Enzymatic Characterization of Dihydrolipoamide Dehydrogenase from *Streptococcus pneumoniae* Harboring Its Own Substrate*

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Anders P. Häkansson†§ and Alexander W. Smith¶

From the †Department of Microbiology and Immunology and the §Witebsky Center for Microbial Pathogenesis and Immunology, University at Buffalo, State University of New York, Buffalo, New York 14214, the ¶New York State Center of Excellence in Bioinformatics & Life Sciences, Buffalo, New York 14203, and the †Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

This study describes the enzymatic characterization of dihydrolipoamide dehydrogenase (DLDH) from *Streptococcus pneumoniae* and is the first characterization of a DLDH that carries its own substrate (a lipoic acid covalently attached to a lipoyl protein domain) within its own sequence. Full-length recombinant DLDH (rDLDH) was expressed and compared with enzyme expressed in the absence of lipoic acid (rDLDH−LA) or with enzyme lacking the first 112 amino acids constituting the lipoyl protein domain (rDLDH−LPOYL). All three proteins contained 1 mol of FAD/mol of protein, had a higher activity for the conversion of NAD⁺ to NADH than for the reaction in the reverse direction, and were unable to use NADP⁺ and NADPH as substrates. The enzymes had similar substrate specificities, with the \( K_m \) for NAD⁺ being ~20 times higher than that for dihydrolipoamide. The kinetic pattern suggested a Ping Pong Bi Bi mechanism, which was verified by product inhibition studies. The protein expressed without lipoic acid was indistinguishable from the wild-type protein in all analyses. On the other hand, the protein without a lipoyl protein domain had a 2–3-fold higher turnover number, a lower \( K_I \) for NADH, and a higher \( K_F \) for lipoamide compared with the other two enzymes. The results suggest that the lipoyl protein domain (but not lipoic acid alone) plays a regulatory role in the enzymatic characteristics of pneumococcal DLDH.

Dihydrolipoamide dehydrogenase (DLDH; EC 1.8.1.4) is a flavoenzyme that constitutes the E3 component or L protein of five characterized 2-oxoacid dehydrogenase complexes (1–4). DLDH is also anticipated to have other functions, as it is present in organisms that do not contain 2-oxoacid dehydrogenase complexes (5–7). In *Escherichia coli*, DLDH stimulates ATP-binding cassette transport of several carbohydrates (8, 9) and ubiquinone-mediated transport of amino acids (10); in fission yeast, it is involved in cell cycle progression (11); and in *Neisseria meningitidis*, DLDH constitutes an immunogenic surface antigen (12, 13). Whether enzymatic activity is required to perform these functions is not known.

In a 2-oxoacid dehydrogenase complex, the DLDH enzyme catalyzes the terminal pyridine nucleotide-linked reoxidation of a protein-bound dihydrolipoic acid using NAD⁺ as an electron acceptor and FAD as a prosthetic group (4). Purified DLDH is capable of catalyzing the reversible NAD⁺-dependent oxidation of free dihydrolipoamide and other reduced lipoic acid derivatives (14–16). The kinetic mechanism of DLDH has been well characterized using enzyme preparations from mammalian cells, yeast, *Escherichia coli*, and mycobacteria (4, 17–24). Catalysis by the DLDH enzyme occurs via a Ping Pong Bi Bi reaction mechanism, which has been verified by both substrate inhibition kinetics and isotope exchange between NADH and NAD⁺ (18, 21, 22). Furthermore, the enzyme shows a strong substrate inhibition by NADH, making it difficult to measure the initial velocity of the forward reaction.

We recently identified DLDH from *Streptococcus pneumoniae* (25). Mutational inactivation of the gene abolishes DLDH activity and results in loss of virulence in both septicaemia and respiratory tract infection models in mice. As *S. pneumoniae* lacks activity associated with all known 2-oxoacid dehydrogenase complexes, alternative functions of the enzyme were investigated. The DLDH-negative strains were found to display impaired galactose and α-galactoside metabolism because of a down-regulation of α-galactosidase and impaired galactose transport into DLDH-negative cells. How these phenotypes relate to a reduction in virulence is under study.

The pneumococcal *dldh* sequence suggests the presence of an N-terminal lipoyl protein domain, which is the physiologic substrate for the enzyme. This study presents the enzymatic characterization of DLDH from *S. pneumoniae* and describes the contribution of lipoic acid and the lipoyl protein domain to the catalytic characteristics of the enzyme. Using recombinant pneumococcal DLDH preparations with and without a functional lipoyl domain, we show that the absence of lipoic acid in the enzyme has no impact on enzyme behavior, but that the
absence of the lipoyl protein domain changes the kinetic characteristics of the enzyme.

EXPERIMENTAL PROCEDURES

Reagents—Protein markers and enhanced chemiluminescence substrate were from GE Healthcare. Plasmid purification reagents, expression vectors, nickel-nitritolactric acid-agarose, and anti-RGS-His antibody were from Qiagen Inc. Horse-radish peroxidase-conjugated antibody was from Invitrogen. Bacto-Todd Hewitt medium, Bacto-yeast extract, Bacto-peptone, Neopeptone, and thioglycolate were from BD Biosciences. 5,5‘-Dithiobis(2-nitrobenzoic acid) (Ellman’s reagent) was from Pierce. The remaining reagents were from Sigma.

Dihydrolipoamide was produced through reduction of lipoamide with borohydride as described (26). Serum from a patient with primary biliary cirrhosis was kindly provided by Dr. Jem Palmer (School of Biochemistry and Genetics, University of Newcastle, Newcastle upon Tyne, UK).

Expression of Recombinant DLDH Proteins—The dldh gene was amplified with and without the initial 112 amino acids (representing the lipoyl protein domain) amplified by PCR using primer pair DLDH-clon-F/DLDH-clon-R or DLDH-DL-F/DLDH-clon-R (see Table 1) and chromosomal DNA from S. pneumoniae TIGR4 (27) as a template. The amplified fragments were cloned into the pQE30 vector and electroporated into TOP10F TIGR4 (27) as a template. The amplified fragments were cloned into the pQE30 vector and electroporated into TOPL0F’ cells (Invitrogen). Clones carrying the insert of interest were selected on LB agar containing ampicillin (100 μg/ml) and verified by restriction digestion and sequencing of the purified plasmid. Verified plasmids were then transformed into E. coli M15 cells for protein expression.

The expression construct containing the full-length DLDH sequence was further used as a template for site-directed mutagenesis of the Lys43 codon. A primer was constructed covering 12 bp in either direction from the Lys43 codon. The codon (AAA in the original sequence) was changed to AGA to make a alanine mutation using primers DLDH-K43R and DLDH-K43A (see Table 1). Site-directed mutagenesis was performed using the U.S.E. site-directed mutagenesis kit (GE Healthcare).

Western Blotting—Enzyme sources (1–4 μg) were run on 10% polyacrylamide gel (Bio-Rad) in the absence of SDS, and the gel was incubated at 37 °C in 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA and 0.4 mM NADH using 0.75 mg/ml nitro blue tetrazolium as the electron acceptor. Activity was detected as purple-colored protein bands.

Expression of plasmid-born protein sequences was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside at room temperature, and the overexpressed proteins were purified by affinity chromatography using nickel-nitritolactric acid-agarose according to the manufacturer’s instructions. The proteins were analyzed by SDS-PAGE and Western blotting and quantified using the Bio-Rad DC protein assay.

DNA Sequencing—Automated sequencing reactions were run on an ABI PRISM 377 sequencer (Applied Biosystems). Each construct was sequenced using the pQE-F or pQE-R primer (Table 1). Sequences were analyzed and edited using the Sequencer software program (Gene Codes Corp.).

The mutated sequence was verified by sequencing and transferred into the E. coli M15 cells for protein expression. Expression of DLDH proteins was conducted in either LB broth or M9 medium (1 g of NH4Cl, 3 g of KH2PO4, 6 g of Na2HPO4, 7H2O, 20 ml of 20% glucose, 1 ml of 1 M MgSO4, and 0.5 g of NaCl per liter of water) with the addition of 300 μg/ml lipoic acid. To express DLDH without covalently attached lipoic acid, bacteria harboring the full-length dldh gene in pQE30 were grown and induced for protein expression in M9 medium without the addition of lipoic acid.

The expression construct containing the full-length DLDH sequence was further used as a template for site-directed mutagenesis of the Lys43 codon. A primer was constructed covering 12 bp in either direction from the Lys43 codon. The codon (AAA in the original sequence) was changed to AGA to make a alanine mutation using primers DLDH-K43R and DLDH-K43A (see Table 1). Site-directed mutagenesis was performed using the U.S.E. site-directed mutagenesis kit (GE Healthcare).
from a patient with primary biliary cirrhosis (1:5000 dilution in PBS-T) or with anti-RGS-His antibody (1:5000 in PBS-T) for 1 h at room temperature and washed three times with PBS-T. The membrane was further incubated with horseradish peroxidase-conjugated goat anti-human or anti-mouse antibody (1:5000 dilution in PBS-T) for 1 h at room temperature. After washing, the membrane was developed on film after exposure to enhanced chemiluminescence substrate.

Flavin Determination—The absorbance spectrum for the recombinant DLDH preparations was measured in an ultraviolet spectrophotometer (GE Healthcare) from 273 to 600 nm. Samples were measured against a reference cuvette containing buffer without protein present. The spectrum of the reduced enzyme was measured in the presence of NADH or dihydrolipoamide.

The enzyme-bound flavins were liberated by thermal denaturation at 100 °C for 15 min, after which the protein was pelletted at 13,000 × g. The flavin concentration of the supernatant was determined spectrophotometrically at 455 nm using an extinction coefficient for FAD of 11.3 mM cm⁻¹. To verify the presence of FAD, a portion of the supernatant was analyzed by thin-layer chromatography on silica plates with 1-butanol/acetone/water (4:3:3) as the solvent using FAD and flavin mononucleotide as references.

Thiol Titration—The number of accessible thiol groups in oxidized and NADH-reduced proteins was determined using 5,5'-dithiobis(2-nitrobenzoic acid) as described (28). To 1 ml of reaction buffer (100 mM potassium phosphate buffer (pH 7.3) with 1 mM EDTA) was added 100 µl of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) solution, and the absorbance at 412 nm was recorded. The enzyme source (4 µM) was added, and the absorbance was recorded until the reaction was complete, usually ~20 min. The reaction was monitored in the presence and absence of 10 mM NADH to determine the reactive thiol concentration of the reduced and oxidized proteins, respectively. The thiol concentration was then determined after correcting for the absorbance of a buffer control using an extinction coefficient for 2-nitro-5-thiobenzoate anion of 14.15 mM cm⁻¹.

RESULTS

Expression of Recombinant DLDH Proteins—The aim of this study was to enzymatically characterize DLDH from the Gram-positive organism S. pneumoniae. Pneumococcal DLDH has the intriguing feature that it harbors an N-terminal protein domain containing a 2-oxoacid dehydrogenase acetyltransferase component lipoyl-binding motif, which is the physiologic substrate for the enzyme (amino acids 27–56) of the DLDH sequence, with Lys⁴³ being the conserved lysine to which lipoic acid can be covalently attached; PROSITE accession number PDOC00168). Although it shares this feature with most Streptococcus and Clostridium and some Mycoplasma species, the lipoyl protein domain is part of the acetyltransferase component in all other species expressing 2-oxoacid dehydrogenases.

Recombinant DLDH (rDLDH) proteins with or without covalently attached lipoic acid and/or the N-terminal lipoyl protein domain were produced. To obtain DLDH enzyme lacking covalently attached lipoic acid, we first attempted to mutate the conserved Lys⁴³ codon to either arginine (rDLDH⁴³⁴³) or alanine (rDLDH⁴³⁴³). Both mutations were postulated to make covalent attachment of lipoic acid impossible. rDLDH⁴³⁴³ expressed well, but still had lipoic acid attached (see below). Several attempts were made to construct the lysine-to-alanine mutation (rDLDH⁴³⁴³); however, all positive clones obtained were unable to express the mutant protein. This suggests that this variant of the protein may not fold correctly or may be toxic to E. coli cells during expression. To obtain non-lipoylated DLDH (rDLDH⁴³⁴³), the wild-type protein sequence was instead expressed in minimal medium lacking lipoyl acid. To obtain DLDH lacking the N-terminal lipoyl protein domain (rDLDH⁻¹⁴⁹⁹⁳⁴³), we expressed the DLDH sequence without the first 112 amino acids that correspond to the lipoyl protein domain.

The full-length protein (rDLDH) had a calculated molecular mass of 61.6 kDa, and a band close to that size was visible on the gel as well as after Western blotting using anti-RGS-His antibody (Fig. 1, A and C). rDLDH⁻¹⁴⁹⁹⁳⁴³ ran identically to wild-type DLDH on the gel, whereas rDLDH⁻¹⁴⁹⁹⁳⁴³ (lacking the N-terminal 112 amino acids and with a calculated molecular mass of 49.8 kDa) ran close to the 50-kDa molecular mass standard. All three recombinant preparations were at least 90% pure as determined by Coomassie Blue staining of the gel as well as by flavin absorbance spectrometry (see below).

The activity of the pure DLDHs was investigated in a native polyacrylamide gel assay using NADH as the substrate and
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FIGURE 2. FAD spectra of the three rDLDH preparations. An absorbance spectrum was determined for each enzyme preparation between 273 and 600 nm (350–550 nm shown here). Each preparation had a peak absorbance value at 454 nm, with a distinct shoulder at 480 nm, consistent with a flavin-containing protein. WT, wild-type. Inset, rDLDH was reduced by the addition of 10 mM NADH, and the spectrum was recorded. Reduction of the protein resulted in reduced peak absorbance and a slight blue shift of the peak absorbance wavelength.

The protein sequence and two thiol groups from the dihydrolipoic acid attached to Lys43. In its oxidized form, only 1 mol (0.94 ± 0.22) of thiol groups/mol of protein was accessible, consistent with intrinsic disulfide bonding between two of the three cysteine residues and oxidation of dihydrolipoic acid to lipoic acid.

rDLDH−LA (grown in the absence of lipoic acid) showed the expected 3 mol (3.20 ± 0.36) of thiol groups/mol of enzyme in its reduced form and 1 mol (0.81 ± 0.29) in its oxidized form. rDLDH−Lipoyl also contained the expected 3 mol (3.09 ± 0.32) of thiol groups/mol of enzyme in its reduced form and 0.83 ± 0.24 mol of thiol groups/mol of protein in its oxidized form. These results show that the N-terminal domain of DLDH is in fact a lipoyl protein domain to which lipoic acid can be attached.

Of related interest, the site-directed mutant rDLDH^K43R (harboring a lysine-to-arginine substitution in the lipoic acid-binding site) showed a similar or (for some batches) higher molar thiol ratio (5.5–7.4) in its reduced state compared with wild-type rDLDH. Whether this results from unspecific lipoylation at other sites in the protein sequence or the tantalizing possibility that arginine in this position can serve as a substrate for ligation of lipoic acid needs to be verified with more specific experiments in the future.

Flavin Determination—At identical concentrations, the rDLDH preparations displayed a yellow color of similar intensity, indicating the presence of a protein-bound flavin. The spectra of the recombinant enzymes were obtained by scanning the absorbance between 273 and 600 nm. All rDLDHs showed the classical flavin-protein absorbance spectra, with a peak at
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454 nm, a distinct shoulder at 480 nm, and a smaller shoulder at
430 nm (Fig. 2). The flavin moiety could be reduced by the
addition of NADH or dihydrolipoamide, resulting in a character-
stic shift in the spectrum (Fig. 2, inset).

After thermal denaturation of the recombinant proteins, the
flavin component was identified as FAD by thin-layer chroma-
tography (data not shown). The molar ratios of FAD were
determined to be 0.98, 0.91, and 0.96 mol of FAD/mol of
enzyme for rDLH, rDLH<sup>−LA</sup>, and rDLH<sup>−LIPOYL</sup>, respec-
tively. This indicated that all of the rDLDH constructs con-
tained 1 mol of FAD/mol of protein.

The FAD molar ratios also indicated that the DLDH prepa-
rations had a high level of purity. This was supported by the
ratios of the absorbance peaks at 273 and 455 nm (an excellent
indicator of protein purity), which were 5.5, 6.0, and 5.2 for
rDLH, rDLH<sup>−LA</sup>, and rDLH<sup>−LIPOYL</sup>, respectively. This indicated that all of the rDLDH constructs con-
tained 1 mol of FAD/mol of protein.

FIGURE 3. Enzyme kinetics for rDLH. Lineweaver-Burk plots of initial velocities for rDLH at varying concen-
trations of NAD<sup>+</sup> and dihydrolipoamide (DLA) were determined in potassium phosphate buffer (pH 8.5) by
measuring the absorbance of NADH at 340 nm. A, enzyme velocities for various NAD<sup>+</sup> concentrations plotted
against the inverse dihydrolipoamide concentration. The plot displays parallel lines indicative of a ping-pong
reaction mechanism. Inset, plot of the inverse V<sub>max</sub> for each NAD<sup>+</sup> concentration as a function of the inverse
NAD<sup>+</sup> concentration. The plot shows linearity and was used to determine the V<sub>max</sub> for the enzyme. B, enzyme
velocities for various dihydrolipoamide concentrations plotted against the inverse NAD<sup>+</sup> concentration. The plot
displays parallel lines indicative of a ping-pong reaction mechanism. Inset, plot of the inverse V<sub>max</sub> for each dihy-
drolipoamide concentration as a function of the inverse dihydrolipoamide concentration. The plot shows
linearity and was used to determine the V<sub>max</sub> for the enzyme.

### TABLE 2

Enzymatic properties of rDLH proteins

Each data point is the mean of at least two and in most cases three or more independent experiments. DLA, dihydrolipoamide.

| Substrate | rDLH | rDLH<sup>−LA</sup> | rDLH<sup>−LIPOYL</sup> |
|-----------|------|-----------------|----------------|---|
| DLA       | 56 ± 18 | 64 ± 25 | 40 ± 29 |
| NAD       | 1037 ± 270 | 985 ± 318 | 820 ± 226 |
| V<sub>max</sub> (μM) | 1.6 ± 0.3 | 2.2 ± 0.5 | 5.1 ± 0.3 |
| k<sub>catalysis</sub> (s<sup>−1</sup>) | 160 ± 31 | 223 ± 54 | 506 ± 34 |
| V<sub>max</sub>/k<sub>catalysis</sub> (s<sup>−1</sup> M<sup>−1</sup>) | (3.31 ± 2.26) × 10<sup>6</sup> | (3.56 ± 0.25) × 10<sup>6</sup> | (17.87 ± 14.03) × 10<sup>6</sup> |

### Inhibitor/variable substrate K<sub>i</sub> (μM)

| Substrate | rDLH | rDLH<sup>−LA</sup> | rDLH<sup>−LIPOYL</sup> |
|-----------|------|-----------------|----------------|---|
| NAD/HAD | 1.5 mM DLA | 14.9 ± 4.2 | 10.7 ± 3.6 | 4.4 ± 1.4 |
|          | 0.03 mM DLA | (Mixed) | (Mixed) | (Mixed) |
| NAD/DLA | 2 mM NAD | 72.2 ± 22.2 | 84.0 ± 25.4 | 35.1 ± 11.1 |
|           | 4 mM NAD | 56.6 ± 9.4 | 48.8 ± 10.7 | 349.0 ± 44.2 |
|           | 0.75 mM NAD | (Mixed) | (Mixed) | (Mixed) |
| LA/DLA | 1.5 mM DLA | 195.3 ± 61.7 | 189.9 ± 55.0 | 879.3 ± 219.1 |

DLDH activity was based on determining the initial velocity of
the reaction, measuring the change in NADH absorbance as described
under “Experimental Procedures.” We first determined the pH at
which the enzymes worked optimally. Initial velocities were determined at
saturating substrate concentrations for each of the recombinant enzymes
in phosphate buffer at pH 5.5–9.8.
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The pH optima for all three enzymes were ~8.5 in the forward reaction and ~8.3 in the reverse reaction (data not shown). Phosphate buffer at pH 8.5 and 8.3 was therefore used for all subsequent analyses in the forward and reverse directions, respectively.

At saturating substrate concentrations, the full-length enzyme (rDLDH) converted NADH to NAD+ with an activity of 11.5 μmol/min/mg of enzyme source and NAD+ to NADH with an activity of 72.6 μmol/min/mg of enzyme source. The corresponding activities were 12.3 and 75.4 μmol/min/mg for rDLDH- LA and 32.4 and 215.4 μmol/min/mg for rDLDH-LIPOLY. The higher conversion activity seen in the forward reaction (Equation 1) correlates well with the trend seen in crude pneumococcal extracts (25) and suggests that the enzyme favors the reaction direction usually seen in 2-oxoacid dehydrogenase complexes. Additionally, the higher conversion activity determined for rDLDH-LIPOLY suggests that the presence of the lipoyl protein domain (but not lipoic acid alone) decreases the activity of pneumococcal DLDH. None of the enzymes could reduce NADP+ or oxidize NADPH (data not shown).

Enzyme Kinetics: $V_{\text{max}}$, $K_m$, and $k_{\text{cat}}$ Values—To further understand the implications of the differences seen in DLDH activity between rDLDH, rDLDH- LA, and rDLDH-LIPOLY, we investigated the kinetic parameters of the rDLDH proteins in more detail. Initial reaction velocities were determined at varying substrate concentrations, and Lineweaver-Burk (double-reciprocal) plots were produced. When the concentrations of NAD+ and dihydrolipoamide were varied, the initial velocities represented a series of parallel lines, suggestive of a ping-pong reaction mechanism. The plots for rDLDH are shown in Fig. 3. The $V_{\text{max}}$ and $K_m$ values were subsequently determined for each substrate after plotting the velocity at each concentration against either the inverse NAD+ or dihydrolipoamide concentration (Fig. 3, insets). The turnover number ($k_{\text{cat}}$) and catalytic efficiency ($k_{\text{cat}}/K_m$) were calculated for each enzyme (Table 2).

Measurements of the reaction parameters in the reverse direction (Equation 1) were also attempted; however, the $K_m$ for NADH was below the concentration at which activity could be measured accurately. We therefore focused our attention on the forward reaction.

Between them, the enzymes had similar $K_m$ values for NAD+ and dihydrolipoamide, suggesting that the substrate specificities are not affected by the presence of the lipoyl protein domain or the lipoic acid substrate. The $K_m$ for NAD+ ranged between 0.82 and 1.04 mM for all three DLDHs. The $K_m$ values for dihydrolipoamide (40–64 μM) were ~20 times lower, suggesting higher substrate specificity for dihydrolipoamide (Table 2). Even though the proteins did not differ in terms of substrate specificity, they differed in their catalytic properties. rDLDH-LIPOLY had approximately three times higher $V_{\text{max}}$ and $k_{\text{cat}}$ values compared with rDLDH and rDLDH- LA. As the $K_m$ values were similar for all enzymes, the catalytic efficiency ($k_{\text{cat}}/K_m$) for the utilization of dihydrolipoamide was greater than that for the utilization of NAD+ for all of the enzymes. Similarly, rDLDH-LIPOLY had a higher catalytic efficiency compared with both other enzymes. This indicates that that the presence of the lipoyl protein domain reduces the catalytic efficiency of the DLDH enzyme, whereas the presence or absence of the lipoic acid substrate does not affect the enzyme’s kinetic properties.

Product Inhibition with NADH—To verify the reaction mechanism of pneumococcal DLDH, the product inhibition patterns for the enzyme preparations were investigated. Inhibition was first measured in the forward direction using NADH as the product inhibitor. At a fixed unsaturated dihydrolipoamide concentration (0.03 mM), NADH showed a mixed inhibition pattern for NAD+ in Lineweaver-Burk plots for all of the DLDHs (Fig. 4A). On the other hand, at a fixed saturated dihydrolipoamide concentration (1.5 mM), the inhibition pattern was shown to be competitive (Fig. 4B), which is consistent with that observed for a Ping Pong Bi Bi reaction mechanism (37). The calculated $K_i$ values for the competitive inhibition (assuming Michaelis-Menten kinetics) were lower for rDLDH-LIPOLY than for the other enzymes (Table 2), indicating that the variant lacking the N-terminal protein domain was more sensitive to inhibition by NADH. There was no difference between the inhibition characteristics of rDLDH and rDLDH- LA, suggesting that the presence of the lipoic acid substrate per se does not affect the enzymatic properties of pneumococcal DLDH.

At a fixed concentration of NAD+, the inhibition with NADH was competitive for dihydrolipoamide for all of the DLDHs, with $K_i$ values again being lower for rDLDH-LIPOLY (Fig. 4C). This type of inhibition pattern is characteristic of a Ping Pong Bi Bi reaction mechanism (22, 37). The $K_i$ values obtained for NADH in this analysis also demonstrated that NADH inhibited rDLDH-LIPOLY more strongly than rDLDH or rDLDH- LA (Table 2), suggesting a role for the lipoyl domain in the catalytic properties of the enzyme.

Product Inhibition with Lipoamide—The product inhibition patterns were then measured using lipoamide as the product inhibitor. At a fixed unsaturated NAD+ concentration (0.5 mM), NADH showed a mixed inhibition pattern for dihydrolipoamide in Lineweaver-Burk plots for all of the DLDHs (Fig. 4D). On the other hand, at a fixed saturated NAD+ concentra-
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...tion (4 mM), the inhibition pattern was shown to be competitive (Fig. 4E), in accordance with the NADH inhibition patterns described above. The calculated $K_i$ values for the competitive inhibition were higher for rDLDH-LIPOYL than for rDLDH and rDLDH-LA (Table 2), indicating that the variant lacking the N-terminal lipoyl protein domain was less sensitive to inhibition by lipoamide than the remaining proteins.

At a fixed concentration of dihydrolipoamide, the inhibition with lipoamide was competitive for NAD$^+$ for all of the DLDHs, with $K_i$ values again being higher for rDLDH-LIPOYL than for the remaining proteins (Fig. 4F). The $K_i$ values obtained for lipoamide, as described above for NADH, indicate that pneumococcal DLDH uses a Ping Pong Bi Bi reaction mechanism and strongly suggest a role for the lipoyl protein domain in regulating the catalytic properties of the enzyme.

**DISCUSSION**

This study describes the enzymatic properties of the DLDH from *S. pneumoniae*. This is the first enzymatic characterization of a DLDH containing an N-terminal lipoyl protein domain. The lipoyl protein domain contains a conserved motif with a lysine residue to which lipoic acid is covalently attached through amide linkage. The lipoic acid is used to shuttle the acyl group from a 2-oxo acid to coenzyme A and is most often associated with the 2-oxoacid acetyltransferase. DLDH takes part in this reaction by reoxidizing the dihydrolipoic acid on the acetyltransferase for the next substrate cycle. In *S. pneumoniae*, the genetic architecture of the 2-oxoacid dehydrogenase locus is different from that in most other species. The sequence encoding the lipoyl protein domain is no longer part of the acetyltransferase gene, but has moved to become the N-terminal portion of the downstream dldh gene. *S. pneumoniae* shares this property with other *Streptococcus* and *Clostridium* species (16, 38, 39). DLDH in some *Mycoplasma* species as well as in *N. meningitidis* (40, 41) also contains an N-terminal lipoyl protein domain. In these cases, however, the acetyltransferase component still retains a similar domain. The focus of this study was to understand how harboring a lipoyl protein domain (the substrate for DLDH) within the DLDH sequence affects the enzyme's properties.

We produced three recombinant forms of the enzyme: 1) rDLDH, the full-length wild-type enzyme; 2) rDLDH-LA, the full-length protein expressed in the absence of lipoic acid, rendering the lipoyl protein domain non-lipoylated; and 3) rDLDH-LIPOYL, the enzyme with a deletion of the first 112 amino acids that encode the lipoyl protein domain. To ensure optimal conditions for lipoylation of the DLDH proteins, they were expressed in the presence of added lipoic acid (except for rDLDH-LA) and expressed at low inducer concentration and low temperature to overcome the rate-limiting steps in lipoylation due to lipoate synthase and lipoate-protein ligase (34).

The proteins showed predicted molecular masses by PAGE and had NADH oxidizing activity on native gels. This suggests that neither lipoic acid nor the lipoyl protein domain are necessary for pneumococcal DLDH activity. Activity required that the proteins form dimers or higher aggregates, as both heat-denatured enzyme and spontaneously dissociating protein in buffer ran as a monomer on native polyacrylamide gels and did not exhibit activity. The need for DLDH dimerization is not surprising, as it has been demonstrated that one enzyme subunit provides a redox-active site and the other provides an active histidine residue in an active enzyme complex (29, 42). It is interesting that the activity gel analysis showed larger DLDH bands that also possessed enzymatic activity, suggesting that the protein is capable of forming aggregates. Whether these aggregates are larger aggregates of DLDH or involve the formation of aggregates with other components in *E. coli* cells remains to be examined.

Full-length DLDH had a functional lipoyl protein domain that could be detected with antiserum from a patient with primary biliary cirrhosis. These patients make autoantibodies that readily react with the lipoyl domain of the E2 component of human pyruvate dehydrogenase (43) and that have been shown to recognize bacterial E2 components of 2-oxoacid dehydrogenases as well (36). The antiserum recognizes the structure of the lipoyl domain, but cannot be used to assess the presence of a covalently attached lipoic acid.

Covalently attached lipoic acid was identified by measuring the molar ratio of reactive thiol groups for the oxidized and reduced forms of the recombinant proteins. rDLDH had a covalently attached lipoic acid, whereas rDLDH-LA and rDLDH-LIPOYL lacked any lipoylation.

Characterization of the three DLDH variants showed that all enzymes contained 1 mol of FAD/mol of enzyme, as reported for all previously characterized DLDHs (44). All three enzymes displayed higher activity for the conversion of NAD$^+$ to NADH than for the conversion of NADH to NAD$^+$ and failed to use NADP$^+$ and NADPH as substrates. The higher reaction activity for the forward reaction (Equation 1) corresponds well with favoring the reaction direction a DLDH would encounter in a 2-oxoacid dehydrogenase complex.

The reaction kinetics of the rDLDHs were then compared to assess the contributions of the lipoyl domain and the lipoic acid substrate to the activity of the enzyme. No differences were seen between the enzymes with regard to substrate specificity. The $K_m$ values for NAD$^+$ (0.8–1.0 mM) were similar for the three DLDHs and were within the range of those reported for mammalian, yeast, and bacterial DLDH preparations (17, 18, 21, 22, 45). The $K_m$ values for dihydrolipoamide (40–60 μM) were also similar for the three preparations and showed a 20-fold higher specificity than those measured for NAD$^+$. These values are similar to those reported for *E. coli* DLDH and ~10 times lower than those reported for most mammalian and fungal enzymes. It is interesting that DLDH from Gram-positive *Mycobacterium smegmatis* shows a $K_m$ of 0.4 mM for dihydrolipoamide, which is closer to the fungal and mammalian enzyme values than to either the *E. coli* or *S. pneumoniae* DLDH value (24).

Even though the enzyme preparations had similar substrate specificities, the presence of a lipoyl protein domain had a marked impact on both the catalytic efficiency and product inhibition kinetics of pneumococcal DLDH. The turnover number of rDLDH and rDLDH-LA was ~200 s$^{-1}$ and was 2.5 times slower than that observed for DLDH lacking the lipoyl protein domain. As the affinities for the sub-
strates were the same for all three enzymes, this suggests that the lipoyl protein domain (but not the presence of the lipoic acid substrate per se) lowers the catalytic efficiency of the DLDH enzyme. The presence of a lipoyl protein domain in pneumococcal DLDH may thus serve as a means of regulating enzymatic activity.

To address the catalytic mechanism of the enzyme, double-reciprocal plots of the initial velocity of the enzymes were produced over a series of substrate concentrations. Initial velocity measurements for the DLDH proteins displayed parallel Lineweaver-Burk plots indicative of a ping-pong reaction mechanism (37). This reaction mechanism was proven by product inhibition studies with NADH and lipoamide. Thus, pneumococcal DLDH utilizes the same reaction mechanism that has been reported for DLDHs in general (4, 18, 21–24, 45). Additionally, when the inhibition was competitive, inhibition constants showed no difference between rDLDH and the variant lacking lipoic acid attached to the lipoyl domain (rDLDH-L1). On the other hand, the protein lacking the whole lipoyl protein domain (rDLDH-L2POY) was inhibited more strongly with NADH and less strongly with lipoamide than the other two proteins. This result further supports the idea that the lipoyl protein domain (but not the lipoic acid substrate per se) changes the properties of pneumococcal DLDH and may be involved in the regulation of its activity.

The role the functional domains of pneumococcal DLDH play in vivo can thus far only be speculated about. S. pneumoniae lacks 2-oxoacid dehydrogenase activity, but requires DLDH for full virulence of the organism, as mutation of the dldh gene renders the bacterium unable to grow or survive in the mouse host environment (25). The virulence attenuation in DLDH-negative bacteria is under study, but may be related to the role of DLDH and lipoic acid in ATP-binding cassette transport of solutes. In S. pneumoniae, mutation of dldh results in an impaired utilization and import of raffinose, galactose, and ribose (25, 46). In E. coli, inhibition of DLDH activity results in drastic inhibition of galactose, maltose, and ribose transport, indicating that enzymatic activity may be important for stimulation of carbohydrate transport. Whether the same is true for S. pneumoniae remains to be seen. Besides a requirement for DLDH activity, carbohydrate transport in E. coli is stimulated by the addition of dihydrolipoate, and mutation of the acetyltransferase gene in the pyruvate dehydrogenase operon that carries a lipoyl protein domain inhibits transport. This suggests an important role also for the presence of functional lipoyl protein domains in ATP-binding cassette transport events (8, 47). In the absence of 2-oxoacid dehydrogenase activity, pneumococcal DLDH may have gained dual functionality by harboring both enzymatic activity and a functional lipoyl protein domain within the same protein molecule. An understanding of how pneumococcal DLDH regulates ATP-binding cassette transport of solutes through the bacterial membrane will be addressed in a future publication.3

3 R. Tyx and A. Häkansson, manuscript in preparation.

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