A Major Surface Protein on Group A Streptococci Is a Glyceraldehyde-3-Phosphate-Dehydrogenase with Multiple Binding Activity

By Vijaykumar Pancholi and Vincent A. Fischetti

Summary

The surface of streptococci presents an array of different proteins, each designed to perform a specific function. In an attempt to understand the early events in group A streptococci infection, we have identified and purified a major surface protein from group A type 6 streptococci that has both an enzymatic activity and a binding capacity for a variety of proteins. Mass spectrometric analysis of the purified molecule revealed a monomer of 35.8 kD. Molecular sieve chromatography and sodium dodecyl sulfate (SDS)-gel electrophoresis suggest that the native conformation of the protein is likely to be a tetramer of 156 kD. NH2-terminal amino acid sequence analysis revealed 83% homology in the first 18 residues and about 56% in the first 39 residues with glycolaldehyde-3-phosphate dehydrogenase (GAPDH) of eukaryotic or bacterial origin. This streptococcal surface GAPDH (SDH) exhibits a dose-dependent dehydrogenase activity on glyceraldehyde-3-phosphate in the presence of β-nicotinamide adenine dinucleotide both in its pure form and on the streptococcal surface. Its sensitivity to trypsin on whole organism and its inability to be removed with 2 M NaCl or 2% SDS support its surface location and tight attachment to the streptococcal cell. Affinity-purified antibodies to SDH detected the presence of this protein on the surface of all M serotypes of group A streptococcal tested. Purified SDH was found to bind to fibronectin, lysozyme, as well as the cytoskeletal proteins myosin and actin. The binding activity to myosin was found to be localized to the globular heavy meromyosin domain. SDH did not bind to streptococcal M protein, tropomyosin, or the coiled-coil domain of myosin. The multiple binding capacity of the SDH in conjunction with its GAPDH activity may play a role in the colonization, internalization, and the subsequent proliferation of group A streptococci.

The initial events governing the colonization of the pharyngeal mucosa by group A streptococci are not understood. To date at least six structurally similar proteins have been identified on the surface of these organisms (1). The similarity among these different molecules is based on the presence of repeat regions in their surface-exposed NH2-terminal half and a highly conserved COOH-terminal portion buried within the cell and responsible for attachment (2). Although the repeat segments of some of these molecules have been shown to perform a particular biological function (1), their role in colonization and infection has not been fully explored. Earlier studies have implicated both the surface M protein (3) and lipoteichoic acid (LTA)1 (4) in the adherence of group A streptococci to epithelial cells, however, more recent data have indicated that neither the M protein nor LTA (5, 6) are directly involved in the colonization process.

In the present communication we purify and characterize a major surface molecule from group A streptococci (termed streptococcal surface dehydrogenase [SDH]) that is distinct from M protein (7), and have determined its NH2-terminal amino acid sequence as a first step in understanding the molecules involved in mucosal colonization. Sequence and enzymatic results reveal that this surface protein is in fact a glycolytic enzyme with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity. In eukaryotes, glycolytic enzymes, of which GAPDH is a key one, are found to be associated with membranes and subcellular cytoskeletal structures (8). Recently, proteins with a similar enzymatic activity have been shown to be membrane bound (9-12) and involved in a variety of functions independent from its catalytic property (9, 13-16). In the case of SDH, we have found that it is able to bind to fibronectin, lysozyme, and cytoskeletal proteins.

Abbreviations used in this paper: DTT, dithiothreitol; G-3-P, glyceraldehyde-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HMM, heavy meromyosin; k, kinetic; LMM, light meromyosin; LTA, lipoteichoic acid; NAD, β-nicotinamide adenine dinucleotide; SDH, streptococcal surface dehydrogenase.

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Fibronectin as well as lysozyme have been shown to serve as important constituents of human saliva (17, 18). The surfaces of both Gram-negative and Gram-positive bacteria have been shown to contain molecules that bind to fibronectin and lysozyme (19–21). While a fibronectin binding protein in *Staphylococcus aureus* has been identified (22), characterized (23), cloned, and sequenced (24, 25), and implicated in the adherence of these organisms, the presence of a similar molecule on group A streptococci is suggested (5) with a probable role in initial adherence events. Talay et al. (6) have recently provided evidence that fibronectin binding molecules found on the streptococcal surface represent a strain-dependent complex of proteins with variable molecular masses distinct from the M protein; however, none have been characterized.

It is clear that the ability to bind to the fibronectin on the surface of mammalian cells may be an important first step in the colonization of mucosal surfaces by microorganisms (5, 26). However, the soluble form of fibronectin found in saliva (17, 27) may also play a key role in the colonization process, since this form would be the first encountered by the incoming bacterium and may serve as a competitive inhibitor. Similarly, lysozyme, an important secretory component of the host's defense against infection by Gram-positive bacteria, is ineffective in the destruction of group A streptococci (28; our personal observation). While saliva (29) and its component lysozyme have been shown to be involved in the aggregation of microorganisms (19), including group A streptococci, in the process of microbial clearance from the oral mucosa, its association with molecules responsible for fibronectin binding has not been described. In our studies, designed to dissect the interactions between the streptococcus and the pharyngeal mucosa, we reasoned that molecules found in human saliva may combine with streptococcal surface molecules and thus influence the colonization process.

In view of the potential multifunctional property of GAPDH-like molecules, the SDH protein found on the streptococcal surface may play a role in the infection process. The present study details the enzymatic and binding characteristics of this major surface molecule.

**Materials and Methods**

**Materials.** Human fibronectin and glutaraldehyde-activated affinity beads were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). Prestained molecule mass standards were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Polyvinylidene difluoride (PVDF) membrane “Immobilon-P” was purchased from Millipore Corp. (Bedford, MA). NaCl was from New England Nuclear (Boston, MA). All other chemicals and reagents, unless otherwise indicated, were purchased from Sigma Chemical Co. (St. Louis, MO).

**Bacteria.** Group A β-hemolytic streptococcal strains of various M types and standard strains used for streptococcal grouping were from The Rockefeller University culture collection (New York, NY) and are listed as follows: M2(D626), M4(D896), M5(M16frando), M6(D471), M24(CS24), M29(D23), M41(C101/103/4), M57(A995), M58(D774), M60(D398), M728/51/4 (30); group A, J17A4 (an M1 strain); group A variant, A486var; group B, O90R; group C, C74; group D, D76; group E, K131; group F, F68C; group G, D166B; group H, F90A; group L, D167B; and group N, C559.

**Lysin Extraction and Location of the 39-kD Protein.** A crude extract containing the major streptococcal surface proteins was prepared using the procedure of lysin extraction to remove the streptococcal cell wall, as described before (31, 32). M6 strain D471 was grown to a stationary phase of culture at 37°C for 18 h in 4-6-liter batches of Todd-Hewitt broth. Bacteria were pelleted by centrifugation and washed in 50 mM sodium acetate buffer, pH 5.5, and suspended in the same buffer containing 30% raffinose and 5 mM EDTA. Lysin was added to the suspension (1:100 dilution; 120 U/ml) and incubated for 90 min at 37°C with end-to-end slow rotation. The resulting protoplasts were sedimented at 15,000 g for 30 min in a Sorvall centrifuge. The supernatant (crude extract) was dialyzed against 25 mM Tris/HCl, pH 8.5, 5 mM EDTA, concentrated 10-fold on a PM-10 membrane (Amicon Corp., Danvers, MA), and used for further purification.

After lysin extraction, the pelleted protoplasts were resuspended and lysed in hypotonic buffer (2 mM sodium acetate, pH 5.5, containing 2 mM PMSF, 1 mM N-p-tosyl-l-lysine chloromethyl ketone [TLCK], 10 mM MgCl2, and 10 μg/ml DNase) followed by three freeze/thaw cycles. The membranes were then sedimented at 100,000 g for 45 min at 4°C. The membrane pellet and cytoplasmic extract in the supernatant were analyzed by Coomassie blue stain after separation on an SDS gel. To determine the nature of association of the 39-kD protein with membranes, equal aliquots of the latter were suspended in 1.5 M sodium chloride or 100 mM sodium carbonate, pH 11.3 (2-3 mg total protein/ml) and placed on ice for 30 min (32). The suspensions were centrifuged (100,000 g, 45 min, 4°C) and the resulting pellets and supernatants were checked for the presence of the 39-kD protein by SDS-PAGE and Western blot.

To determine whether the 39-kD protein is surface exposed, lysin extraction of trypsinized bacteria was carried out as described earlier (31, 32). Briefly, washed bacteria were suspended in 100 mM NH4HCO3, and digested with trypsin (250 μg/ml) at 37°C for 3 h, after which the trypsin was inactivated by the addition of soybean trypsin inhibitor (200 μg/ml). Lysin extracts of trypsinized and control nontrypsinized bacteria were compared for the loss or reduction in the size of the 39-kD protein after SDS-PAGE and Western blot.

**Purification of 39-kD Protein.** The dialyzed and concentrated crude lysin extract was precipitated at a 60% saturation of ammonium sulfate at 4°C for 18 h. The precipitate was centrifuged at 6,000 g for 20 min and the supernatant was brought to 85% saturation of ammonium sulfate. The resultant precipitate was again collected by centrifugation, resuspended in 50 ml 25 mM Tris/HCl buffer, pH 8.5, dialyzed against the same buffer, and stored at −70°C until further use. A 10-ml aliquot of this dialyzed precipitate was applied to a Mono Q FPLC column (1-ml bed volume) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated with the same buffer. After washing with five-column volumes of the starting buffer, bound proteins were eluted with a 50-ml linear NaCl gradient from 0 to 300 mM. Fractions containing the 39-kD protein, as determined by SDS-PAGE, were pooled and dialyzed against 25 mM Tris/HCl/EDTA starting buffer and rechromatographed on the Mono Q column. The pooled fractions were concentrated to a volume of <1.0 ml using a Centricon concentrator (cut off, 10,000 kDa, Amicon Corp.). The concentrated sample (200–300 μl) was applied to a Superose 12 FPLC column (Pharmacia LKB Biotechnology Inc.) preequilibrated with 50 mM Tris/HCl, pH 8.5, containing 300 mM NaCl and 5 mM EDTA. Fractions containing the 39-kD protein were pooled, dialyzed against...
25 mM Tris/HCl buffer, pH 8.5, containing 1.0 M (NH₄)₂SO₄, and applied to a TSK-phenyl HPLC column (Bio-Rad Laboratories, Richmond, CA) prequillibrated in the same buffer. The protein was eluted by a decreasing linear gradient of (NH₄)₂SO₄ from 1.0 to 0.0 M. The 39-kD protein was eluted at 200 mM (NH₄)₂SO₄. The purity of the final product was determined by Coomassie blue stain of the purified protein after SDS-PAGE and by analytical procedures described below. The purified protein was either stored at 4°C after dialyzing against 25 mM Tris/HCl, pH 8.5, for various protein binding experiments or at -70°C for longer storage.

Analytical Procedures (NH₂-terminal Sequence and Amino Acid Composition). NH₂-terminal amino acid sequence was determined according to the method of Matsudaira (33). Briefly, the purified 39-kD protein was separated on a preelectrophoresed 10% SDS-PAGE gel under nonreducing conditions and transferred to a PVDF Immobilon-P filter presoaked in methanol. Protein was visualized by staining with 0.05% Coomassie blue in a methanol/water/acetic acid (50:40:10) solvent mixture. The blots were destained in the same ratio of methanol/water/acetic acid. The portion of the membrane containing the 39-kD protein band was excised and subjected to automated Edman degradation on a sequenator (A470; Applied Biosystems, Inc., Foster City, CA). Each sample applied to the SDS gel contained ~2–3 µg of protein as determined by the BCA protein estimation method (Pierce Chemical Co., Rockford, IL).

For amino acid composition, the PVDF membrane containing the 39-kD protein was stained with 0.1% Ponceau-S (Sigma Chemical Co.) in 1% acetic acid. The section of the membrane containing the protein band was excised and destained with water. This section of membrane was hydrolyzed in 6 N HCl/0.1% phenol at 110°C for 22 h. Amino acids were separated on a Novapak C8 column and analyzed using the Maxima software, 510 pump, and 490 detector (Waters Associates, Milford, MA). Cysteine content was also analyzed from a PVDF membrane with bound 39-kD protein carboxy amidomethylated with mercaptoethanol as described (34), and hydrolyzed and analyzed as described for amino acid analysis. All analyses were performed by the Protein Biotechnology Facility of The Rockefeller University.

Molecular Mass Determination. Molecular mass of the purified protein was determined in the laboratory of Mass Spectrometry and Gas Phase Ion Chemistry of The Rockefeller University using a modified method of the matrix-assisted laser desorption technique (35).

GAPDH Activity. The assay for GAPDH activity was carried out according to the method originally described by Ferdinan (36) with minor modifications. Since GAPDH catalyzes the oxidative phosphorylation of D-glyceraldehyde-3-phosphate to form 1,3-diphosphoglycerate in the presence of β-nicotinamide adenine dinucleotide-positive (NAD⁺) and inorganic phosphate, the assay buffer was composed of triethanolamine (40 mM), Na₂HPO₄ (50 mM), and EDTA (5 mM), pH 8.6. Disposable semi-micro (1.5-ml capacity) spectrophotometer cuvettes (VWR Scientific) contained 7 µl glyceraldehyde-3-phosphate (G-3-P) (49 mg/ml; Sigma Chemical Co.), 100 µl NAD (10 mM; Boehringer Mannheim Biochemicals), and assay buffer to a final volume of 1.0 ml after the addition of enzyme source resulting in a pH of 8.6. Different concentrations of the 39-kD protein were used to plot a standard curve for the absorbance at 340 nm/min as a measure of conversion of NAD to NADH using a Spectronic 3000 array spectrophotometer (Milton Roy, Rochester, NY).

Enzyme Kinetics. Kinetics of the enzymatic activity of the 39-kD protein (25 µg) was determined with varying concentrations of G-3-P with a fixed concentration of NAD and vice versa to, respectively, determine the Km and Vmax for G-3-P and NAD. The results were recorded as the rate analysis of NADH release at 0.5-s intervals for a period of 1 min at 340 nm. The molar extinction coefficient of NADH (6.22 × 10³ [37]) was used to convert absorbance (340 nm)/min to M NADH/min. The kinetics coefficients (Km) were calculated from the values obtained for the intercepts and slopes of the double reciprocal plots of Lineweaver and Burk (38) with respect to each substrate. The specific activity of the enzyme (U/mg) was measured using the equation: specific activity = v(1 + Km/S)NAD (1 + Km/S)G-3-P, where v = µmol NADH/min/mg of enzyme.

The GAPDH activity for the 39-kD protein was measured in the lysin extract, dialyzed ammonium sulfate precipitate, and pooled fraction at various purification stages.

Rabbit Immunization and Affinity Purification of Immune Sera. New Zealand white rabbits were immunized subcutaneously with 200 µg of the purified 39-kD protein emulsified in CFA (1:1) at multiple sites. Rabbits were boosted once with 200 µg of the protein inIFA (1:1) 30 d later. Rabbits were bled 10 d after the second immunization. All sera were filter sterilized and stored at 4°C.

39-kD-specific antibodies were affinity purified from the polyclonal sera on columns containing 2.0 mg of the purified 39-kD protein linked covalently to glutardialdehyde activated beads as described (39). Anti-39-kD protein antiserum (2–3 ml) was adsorbed to and eluted from the immunoabsorbent column, dialyzed, concentrated, and stored as described (39). The antibodies were further purified on a protein A-Sepharose column (Pharmacia LKB Biotechnology Inc.) essentially as described (39). The monospecificity of the antibody was checked against crude lysin extracts by Western blot as described below.

Dot-Blot Immunoassay to Determine the Location of the 39-kD Protein. The surface location of 39-kD protein was determined with the monospecific antibodies using a bacterial dot-blot immunobassay as previously described (31, 40). Essentially, an overnight culture of strain D471 was adjusted to OD₆₀₀ 1.0 with 50 mM Tris/HCl buffer, pH 8.5. Aliquots of this suspension were centrifuged and resuspended to the same volume of buffer containing either 2 M NaCl or 2% SDS, and rotated at room temperature for 4 h, centrifuged, and the respective supernatants were saved. After washing, the pellets were again adjusted to OD₆₀₀ 1.0 with 50 mM Tris/HCl buffer, pH 8.5. In a separate experiment, the bacterial suspension in the Tris/HCl buffer was centrifuged, and the bacteria were suspended in 100 mM NH₄HCO₃ to OD₆₀₀ 1.0 and treated with trypsin (250 µg/ml) for 3 h at 37°C. Trypsin activity was inhibited with trypsin inhibitor as described above, and the bacteria were pelleted and resuspended in the Tris/HCl buffer to OD₆₀₀ 1.0. 50 µl of each bacterial suspension was transferred to nitrocellulose paper using dot-blot assembly (Bio-Rad Laboratories, Richmond, CA). Reactivity of surface-exposed epitopes of the 39-kD protein was determined using affinity-purified anti-39-kD protein antibodies (1:1,000 dilution of 0.5 mg/ml stock) as described previously (31). For densitometric analysis of the dot blot, a duplicate blot was developed with Lumi-Phos™ 530 (adamantyl-1,2-dioxetane phenyl phosphate; Lumigen Inc., Detroit, MI), which undergoes enzyme (alkaline phosphatase)-catalyzed diphosphorylation to form a dioxetane anion that is converted ultimately into an excited state of the methyl meta-oxybenzoate anion, the light emitter (41). The developer was then drained off, and the wet blot wrapped in Saran Wrap was exposed to x-ray film for 20 min and developed using conventional procedures. Densitometric analysis of each spot on the x-ray film was carried out on an image analyzer using the Dumas program (Drexel University, Philadelphia, PA) interfaced with IBM computer.
GAPDH Activity of Whole Streptococci. A whole cell assay was developed to determine whether the 39-kD protein on the surface of streptococci functions as an active GAPDH enzyme. Different concentrations of trypsinized (as described above) and nontrypsinized streptococci were incubated with and without G-3-P in the presence of NAD in triethanolamine-phosphate-EDTA dithiothreitol (DTT) buffer as described above. The reaction was allowed to occur in a final volume of 1.0 ml for a period of 2 min at room temperature, after which the bacteria were removed by centrifugation. The supernatants were analyzed for the conversion of NAD to NADH by recording the absorbance at 340 nm as described above. This enzymatic activity was also determined on streptococci preincubated with a 1:50 dilution of a 0.5 mg/ml stock of affinity-purified anti-39-kD protein antibodies to examine the inhibition of enzymatic activity.

PAGE and Western Blotting. Electrophoresis and Western blotting of protein samples were carried out as described before (31, 32). Specific proteins bound to the nitrocellulose membrane were probed and visualized with affinity-purified anti-39-kD protein antibodies (1:2,000 dilution of 0.5 mg/ml stock) as described previously (31, 32).

Presence of a 39-kD Protein on Different Streptococcal M Serotypes and Streptococcal Groups. Lysin extracts of M serotypes 2, 4, 5, 6, 24, 29, 41, 57, 58, 60, and M+ were prepared as described (31, 32). The murexyl enzyme mutanolysin (20 μg/ml; Sigma Chemical Co.) was used to prepare cell walls: extracts of each grouping strain suspended in 50 mM Tris/HCl buffer, pH 6.8, containing 5 mM EDTA, 5 mM MgCl2, and 30% raffinose, and incubated at 37°C for 60 min under constant end-to-end rotation. Proteins in all the extracts were separated on SDS-PAGE and transferred to nitrocellulose. The blots were probed with affinity-purified anti-39-kD protein antibodies as described above.

Relationship of GAPDH from Bacterial and Mammalian Origins with the 39-kD Protein. The crossreactivity of GAPDHs isolated from rabbit skeletal muscle, human erythrocytes, and Bacillus stearothermophilus were determined both by Western blot and competitive ELISA (described below).

ELISA and Competitive Inhibition. ELISA was performed as described earlier (42), except that ELISA plates were coated with 100 μl/well of 1 μg/ml of purified 39-kD protein for 3 h at 37°C and then overnight at 4°C.

Competition ELISA was used to determine the relationship of GAPDH molecules from bacterial and mammalian origin with the 39-kD protein by methods described previously (40). Briefly, competing GAPDHs were serially diluted in 10 mM sodium phosphate, pH 7.4, containing 0.05% Brij-35 (40) starting with 100 times molar excess relative to the 39-kD protein coating the plates. Affinity-purified antibodies to the 39-kD protein were then diluted to a dilution resulting in an ELISA reading of 1.0 at 405 nm after 60 min. Antibodies diluted in this way were then added to each well and the plates processed for kinetic ELISA analysis as described (42) using an ELISA-5 microtiter plate reader (Pierce Chemical Co., Rockford, IL).

Radioiodination of Proteins. The 39-kD protein (400 μg) was labeled with 125I by the chloramine-T method using iodobeads (Pierce Chemical Co., Rockford, IL). The labeled protein was separated from free iodine by filtration over a column of Sephadex G-25 (PD-10; Pharmacia LKB Biotechnology Inc.) and collected in 100 mM phosphate buffer saline, pH 6.5. The labeled protein was stored at −20°C in aliquots containing 0.02% Na3HCO3. Fibronectin and plasmin were labeled essentially by the same method. The specific activity of the 39-kD protein, fibronectin, and plasmin after labeling was 2 × 106, 1.0 × 106, and 1.21 × 106 cpm/μg, respectively.

Binding Studies. The binding activity of the 39-kD protein, fibronectin, and plasmin was determined by the use of the 125I-labeled proteins. Egg white-lysozyme and/or cytoskeletal proteins (myosin, heavy meromyosin [HMM], light chain myosin [LMM], tropomyosin, and actin) and streptococcal M protein (43) were separated on 10% SDS-PAGE gels and electrophobted onto nitrocellulose paper. The blots were blocked in 10 mM Hepes buffer containing 150 mM NaCl, 10 mM MgCl2, 2 mM CaCl2, 50 mM KCl, 0.5% Tween-20, 0.04% NaN3, and 0.5% BSA, pH 7.4, for 2–3 h at room temperature, and probed for 3–4 h at room temperature in the same buffer containing 125I-fibronectin, 125I-39-kD protein, or 125I-plasmin adjusted to 3 × 106 cpm/ml. The probed blots were then washed three to four times with blocking buffer, dried, and exposed to Kodak Blue Brand film with an intensifying screen for 36–48 h at −70°C.

Results

Digestion of the streptococcal cell wall with lysozyme at pH 6.1 in the presence of 30% raffinose results in the release of M protein (31, 32). SDS-PAGE of an M6 streptococcal lysin extract indicates that in addition to the M protein, a number of other proteins are found in the cell wall digest, some at an apparent higher concentration than the M protein molecule based on Coomassie blue staining (Fig. 1, lane a). To further characterize these other wall-associated proteins, we first selected the molecule at 39 kDa.

Purification of the 39-kD Protein. The 39-kD molecule was partially purified from the crude lysin extract (Fig. 1, lane a) by ammonium sulfate precipitation (lanes b and c). Mono Q ion-exchange chromatography (lane d), and molecular sieving chromatography on a Superose 12 column (lane e). Remaining contaminants were separated from the 39-kD molecule by hydrophobic chromatography. SDS-PAGE of the final purified preparation revealed a homogeneous protein with a molecular mass of 39 kDa (Fig. 1, lane f). The total yield of purified protein from 4 liters of culture representing 6–8 g wet weight of bacteria was 800 μg. The peak elution volume of the 39-kD protein on a Superose 12 column was the same for IgG (150 kDa) (data not shown), suggesting that either the molecule...
exists as a tetramer or it behaves anomalously on a molecular sieve.

NH₂-terminal Sequence and Amino Acid Composition. 
NH₂-terminal amino acid sequence analysis of the purified 39-kD protein confirmed the homogeneity of the preparation resulting in a single amino acid at nearly all positions (Fig. 2 a). Except for positions 31 and 35, a single amino acid was identified in the first 35 residues with the remaining four tentatively identified because of the low yield.

The amino acid composition of the purified protein indicated a high content of Asp/Asn (12.1%), followed by Ala (10.7%), Gly (10.3%), Val (10.2%), and Glu/Gln (8.4%).

The mass of the purified protein (35,882 daltons) as determined by laser desorption mass-spectrometry was used to more precisely assign the number of residues/mol (Table 1).

Amino Acid Sequence and Composition Comparison. When the sequence of the first 39 amino acids of the 39-kD protein was compared to known sequences in the translated GenBank database (Fig. 2 b), significant identity was found with bacterial and eukaryotic GAPDHs. The identity within the first 18 residues was 77-83% with bacterial, eukaryotic, or fungal GAPDHs. This strong homology decreased over the remaining 21 residues with an overall identity of 41-56% (Fig. 2 b).

When the amino acid compositions of the various GAPDHs were compared, the methionine content of the 39-kD protein was found to be significantly low (1.8 residue/mol) with relation to the eukaryotic (8.4 residue/mol) or other bacterial GAPDHs (7 residues/mol) (Table 1). Although the amino acid compositions of the remaining residues of the 39-kD protein were found to be relatively close to that of the other GAPDHs, sufficient differences were found that suggest that, except for the NH₂-terminal sequence, the streptococcal protein may be structurally different from reported GAPDHs.

GAPDH Activity of the 39-kD Protein. The strong NH₂-terminal sequence homology of the purified 39-kD protein with GAPDH prompted us to check whether the protein possessed any GAPDH activity. In the presence of G-3-P in triethanolamine buffer at pH 8.6, the 39-kD protein showed a dose-dependent conversion of NAD to NADH, as observed by absorbance of the latter at 340 nm. A variation of the enzyme reaction rate with different concentrations of G-3-P and NAD was determined using 25 µg of the purified 39-kD protein. The results were analyzed both as Michaelis-Menten and double reciprocal plots according to Lineweaver and Burk (38) as shown in Fig. 3, a and b. From these plots the Km for G-3-P and NAD was estimated to be 1.33 mM and 156.7 µM, respectively, and Vm, was 0.487 x 10⁻³ M NADH/min. The specific activity (34 U/mg) of a typical run of purified 39-kD protein was calculated using Ferdinand's equation (36): 1.91 x v, where v is µM NADH/min/mg.

Tetrameric Structure. From the anomalous behavior of the 39-kD protein on a Superose 12 molecular sieve suggesting a molecule four times larger than that observed by SDS-PAGE and the fact that functional GAPDH molecules are tetrameric structures, we reasoned that the 39-kD protein may be seen under mild denaturing condition. To examine this, SDS-PAGE was performed with the 39-kD protein saturated with NAD to stabilize the complex in a sample buffer containing no SDS or β-mercaptoethanol and without boiling; however, SDS was present in the gel and running buffer. Under these conditions, a major protein band was found at 39 kD with a minor band at 150 kD by Coomassie stain (Fig. 4 A). This finding was further confirmed by Western blot using affinity-
Table 1. Comparison of Amino Acid Composition of SDH Protein from M Type 6 Streptococci with That of GAPDH from Different Species

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<td>21.4</td>
<td>23</td>
<td>23</td>
<td>23.9</td>
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<tr>
<td>Cys</td>
<td>3.1$^a$</td>
<td>2</td>
<td>1</td>
<td>3.0</td>
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</tr>
<tr>
<td>Trp</td>
<td>ND</td>
<td>2</td>
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</tr>
</tbody>
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Bst, B. stearothermophilus (52); Thaq, Thermus aquaticus (52); RSM, Rabbit skeletal muscle (9).

* Mean of three determinations.
† Molecular mass of SDH protein (35,882) was measured by laser desorption mass spectrometry.
§ Determined by carboxy amidomethylation method (34).

purified anti-39-kD protein antibodies, which reacts only with the 39-kD protein in a crude lysin extract (Fig. 4 B). Taken together, these findings suggest that a tetrameric form of the 39-kD protein may be the native state of the molecule on the streptococcal cell surface.

Location of the 39-kD Protein on the Streptococcal Cell.

Affinity-purified anti-39-kD protein antibodies were used in a dot-blot immunoassay using whole streptococci to determine the location of the protein. Trypsin treatment of whole streptococci resulted in a marked reduction in their reactivity to anti-39-kD antibodies (Fig. 5). The inability to remove the 39-kD protein from the streptococcal surface after washing in 2 M NaCl or 2% SDS (Fig. 5) indicates that the protein is not peripherally associated but tightly bound to the cell. These data indicate that the 39-kD protein is a surface GAPDH molecule on the streptococcal cell, which we hereafter term SDH.

GAPDH Activity of Whole Streptococci.

To determine if the GAPDH activity of SDH was also present on the streptococcal surface, enzymatic studies were carried out using whole streptococci. The same concentration of substrates (G-3-P and NAD) employed with the purified SDH protein were used with whole streptococci. Data presented in Fig. 6 a reveal a dose-dependent GAPDH activity catalyzed by the whole organisms. As found with the purified SDH protein, the intact bacteria also did not catalyze the reaction in the absence of the substrate G-3-P in the presence of NAD (100 µM) in triethanolamine-phosphate-EDTA-DTT buffer at pH 8.6. The Km for G-3-P was estimated to be 1.33 mM; Vmax, 0.487 × 10⁻³ M NADH/min; intercept on y-axis(Vmax), 2.05; and slope (Km/Vmax), 2.73. The inset shows the analysis based on the method of Michaelis-Menten. Km, 1.22 mM; and Vmax, 0.466 × 10⁻³ M NADH/min. (b) 25 µg of the 39-kD protein was assayed as a function of NAD in the presence of G-3-P (2 mM) in the buffer system as described in a. The Km for NAD was estimated to be 156.7 µM; Vmax, 0.459 × 10⁻³ M NADH/min; intercept on y-axis(Vmax), 2.18; and slope (Km/Vmax), 341.74. Km for NAD by the method of Michaelis-Menten as shown in the inset was estimated to be 148.86 µM; and Vmax, 0.445 × 10⁻³ M NADH/min.

Figure 3. Lineweaver-Burk's double reciprocal kinetic analysis of GAPDH activity of the 39-kD protein. (a) 25 µg of 39-kD protein was assayed as a function of G-3-P in the presence of NAD (100 µM) in triethanolamine-phosphate-EDTA-DTT buffer at pH 8.6. The Km for G-3-P was estimated to be 1.33 mM; Vmax, 0.487 × 10⁻³ M NADH/min; intercept on y-axis(Vmax), 2.05; and slope (Km/Vmax), 2.73. The inset shows the analysis based on the method of Michaelis-Menten. Km, 1.22 mM; and Vmax, 0.466 × 10⁻³ M NADH/min. (b) 25 µg of the 39-kD protein was assayed as a function of NAD in the presence of G-3-P (2 mM) in the buffer system as described in a. The Km for NAD was estimated to be 156.7 µM; Vmax, 0.459 × 10⁻³ M NADH/min; intercept on y-axis(Vmax), 2.18; and slope (Km/Vmax), 341.74. Km for NAD by the method of Michaelis-Menten as shown in the inset was estimated to be 148.86 µM; and Vmax, 0.445 × 10⁻³ M NADH/min.
Prevalence of SDH in Other M Serotypes and Groups of Streptococci. The presence of the SDH protein in different streptococcal M serotypes was determined by Western blot analysis of lysin extracts using affinity-purified anti-SDH antibodies. As shown in Fig. 7 A, a similar high concentration of the SDH protein was found in all the serotypes tested, even a strain defined as M protein negative based on an extensive genetic deletion (30). Furthermore, all reactive bands were found to be of the same apparent molecular size as the purified SDH molecule without any obvious indication of heterogeneity.
Figur| 8. Competition kELISA with immobilized SDH protein. Com-mercially available purified GAPDH from B. stearothermophilus, human erythrocytes, and rabbit skeletal muscle were used to compete for the binding of affinity-purified anti-SDH antibodies (1:1,000 dilution of 0.5 mg/ml stock). Each curve represents the mean of three separate experiments with <5% SD (not shown). Inset shows the Western blot of the reactivity of affinity-purified anti-SDH antibodies with (a) streptococcal SDH and GAPDHs of (b) bacterial (B. stearothermophilus), (c) rabbit skeletal muscle, and (d) human erythrocytes.

Since the lysin enzyme does not act on all streptococcal cell walls, the presence of SDH protein in different streptococcal grouping strains was seen in their respective mutanolysin extract. The results of Western blot analysis shown in Fig. 7 B reveal that the SDH protein was also found in mutanolysin extracts of all the grouping strains examined, except groups D, F, and N. The reactivity of an SDH-like protein in the group H extract was found to be weaker when compared with that of the others.

Relationship of SDH with GAPDHs of Bacterial, Animal, and Human Origin. The relationship of SDH with known GAPDHs was determined by both Western blot and competitive kinetic (k) ELISA using affinity-purified anti-SDH antibodies. Western blot analysis with SDH-specific antibodies revealed that GAPDH from bacillus, human RBCs, and rabbit muscle reacted weakly or not at all (Fig. 8, inset). This finding was further confirmed by competition ELISA (Fig. 8) showing that only a maximum of 20–25% inhibition of binding of anti-SDH antibodies to SDH could be achieved with 100 molar excess of these proteins, with the rabbit muscle GAPDH exhibiting the least activity. The fact that almost 20% inhibition is observed with <20 times molar excess of bacillus and human GAPDH may reflect the sequence homology observed at the NH₂ termini of these molecules (Fig. 2).

Binding of SDH to Cytoskeletal Proteins, Lysozyme, and Fibronectin. Since many glycolytic enzymes have been shown to specifically bind cytoskeletal proteins (8, 44), we examined whether SDH has a similar property. ¹²⁵I-SDH was used to probe a Western blot containing several cytoskeletal proteins. The results revealed a specific binding to myosin (Fig. 9, A and B, lanes a) and its globular domain (heavy meromyosin) (lanes b) and actin (lanes d), but not to the α-helical domain of myosin (light meromyosin) (lanes c), tropomyosin (lanes e), or streptococcal M protein (lanes f). In addition to the above proteins, the ¹²⁵I-SDH was also found to strongly bind to lysozyme (Fig. 9, lanes g). Since a fibronectin binding property has been reported to be present on this streptococcal strain (9), we tested the fibronectin binding activity of the SDH protein using either ¹²⁵I-labeled fibronectin or fibronectin-anti-fibronectin on a Western blot.

Figure 9. Binding of ¹²⁵I-SDH to cytoskeletal proteins. (A) Coomassie blue-stained SDS-PAGE gel (10%) containing 5 µg protein of various cytoskeletal proteins, lysozyme, and M6 protein (43). Lane a, rabbit skeletal myosin; lane b, heavy meromyosin; lane c, light meromyosin; lane d, actin; lane e, M6 protein; lane f, S-2 fragment of heavy meromyosin; lane g, egg white lysozyme. (B) Autoradiograph of proteins in a duplicate gel after transfer onto nitrocellulose and incubation with the ¹²⁵I-SDH. The proteins in each lane are described in A. For details of each molecular mass marker on the left, see Fig. 1.
Figure 10. Binding activity of SDH to fibronectin. (A) Coomassie stain of an SDS gel containing 5 μg of SDH and BSA. (B) Western blot analysis of a duplicate gel showing the binding of fibronectin followed by antifibronectin to the SDH molecule. (C) Autoradiograph of a similar Western blot showing the binding of 125I-fibronectin to the SDH protein. Lanes a, c, and e, SDH; lanes b, d, and f, BSA.

The results revealed that fibronectin bound specifically to SDH and not to BSA in both assays (Fig. 10).

Discussion

Because one of the sites of the primary infection by group A streptococci is the pharynx, it is likely that initial events involve an interaction of the organism with salivary components before attachment to pharyngeal epithelial cells, particularly the tonsillar mucosa. These initial events ultimately result in the colonization and subsequent infection of the target tissue. It is, however, unknown which surface molecule(s) play a role in one or more of these early events. In the present study, we have focused our attention on a 39-kD surface protein that comprises a significant amount of the total protein found in a lysin extract of whole streptococcal cells. This molecule is found at concentrations higher than the surface M protein in this and other streptococci examined. Because of its relatively high level of expression on the cell surface, and recent evidence (5, 6) indicating that neither M protein nor LTA alone or in combination take part in adherence to pharyngeal epithelial cells, we examined the characteristics of this surface protein in the hope to understand its role (if any) in the colonization and/or infection process.

The 39-kD protein was purified to homogeneity from streptococcal cell wall extracts and its Nt-terminal sequence compared with sequences collected in the translated GenBank database. Over 80% of the Nt-terminal 18 of 39 amino acids were identical to the GAPDH family of enzymes. The enzymatic activity of the purified protein was verified by standard assays for GAPDH activity. A specific activity of 34 U/mg of purified protein was found to be comparable to other reported GAPDH enzymes (11, 45, 46). SDS gel electrophoresis and Western blot analyses of the unboiled SDH protein sample containing no SDS and saturated with NAD to stabilize the tetrameric structure (47) indicated the presence of a protein band in a position consistent with a molecule of 150 kD, as found for the SDH by molecular sieve chromatography (Fig. 4). This molecular form could not be seen using a standard SDS sample buffer without reducing agent, suggesting that the subunits are not held by sulfhydryl bonds despite the presence of three cysteine residues/mole (Table 1). The heat stability of the tetrameric structure of GAPDHs from thermophilic bacillus species compared with those from eukaryotes and SDH can be attributed to the presence of extra ionic bonds located between the enzyme subunits of the bacillus GAPDH enzymes (48). Since the enzymatic activity of GAPDH is dependent upon its tetrameric structure, the GAPDH activity of whole streptococci support the idea that the SDH may also exist as a tetramer on the streptococcal surface.

The Km for G-3-P (1.33 mM) in the present investigation is about the same magnitude as was found for GAPDH from B. stearothermophilus (1.0 mM) (46), but 22 times higher than rabbit muscle GAPDH (60 μM) (45) and up to 238 times higher than that reported for GAPDH from human erythrocytes (5.6 μM) (11). Similarly, the Km for NAD (156.7 μM) of SDH was ~2.4 times that for B. stearothermophilus (66 μM) (46) or only 1.5–4-fold higher than reported for GAPDH enzymes from rabbit muscle (45) and human erythrocytes (11). This finding possibly reflects a conservation within the NAD binding domain, which is located at the NH2 terminus of the GAPDH molecule, and limited conservation within the catalytic domain as reported recently by Nelson et al. (49). The strong NH2-terminal sequence similarity of the SDH protein with other GAPDHs supports the idea. It is not possible to make further predictions regarding the structure of this protein without the complete amino acid sequence. However, amino acid composition comparison with other GAPDH molecules reveals sufficient homology to predict a certain degree of relatedness but indicates that SDH is likely to be structurally distinct from other known GAPDH molecules. This difference was verified by Western blot indicating a weak reactivity of anti-SDH antibodies with known GAPDH molecules. Furthermore, solid-phase competitive kELISA, at up to 100 times molar excess of bacillus and eukaryotic GAPDHs, revealed only 20–25% inhibition of reactivity of anti-SDH with SDH, confirming its limited relatedness with GAPDHs of both bacillus and eukaryotic origin, possibly confined to the NH2-terminal region of these molecules. The NH2-terminal sequence of the SDH protein revealed the presence of a motif (Gly-X-Gly10-X-X-Gly10) (Fig. 2) found in other GAPDHs and shown to be a putative ATP-binding domain (50). By Western blots, [32P]ATP was found to bind to eukaryotic GAPDHs but not bacillus GAPDH or the SDH protein (data not shown). Since both eukaryote and prokaryote GAPDH molecules have the same motif, the difference in ATP binding may reflect variations found in the regions flanking the motif (Fig. 2 b).

Historically, GAPDH is known to be a cytoplasmic enzyme. However, recent reports (9–12) have shown that...
GAPDH from eukaryotes may also be membrane bound. SDH was isolated from the lysin extract of streptococci that represented mainly surface located proteins. This was confirmed by its sensitivity to trypsin digestion of whole streptococci resulting in both the loss of the 39-kD band from the lysin extract, and a significant decrease in the anti-SDH antibodies reactivity of whole streptococci. The inability of 2% SDS or 2 M NaCl to remove the SDH protein from intact streptococci suggests that, like M protein (51), SDH is firmly bound to the cell and is not peripherally associated, however, the nature of its cell attachment is not apparent. In bacteria, GAPDH has been reported and purified from cytoplasmic fractions (52), however, there have been no reports of an active enzyme on a bacterial surface. Detection of a dose-dependent GAPDH activity of intact streptococci in presence of the appropriate substrates clearly indicates that SDH is enzymatically active on the streptococcal surface. Only a small quantity of protein reactive with the anti-SDH antibodies by Western blot was seen in the growth supernatant after 50-fold concentration (data not shown), demonstrating that the SDH is not a secretory product of the group A streptococcus.

Recently, a strong plasmin binding protein from group A streptococci (53) was reported to have extensive sequence homology with GAPDH (54). Using radioiodabeled plasmin, we were able to detect weak binding to the SDH molecule by Western blot, indicating that SDH may be different from the reported strong plasmin binding molecule (data not shown). Although the GAPDH enzymatic activity of the strong plasmin binding molecule was not determined (53, 54) its homology with GAPDH may suggest that a family of related molecules may exist on the streptococcal surface. It is conceivable that the surface-bound forms of GAPDHs are subject to sequence variation to perform functions in addition to their NH2-terminal dehydrogenase activity, while cytoplasmic forms of GAPDH may remain more conserved throughout the molecule.

Goudot-Crozel et al. (55) recently presented evidence for an active GAPDH on the surface of Schistosoma mansoni. They showed that sera from subjects with low susceptibility to infection by this parasite reacted with this protein while sera of susceptible individuals had little or no activity. They proposed that human T cell response to GAPDH would be genetically restricted resulting in a population of nonresponders to this protein, allowing the parasite to have an increased chance to infect. Although this hypothesis requires further experimental support, in terms of the current finding it may shed some light on the pathogenesis of streptococcal infections and associated sequelae.

Western blot analysis of lysin extracts from different streptococcal M types revealed the presence of SDH in all the M types tested with no observed size heterogeneity as found with the M protein. The ubiquitous nature of the SDH protein among group A streptococci along with its relatively high concentration may reflect a common and perhaps important function for the organism. This ubiquitous nature was found to a limited extent among different groups of streptococci. The absence of anti-SDH-reactive protein in the mutanolysin extracts of streptococcal groups D, F, and N indicates either the complete lack of this protein or presence of antigenically unrelated SDH-like molecule.

To determine if the SDH protein plays a role in the early events of infection, we examined its relationship to a recently reported fibronectin binding protein found on the surface of group A streptococci (6). We discovered that 125I-SDH is able to bind to both fibronectin and lysozyme by Western blot. Whether this attachment to fibronectin is mediated by an RGD sequence or another specific sequence motif (56) will demand additional sequence data. Because lysozyme is a polycationic molecule, the observed binding of SDH to this protein may be charge dependent. However, the possibility that the strong binding between these two molecules may be mediated by a specific sequence motif can not be excluded.

Since SDH binds to both fibronectin and lysozyme, and both are found in soluble form in saliva as well as plasma, they may influence the streptococcal surface during colonization and/or infection.

Many glycolytic enzymes such as aldolase, lactate dehydrogenase, and GAPDH have been found to bind with high affinity to structural proteins of the muscle such as F-actin, myosin, and actomyosin (8, 44). However, information regarding the binding domains within these proteins is not known. In the present study, we have localized the binding site of myosin for SDH to be in the myosin globular domain (S-1 fragment of HMM) and not the α-helical portion (S-2 fragment of HMM and LMM). Similarly, SDH did not bind to the α-helical coiled-coil molecules tropomyosin, M6 protein, or the light chain of myosin. Since cytoskeletal molecules are restricted to the cytoplasmic side of the cell membrane and are not located on the cell surface, the significance of the interaction of SDH with the ATPase domain of myosin and to actin is not readily apparent.

It is not surprising that SDH has both enzymatic and multiple binding activity since several recent reports have suggested an involvement of eukaryotic membrane-bound GAPDH in a variety of cellular events. These include: (a) a regulatory role in the bundling/unbundling of microtubules isolated from brain by a mechanism involving conversion of enzyme tetramers (which catalyze the bundling of microtubules) into dimers (which unbundle microtubules) in an ATP-dependent manner (13); (b) involvements in the assembly of junctional triads from transverse tubes in skeletal muscle cells (9); (c) protein kinase activity of GAPDH to phosphorylate transverse-tubule protein (14); (d) single-stranded DNA-binding activity regulating transcription similar to that observed for nonhistone protein (15, 57); and (e) human nuclear uracil DNA glycosylase activity (16). Thus, in the case of SDH, its GAPDH activity coupled with its multiple binding capacity for fibronectin, lysozyme, and cytoskeletal proteins may reflect the complexities involved in the infection process.
We thank Emil C. Gotschlich for his helpful comments on the manuscript.

This work was supported by Public Health Service grant AI-11822 from the National Institutes of Health.

Address correspondence to Vijaykumar Pancholi, Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University, 1230 York Avenue, New York, NY 10021.

Received for publication 8 April 1992.

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