to pump protons out of the cell, preventing cell death by acid denaturation.

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APPENDIX 1

Microtiter-Plate Assay for Rapid Detection of Membrane-Bound [NAD(P)-Independent] Dehydrogenase Activity from *Gluconobacter oxydans*.

Bacteria of the genus *Gluconobacter* are well known for their rapid limited oxidations of a large number of different hydroxyl-containing compounds (2,3). These oxidations are catalyzed by plasma membrane-bound [NAD(P)-independent] dehydrogenases (8). The genus *Gluconobacter* is industrially significant, since (*i*) many of its oxidation products are industrially useful (4,6,10,13,14); (*ii*) they perform these oxidations very rapidly (2,3,8); and (*iii*) the oxidation products accumulate in stoichiometric amounts in the spent growth medium (2).

The enzymatic activity of these membrane-bound dehydrogenases (MBDHs) can be assayed by several different methods, such as by oxygen consumption (7,11,15), product formation (5), or substrate disappearance (9). However, these assays are time consuming and may require large amounts of enzyme sample.

More common methods for detection of MBDHs from *Gluconobacter* involve the use of artificial electron acceptors, such as potassium ferricyanide, dichlorophenol indophenol, or tetrazolium salts (1,3,16). These artificial electron acceptors undergo absorbance changes when reduced by electrons (or

hydrogens) removed from the substrate by the MBDHs. These absorbance changes are monitored spectrophotometrically.

In my studies on the role of MBDHs in the metabolism of *Gluconobacter*, I needed a rapid method for detecting MBDH activity. Such an assay could be used for (*i*) screening large numbers of bacterial isolates for their ability to oxidize a particular substrate, (*ii*) checking a particular strain's ability to oxidize many different substrates, and (*iii*) determining optimal conditions for substrate oxidation. This communication describes a rapid, semi-quantitative microtiter-plate assay for detecting of MBDH activity from membrane and detergent-solubilized membrane protein fractions from *Gluconobacter*.

Enzyme fractions were obtained from *Gluconobacter oxydans* ATCC strain 621 as previously described (12). Briefly, *Gluconobacter* was grown to early stationary phase in a complex sorbitol medium. Washed cells were broken by sonic oscillation and membrane fractions isolated by ultracentrifugation. The membrane fractions were solubilized with Triton X-100 to obtain the detergent-solubilized membrane-protein fraction (DSMP) fractions.

The microtiter-plate assay solution used for detecting MBDH activity of the DSMP fractions, contained the following: 0.6 mM methylphenazonium methosulfate (MPMS); 0.4 mM potassium ferricyanide (PFC); 250 mM sorbitol; 5 mM $CaCl_2$; and 10 mM $MgCl_2$; all dissolved in 200 mM succinate-NaOH buffer at pH 5.2. For detection of MBDH activity of the membrane fraction, the

microtiter-plate assay solution was similar to above, except that MPMS was excluded and the PFC concentration was raised to 1 mM.

Assays were started with the addition of 7μl of enzyme fraction and 35 μl of the appropriate microtiter-plate assay solution to the wells of a microtiter-plate. These were then incubated at 28°C for 10 min. After incubation, each well received 63 μl of a ferric sulfate-Dupanol reagent (8) (5 g of ferric sulfate, 3 g of SDS, and 95 ml of 85% phosphoric acid in one liter of ultrapure water) was added. Twenty minutes after addition of the ferric sulfate-Dupanol reagent, 252 μl of ultrapure water was added to each well. The color intensity was then measured at 600 nm with a Dynatechs MR 350 microtiter-plate reader. The absorbance of the microtiter-plate reader was set to zero with the number one well which contained the assay solution plus 7 μl of water instead of enzyme sample, and otherwise treated as described above.

The theory behind the color development is as follows: The MBDHs remove electrons (hydrogens) from the substrate, which are then passed either directly to PFC, if the membrane fraction is used, or to MPMS which subsequently reduces PFC, if the DSMP fraction was used (12). After the reduction of PFC, the SDS and phosphoric acid in the ferric sulfate-Dupanol reagent stops the reaction by denaturing the proteins of the enzyme fraction. The cupric sulfate, in the ferric sulfate-Dupanol reagent, reacts with the reduced

potassium ferrocyanide to form a Prussian blue color. The color intensity must then be diluted to a measurable range by the addition of water.

To determine if this microtiter-plate assay can detect oxidation of different chemical substrates, I tested the DSMP fraction for MBDH activity using substrates from five different chemical classes. Enzymatic activity was detected in the presence of (but not the absence of) each substrate (Fig. A1.1). Enzyme activities detected with the microtiter-plate assay were comparable to those obtained with an established (1) PFC spectrophotometric assay using much less volume (Fig. A1.1)

To determine that color development was indeed due to enzyme activity several controls were performed. Wells containing water instead of enzyme samples were examined and found to exhibit less than 2% of the color development which occurred in the presence of enzyme (Fig. A1.2). Wells containing no added substrate also resulted in minimal color development (Fig. A1.2). To determine that color development was not due to a non-enzymatic catalyst in the enzyme samples, samples were denatured by boiling prior to addition to the microtiter-plate assay solution. Boiled enzyme samples also resulted in minimal color development compared to wells containing non-denatured enzyme fractions (Fig. A1.2). This same minimal color development was also detected if the terminal electron acceptor (PFC) was not included in the assay solution (Fig. A1.2).

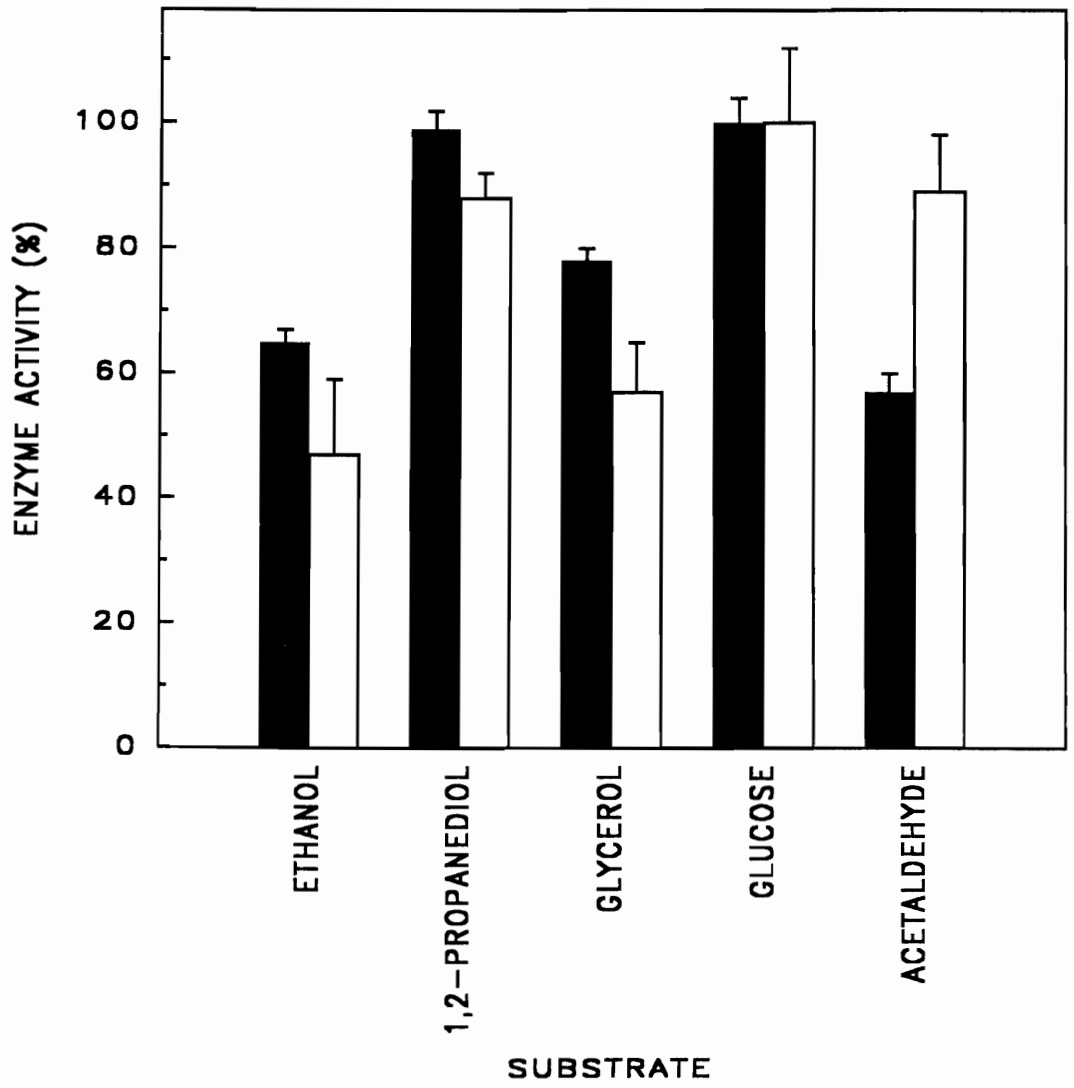

Figure A1.1. Comparison of enzymatic activity detected by the microtiter-plate assay (open bars) and ferricyanide spectrophotometric assay (solid bars). A membrane fraction was assayed for enzymatic activity with the indicated substrates, without MPMS, using both assays. Enzyme activity detected using glucose as the substrate was arbitrarily designated as 100% for each assay. The height of each bar represents the average value obtained from 3 replicates using the same membrane fraction (41 μg of protein ml^{-1}). Standard deviations are shown.

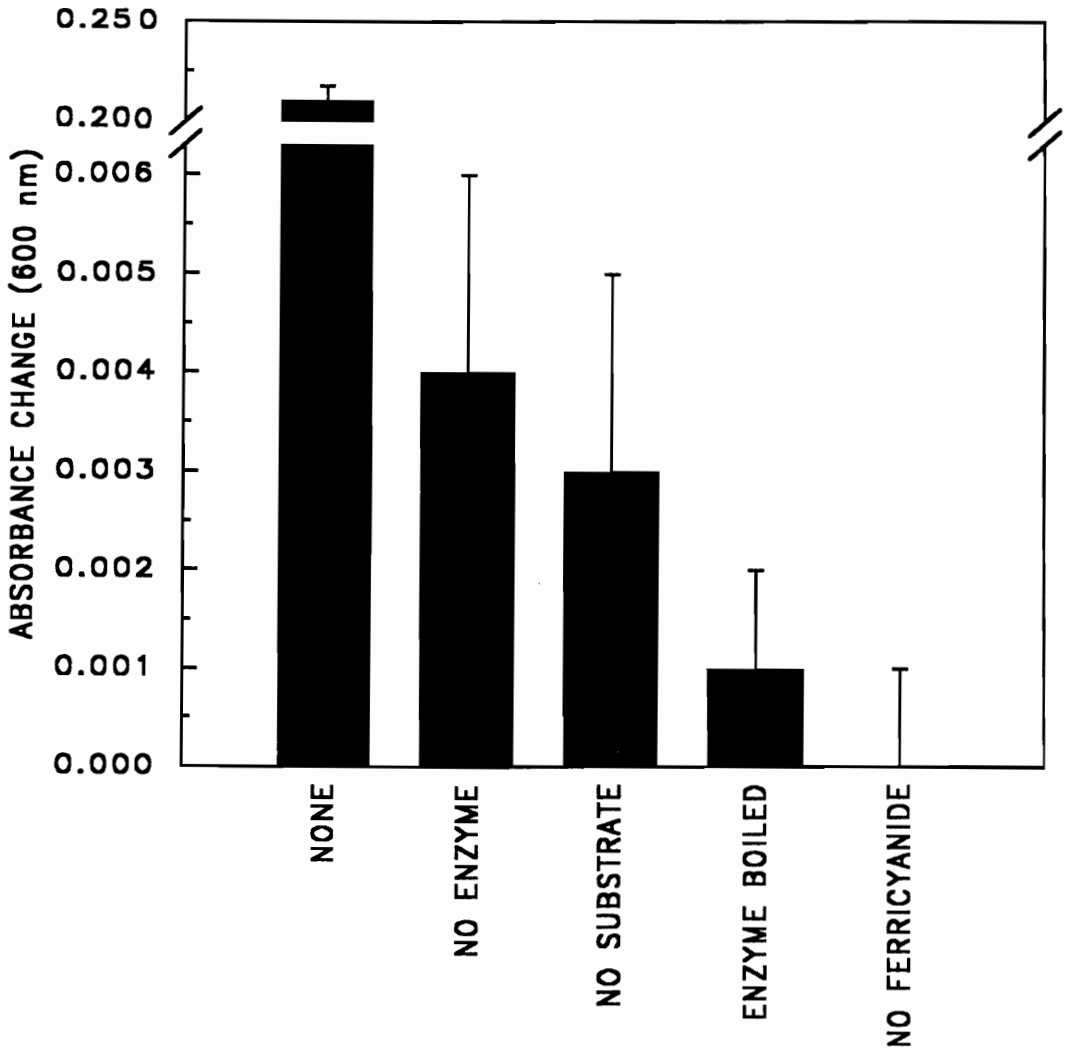

Figure A1.2. Microtiter-plate assay controls. The column on the left ("none") shows the change in absorption caused by 7 μl of the DSMP fraction when assayed with the microtiter-plate assay. The remaining columns represent the following variations from the standard microtiter-plate assay procedure: "No enzyme" – water was added to the assay solution instead of the DSMP fraction; "No substrate" – water was added to the assay solution in place of the substrate sorbitol; "Enzyme boiled" – the DSMP fraction was incubated in a boiling water bath for 20 min prior to addition of the microtiter-plate assay solution; and "No ferricyanide" -- water was added to the microtiter-plate assay solution instead of the electron acceptor PFC. The height of each bar represents the average value obtained from 3 replicates using the same DSMP fraction (28 μg of protein ml^{-1}). Standard deviations are shown.

Color development in the microtiter-plate assay solutions were proportional to the time of incubation with the enzyme fraction (Fig. A1.3) and also to the amount of enzyme fraction added (Fig. A1.4).

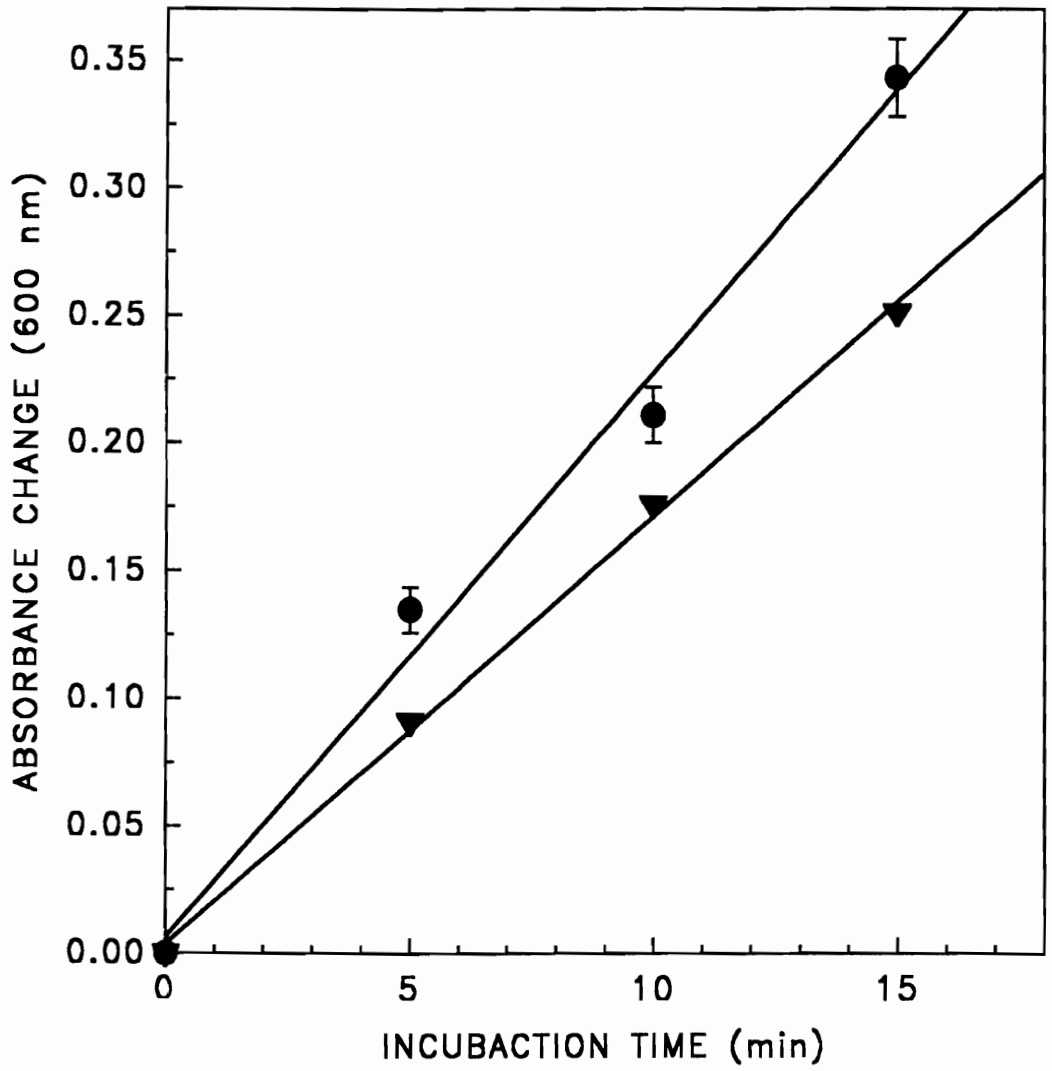

Figure A1.3. Correlation of color development with incubation time of microtiter-plate assay. The membrane fraction (▼) or the DSMP fraction (●) was added to the standard microtiter-plate assay solution and incubated at 28°C for the indicated times. Samples were otherwise treated as described in the text. Color development was measured at 600 nm. Each data point represents the average value obtained from 3 replicates using the same membrane fraction (41 μg of protein ml^{-1}) or the DSMP fraction (28μg of protein ml^{-1}). Standard deviations are shown for values lying outside the data point.

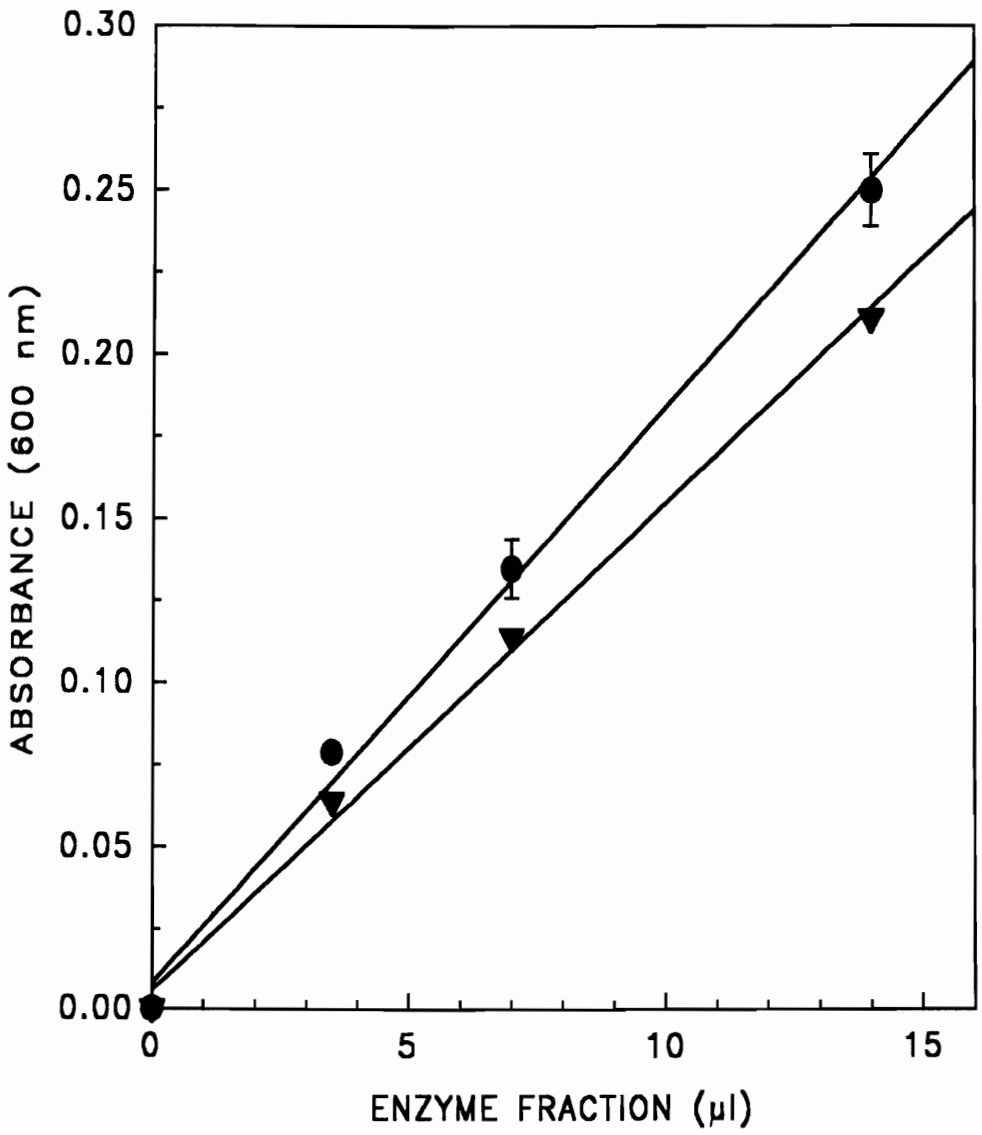

Figure A1.4. Correlation of color development with volume of enzyme fraction in the microtiter-plate assay. The indicated volumes of the membrane fraction (▼) or DSMP fraction (●) were separately added to the standard assay solution. Samples were then otherwise treated as described in the text. Color development was measured at 600 nm. Each data point represents the average value obtained from 3 replicates using the same membrane fraction (41 µg of protein ml^{-1}) or the DSMP fraction (28 µg of protein ml^{-1}). Standard deviations are shown for values lying outside the data point.

This Appendix describes a rapid, semi-quantitative assay for detecting membrane-bound dehydrogenase activity. The advantages of this assay are: (*i*) miniaturization so that 96 assays can be accomplished at one time;,(*ii*) completion of 96 assays with in one hour; (*iii*) many different substrates or enzyme fractions many be tested with one microtiter plate; (*iv*) only small amounts of enzyme fractions are needed; and (*v*) the assay reagents are stable for several months.

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APPENDIX 2

NAD or NADP-Dependent Oxidations Accomplished by the Cytoplasmic Fraction of *Gluconobacter oxydans*.

Gluconobacter constituitively synthesize membrane-bound dehydrogenases that oxidize over 100 different hydroxyl-containing compounds. These membrane-bound dehydrogenases have been extensively studied, however little is known about the diversity of oxidations accomplished by the cytoplasmic fraction. In this study, the cytoplasmic fraction isolated and described below was tested for its ability to oxidize 48 different substrates from nine different chemical classes.

Gluconobacter oxydans ATCC strain 621 was grown to early exponential phase in a complex sorbitol medium as described in Chapter 1. Cells were harvested, suspended in 50 mM Tris-succinate buffer (pH 8.0) and broken by ultrasonic disruption. The suspension was then ultracentrifuged (120,000 x *g* for 90 min) to remove unbroken cells and cell envelope fractions. The supernatant fluid was called the cytoplasmic fraction. No NAD(P)-independent dehydrogenase activity was detected in the cytoplasmic fraction (data not shown).

The cytoplasmic fraction was assayed for NAD and NADP-dependent oxidations against 48 different substrates. The assay solution contained 100 µl

of either 10 mM NAD or 10 mM NADP, 250 μl of 1 M substrate, and 550 μl of 50 mM Tris-succinate buffer (pH 8.0). Enzyme reactions were started by adding 100 μl of the cytoplasmic fraction. The cytoplasmic fraction prepared for the first experiment had 34.9 mg of protein ml^{-1} and that prepared for the second experiment had 20.5 mg of protein ml^{-1} . Change in absorbance due to NAD(P) reduction was monitored at 340 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer.

The cytoplasmic fraction oxidized 38 of the 48 substrates tested with NAD-dependent enzymes (Table A2.1). NADP-dependent oxidations occurred with 30 of the 48 substrates tested. Each chemical class except one contained substrates oxidized by NAD and NADP dependent enzymes. No oxidation was detected using one trisaccharide. Overall, the NAD-dependent enzymes performed these oxidations at a higher rate than the NADP-dependent enzymes, or NAD was the preferred electron acceptor for the cytosolic dehydrogenases.

One aspect of *Gluconobacter* metabolism that these experiments did not address was the direction of the reactions catalyzed by the cytosolic enzymes in vivo. It is possible that at physiological concentrations of substrate in the cell's cytoplasm, these enzymes may carry out the reverse reactions. In other words, it is not known if the enzymes detected in this study serve as reductases or oxidases.

TABLE A2.1. NAD or NADP-dependent oxidations performed by the cytoplasmic fraction of *Gluconobacter oxydans* ATCC strain 621.

Substrate	Specific Activity (mkat kg protein^{-1})			
	Experiment 1		Experiment 2	
	NAD	NADP	NAD	NADP
Aliphatic Monoalcohols				
1-Butanol	13,625 ± 1442	473 ± 60	22,602 ± 2010	938 ± 90
2-Butanol	12,978 ± 433	500 ± 25	19,918 ± 82	912 ± 42
Ethanol	10,595 ± 822	58 ± 5	16,503 ± 518	243 ± 12
1-Hexanol	9,517 ± 0	12,023 ± 60[a]	1,158 ± 12	14,057 ± 87[a]
Methanol	217 ± 17	nd[b]	nd	nd
2-Methyl-1-propanol	10 ± 2	22 ± 2	15 ± 0	7 ± 0
3-Methyl-1-butanol	423 ± 32	257 ± 28	514 ± 28	581 ± 17
2-Propanol	5,137 ± 523	nd	5,772 ± 0	nd
Aliphatic Dialcohols				
1,3-Butanediol	6,013 ± 31[c]	nd	nd	nd
1,2-Ethanediol	597 ± 20	tr[d]	766 ± 13	tr
1,6-Hexanediol	4,750 ± 72	127 ± 2	5,839 ± 41	198 ± 2
1,2-Propanediol	1,257 ± 13	4 ± 1[c]	1,418 ± 23	4 ± 2[c]
Aliphatic Polyalcohols				
Adonitol	579 ± 13[a]	nd	613 ± 41[a]	nd
L(-)Arabitol	1,300 ± 8	321 ± 48[d]	277 ± 40	432 ± 53
Glycerol	nd	nd	nd	nd
D-Mannitol	3,033 ± 25	9,027 ± 192	3,903 ± 81	11,627 ± 163
D-Sorbitol	5,110 ± 48	5,045 ± 100	6,383 ± 42	6,477 ± 77
Trihydroxyhexane	472 ± 22	10 ± 2[g]	652 ± 21	nd
Xylitol	3,252 ± 70	-[f]	5,273 ± 43	-[f]

TABLE 1. cont.

Substrate	Specific Activity (mkat kg protein^{-1})			
	Experiment 1		Experiment 2	
	NAD	NADP	NAD	NADP
Aldehydes				
Acetaldehyde	5 ± 0	nd	8 ± 0	nd
Formaldehyde	252 ± 5	220 ± 12	423 ± 5	374 ± 21
Glutaraldehyde	67 ± 12[c]	12 ± 2[c]	74 ± 14[c]	8 ± 2[c]
Isobutyraldehyde	tr	tr	tr	tr
Propionaldehyde	133 ± 23	117 ± 12	58 ± 18	480 ± 35
Cyclic Alcohols				
Benzyl alcohol	62 ± 27	115 ± 0.0	332 ± 93	353 ± 28
Cycloheptanol	5,013 ± 68	253 ± 13	4,793 ± 31	418 ± 12
Cyclohexanol	1,153 ± 45	215 ± 10	1,302 ± 62	388 ± 20
Cyclohexanone	nd	nd	nd	nd
Cyclooctanol	1,465 ± 60	347 ± 50	1,667 ± 42	618 ± 17
Cyclopentanol	1,962 ± 25	833 ± 25	1,653 ± 38	1,541 ± 87
Fucose	13 ± 3[g]	nd	21 ± 5[g]	nd
meso-Inositol	24 ± 7[h]	nd	31 ± 4[h]	nd
Carboxylic Acids				
Sodium Acetate	35 ± 2	nd	10 ± 2	nd
Ethyl-acetate	362 ± 8	27 ± 0	288 ± 8	15 ± 3
Formic acid	148 ± 13	178 ± 35	187 ± 17	203 ± 25
Gluconic acid	428 ± 22	1370 ± 95	312 ± 40	2035 ± 147
Lactic acid	78 ± 5	47 ± 3	112 ± 12	71 ± 5

TABLE 1. cont.

Substrate	Specific Activity (mkat kg protein^{-1})			
	Experiment 1		Experiment 2	
	NAD	NADP	NAD	NADP
Monosaccharides				
L(+)Arabinose	73 ± 8[e]	nd	66 ± 4[e]	60 ± 3
D(-)Arabinose	41.6 ± 5	ul	72.3	1,208 ± 82
Erythrolose	nd	nd	nd	nd
Glucose	122 ± 7	8,103 ± 410	411 ± 23	10,813 ± 230
Mannose	285 ± 92	9,242 ± 120	180 ± 33	11,328 ± 812
Ribose	nd	nd	nd	nd
Sorbose	nd	nd	nd	nd
Xylose	841 ± 13	943 ± 47	744 ± 12	1500 ± 77
Dissacharides				
Maltose	4123 ± 72	1147 ± 10	6857 ± 167	1843.2 ± 102
Sucrose	nd	nd	nd	nd
Trisaccharides				
Raffinose	nd	nd	nd	nd

[a]Enzymatic activity was unstable. There was an approximate 25% reduction in activity each minute. Specific activity was calculated from the first minute.

[b]nd = No detection of enzyme activity.

[c]Enzymatic activity was unstable. There was an approximate 75% reduction in activity each minute. Specific activity was calculated from the first minute.

[d]tr=trace. Under 1 mkat kg protein^{-1}.

[e]Enzymatic activity was unstable. There was an approximate 40% reduction in activity each minute. Specific activity was calculated from the first minute.

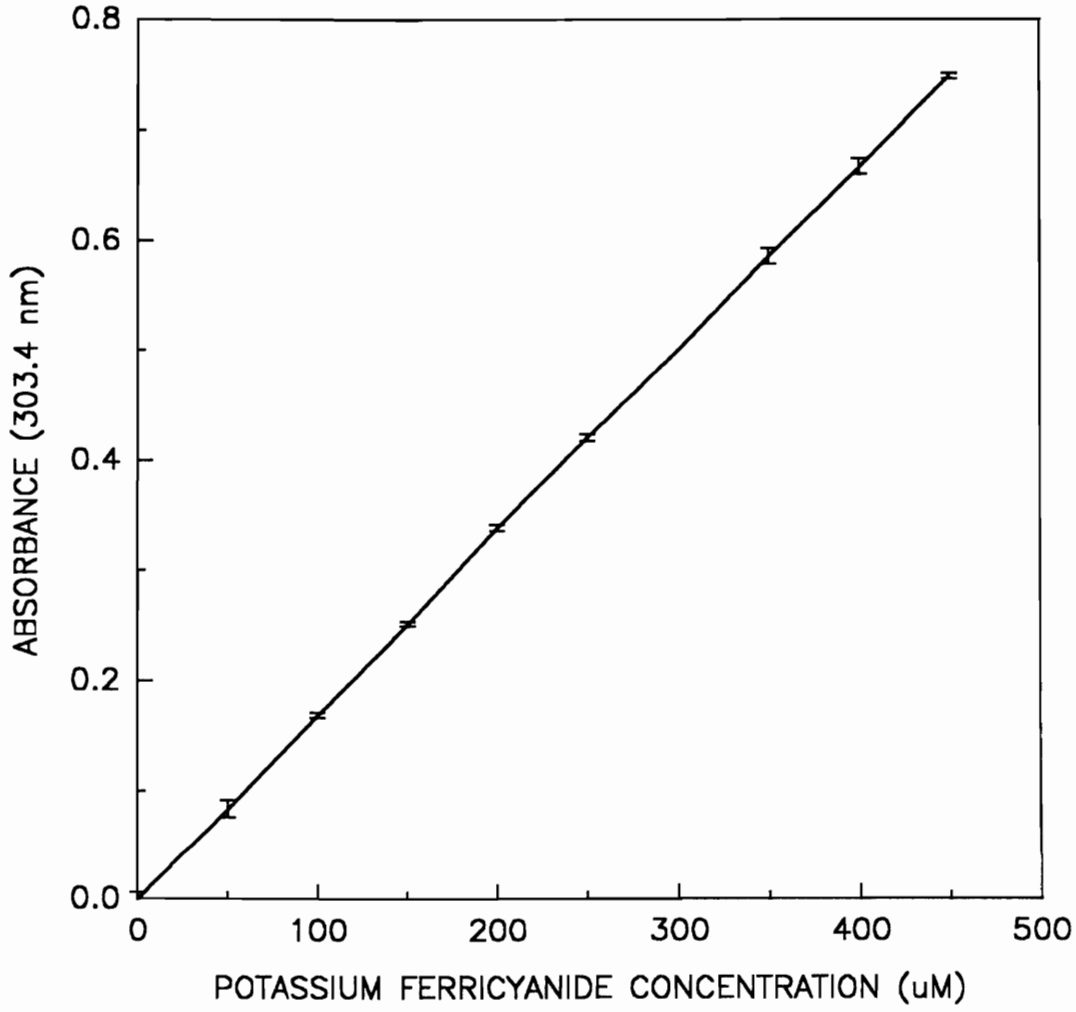
[f]Substrate was not tested.

[g]Enzymatic activity was unstable and decreased to zero with in one minute. Specific activity was calculated from the first minute.

[h]Enzymatic activity was unstable and decreased to zero in 3 min. Specific activity was calculated from the first minute.

APPENDIX 3

Absorbencies of Known Concentrations of Potassium Ferricyanide.

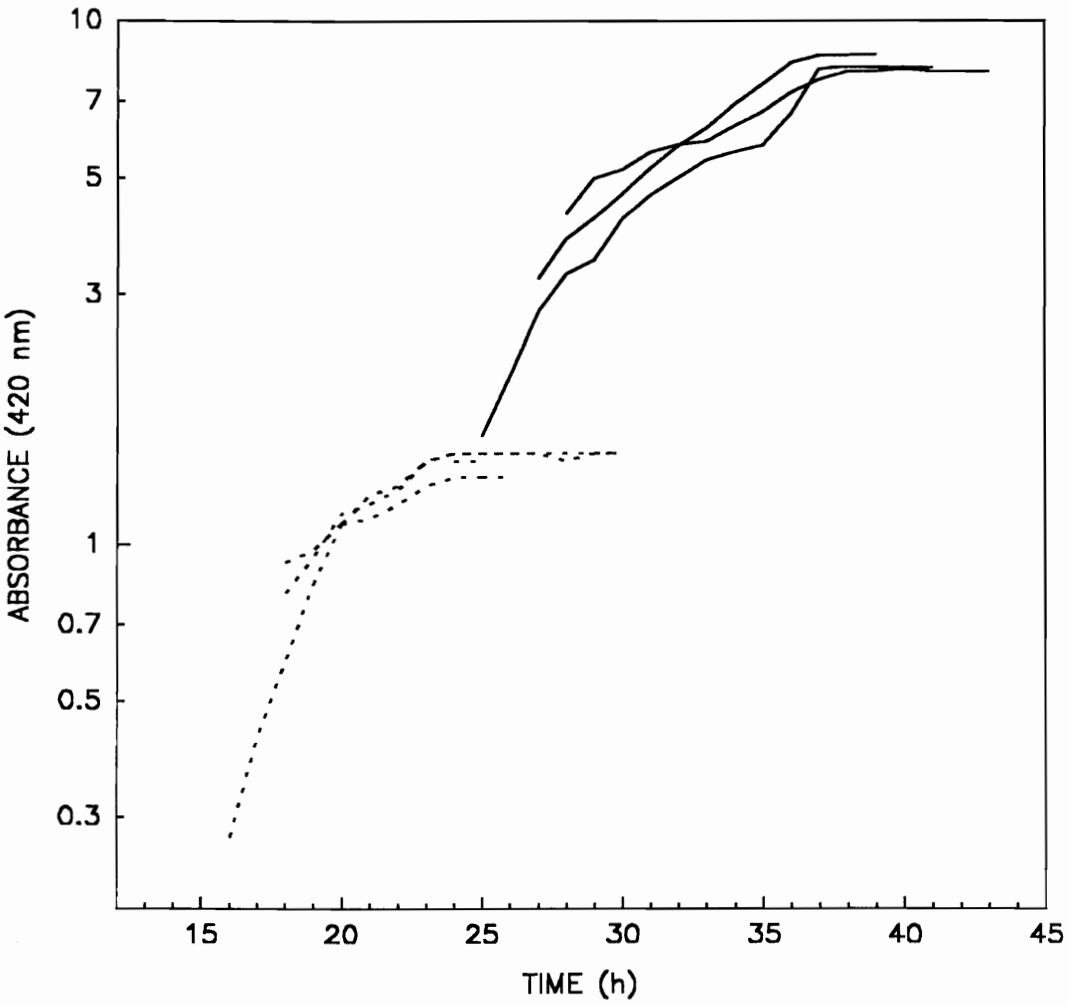


Potassium ferricyanide was dissolved in dddH_2O at the indicated concentrations. Absorbance was measured at 303.4 nm with a Milton Roy Spectronic 1201 spectrophotometer. Data represent the average of 3 replicates from separately prepared solutions. Standard deviations are shown. This curve was used to determine that the extinction coefficient for potassium ferricyanide was 1670.

Potassium ferricyanide standard curve.

APPENDIX 4

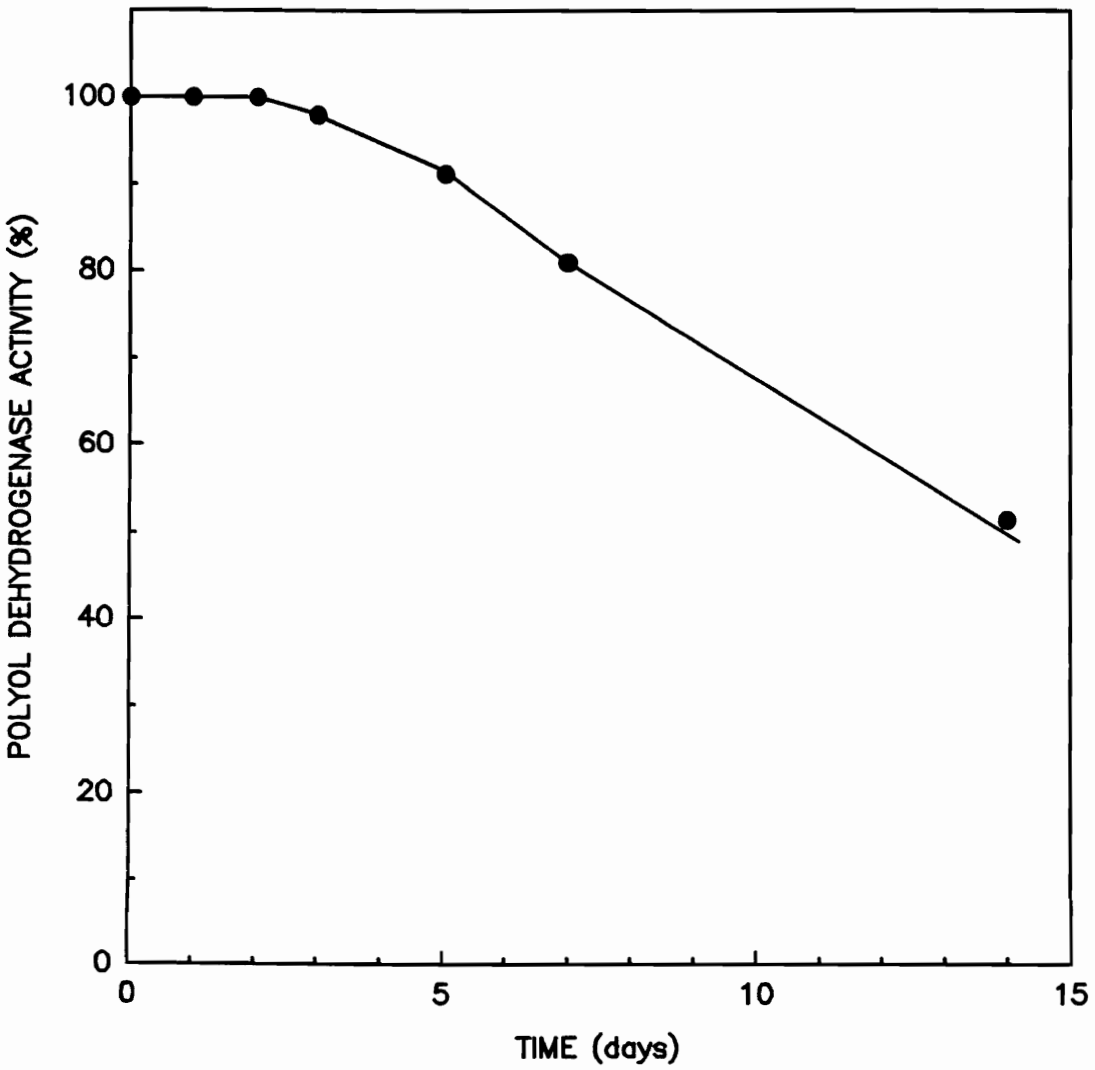
Growth of *Gluconobacter oxydans* in a Complex Sorbitol Medium.



A three milliliter inoculum of sorbitol stock solution of *Gluconobacter oxydans* ATCC strain 621 was inoculated into 2.5 liters of a medium containing 5% (wt/vol) sorbitol (Sigma), 1% (wt/vol) yeast extract (DIFCO), 1% (wt/vol) peptone (DIFCO), and 3.0 ml (vol/vol) of GE60 antifoam (General electric). Cultures were incubated at 28°C with agitation set at 400 rpm with an air flow rate of 3.5 liter min^{-1}. The dotted lines represent three separate experiments in which the absorbance was monitored at 420 nm with a Bausch and Lomb Spectronic 2001 spectrophotometer. Solid lines represent three separate experiments in which samples were diluted 10-fold and the diluted absorbance values multiplied by 10.

APPENDIX 5

Stability of Polyol Dehydrogenase Activity on Ice.



The Triton-DSMP fraction was obtained by Triton-treatment of the membrane fraction as described in Chapter 1. Proteins of the Triton-DSMP fraction were precipitated with 18% PEG and resuspended in 10 mM Na-acetate buffer (pH 5.0) containing 0.01% (vol/vol) Triton X-100. This sample was held on ice and PDH activity measured spectrophotometrically on days 1, 2, 3, 5, 7, and 14. Data points represent the averages of three replicated from a single experiment.

VITA – IAN VANLARE

PERSONAL

Birth: 18 October 1965, Albany, New York

Home: *Address*
702 Westchester Drive, Apt. O
High Point, North Carolina, 27262
(910) 886-3145

Office: Department of Biology
High Point University
High Point, North Carolina 27262-3598
ivanlare@acme.highpoint.edu

EDUCATION

1989 - 1997 Virginia Polytechnic Institute and State University
Blacksburg, Virginia
Ph.D. program

1983 - 1987 Virginia Intermont College
Bristol, Virginia
Bachelor of Arts degree in Biology

POSITION

1993 - present Assistant Professor of Biology
High Point University
High Point, NC

TEACHING ACTIVITIES

Costa Rica Field Biology, Molecular Biology Lecture, Molecular Biology Laboratory, Immunology, General Biology Lecture--A Human Perspective, General Biology Laboratory--A Human Perspective, General Biology Lecture: Principles, General Biology Laboratory--Principles, General Biology Lecture: Organisms, General Biology Laboratory--Organisms , Microbiology Lecture, Microbiology Laboratory, Senior Research, Senior Seminar, DNA Technology Lecture, DNA Technology Laboratory