Spr1479 from *Streptococcus pneumoniae* R6 is a 33-kDa hypothetical protein of unknown function. Here, we determined the crystal structures of its apo-form at 1.90 Å and complex forms with inorganic phosphate and AMP at 2.30 and 2.20 Å, respectively. The core structure of Spr1479 adopts a four-layer αββα-sandwich fold, with Fe^{3+} and Mn^{2+} coordinated at the binuclear center of the active site (similar to metallophosphohydrolases). Enzymatic assays showed that, in addition to phosphodiesterase activity for bis(3-nitrophenyl) phosphate, Spr1479 has hydrolase activity for diadenosine polyphosphate (Ap_{n}A) and ATP. Residues that coordinate with the two metals are indispensable for both activities. By contrast, the streptococcus-specific residue Trp-67, which binds to phosphate in the two complex structures, is indispensable for the ATP/Ap_{n}A hydrolase activity only. Moreover, the AMP-binding pocket is conserved exclusively in all streptococci. Therefore, we named the protein SapH for *streptococcal ATP/Ap_{n}A and phosphodiester hydrolase.*

The commensal Gram-positive bacterium *Streptococcus pneumoniae* is a major human pathogen that is responsible for many infectious diseases such as pneumonias, sepsis, otitis media, and meningitis (1). Beyond its capsule, the virulence factors of this pathogen are not fully understood. Since the release of the complete genome sequences of virulent (TIGR4) and non-virulent (R6) isolates of *S. pneumoniae*, the identification of virulence factors and their characterization as new potential antibiotic and/or vaccine targets have been in progress (2–4). In addition to the surface-exposed proteins, a large array of hypothetical proteins have been proposed to contribute to the streptococcal pathogenesis (4, 5).

The 33-kDa hypothetical protein Spr1479 from *S. pneumoniae* R6 was predicted to be a virulence factor. It belongs to the core genome, and its orthologs have also been found in other streptococci (5). Recently, the *Mycobacterium tuberculosis* cyclic nucleotide phosphodiesterase Rv0805, a distant homolog of Spr1479, was reported to play a key role in the pathogenicity of mycobacteria not only by hydrolyzing bacterial cAMP but also by moonlighting as a protein that can alter cell wall functioning (6). A sequence homology search against the Pfam Database (7) indicated that Spr1479 belongs to the calcineurin-like phosphoesterase (also referred to as metallophosphodiesterase (MPP)^{4} superfamily. Members of this superfamily, which are widespread in all organisms, include protein phosphoserine phosphatases, nucleotidases, sphingomyelin phosphodiesterases, 2',3'-cAMP phosphodiesterases, and nucleases, as well as human VPS29 (vacuolar protein-sorting protein 29). Previous structures have shown that these members share a common αββα-sandwich fold, with an active site composed of two metal ions (typically manganese, iron, or zinc) that are coordinated in an octahedral geometry with the residues His, Asp, and Asn. This highly conserved active site involves a common catalytic mechanism: a hydroxide anion, activated by the binuclear metals, attacks the phosphorus atom of the substrate (8–12).

A sequence homology search (blast.ncbi.nlm.nih.gov/Blast.cgi) of Spr1479 against the Swiss-Prot Database (ExPaSy) gave only hits of low identity. The top hit is an uncharacterized protein, MJ0912 (accession number Q58322) from *Methanococcus jannaschii*, which has a 27% sequence identity of over 253 residues with Spr1479. Because the next closest hits are ApaH-related symmetric diadenosine 5',5''-P_{1},P_{4}-tetraphosphate (Ap_{4}A) hydrolases (EC 3.6.1.41; 21% sequence identity), which hydrolyze one Ap_{4}A into two ADP molecules (13), we analyzed the *in vitro* hydrolase activity of Spr1479 for Ap_{4}A, as well as Ap_{3}A and Ap_{5}A. Against our expectation, Spr1479 catalyzed cleavage of these molecules asymmetrically with AMP as the common product. This resembles the activity of Nudix (nucleoside diphosphate linked to X) proteins, which catalyze the hydrolysis of NDP linked to another moiety such as NTP.
Ap$_2$A, NDP-sugar, NADH, and coenzyme A, etc. (14). Members of this superfamily have an $\alpha$-$\beta$-sandwich fold (15–19) and the Nudix signature motif GX$_2$EX,REUXXEEXG (where X stands for any residues, and U is a bulky aliphatic residue, usually Ile, Leu, or Val) (20). This motif forms a loop-helix-loop structure that is involved in substrate binding and catalysis (21). It has been proposed that the physiological function of Nudix proteins is “housecleaning” to eliminate potentially toxic nucleotide metabolites from cells and to regulate the concentrations of NDP derivatives (20).

To decipher the molecular function of Spr1479, we solved its crystal structures in the apo-form at 1.90 Å and complex forms with inorganic phosphate and AMP at 2.30 and 2.20 Å, respectively. The core structure and binuclear active site of Spr1479 resemble those of the MPP superfamily. In addition to phosphodiesterase activity for the generic substrate bis(p-nitrophenoxy) phosphate (bis-pNP), Spr1479 has hydrolase activity for Ap$_2$A and ATP. Thus, Spr1479 might function not only as a phosphodiesterase but also as a housecleaning protein to keep the homeostasis of intracellular nucleotides.

**EXPERIMENTAL PROCEDURES**

Cloning, Expression, and Purification of SapH and Its Mutants—The coding regions of Spr1479/SapH (residues 1–262) and full-length SapH (SapH-FL; residues 1–282) were amplified from genomic DNA of *S. pneumoniae R6* and individually cloned into a PET28a-derived vector and overexpressed in the *Escherichia coli* Rosetta(DE3) strain (Novagen, Madison, WI) using 2×YT culture medium (5 g of NaCl, 16 g of Bacto-Tryptone, and 10 g of yeast extract per liter). A hexahistidine tag was added to the N terminus of each of the recombinant proteins. The cells were grown at 37 °C to an A$_{600}$nm of 0.6. Expression of the recombinant protein was induced with 0.2 mM isopropyl $\beta$-d-thiogalactoside for another 20 h at 16 °C before harvesting. Cells were collected by centrifugation at 4000 × g for 20 min and resuspended in 40 ml of lysis buffer (20 mM Tris-Cl (pH 8.0) and 200 mM NaCl). After 2.5 min of sonication and centrifugation at 12,000 × g for 25 min, the supernatant containing the target protein was collected and loaded onto a nickel-nitrilotriacetic acid column (GE Healthcare) equilibrated with the binding buffer (20 mM Tris-Cl (pH 8.0) and 200 mM NaCl). The target protein was eluted with 300 mM NH$_4$Ac). The resultant model was built automatically with the program phenix.solve implemented in PHENIX (24). The initial model was built automatically with the program AutoBuild in PHENIX. The resultant model was subsequently used as a search model against the 1.90 Å data of SapH. Using the SapH structure as the search model, the structures of SapH-PO$_4$ and SapH-AMP were determined by the molecular replacement method with the program MOLREP (25) implemented in CCP4i (26). All initial models were refined using the maximum likelihood method implemented in REFMAC5 (27) as part of the CCP4i program suite and rebuilt interactively using the program COOT (28). The final models were evaluated with the programs MOLPROBITY (29) and PROCHECK (30). Crystallographic parameters are listed in Table 1. All structure figures were prepared with PyMOL (31).

Determination of Active-site Metals—Atomic absorption spectroscopy (Atomicscan Advantage, Thermo Ash Jarrell Corp.) was performed to determine the metal content. Prior to analysis, purified SapH-FL in 20 mM Tris-Cl (pH 8.5) and 150 mM NaCl was concentrated to ~8 mg/ml.

Enzymatic Assays—All enzymatic assays were performed at 37 °C in buffer containing 100 mM Tris-Cl (pH 7.0), 0.2 mM FeCl$_3$, and 0.2 mM MnCl$_2$. Using a DU800 spectrophotometer (Beckman Coulter), phosphatase and phosphodiesterase activities were measured with $p$NP and bis-pNP (Sigma) as substrates, respectively, by following the absorption increase at 405 nm. The kinetic parameters of wild-type SapH and its mutants were measured using bis-pNP as the substrate to a standard curve of 4-nitrophenol. Reactions were initiated by the addition of SapH or its mutants.

The hydrolysis of nucleotides/derivatives (Sigma) was measured by HPLC (Agilent 1200 series). SapH and its mutants were incubated with a range of substrate concentrations in a volume of 25 μl. The reaction was terminated after 15 min of incubation by the addition of 50 μl of 20 mM NH$_4$H$_2$PO$_4$ (pH 6.2). As a control, an assay mixture without any protein added was used. NMP standards were quantified by HPLC analysis using a series of concentrations ranging from 0.1 to 5 mM. 20 mM NH$_4$H$_2$PO$_4$ was used for equilibration of the column (Eclipse XDB-C18 column (4.6 × 150 mm), Agilent) and separation of the components at a flow rate of 1 ml/min. Samples were injected in a volume of 10 μl. The parameters $K_m$ and $k_{cat}$ were calculated by nonlinear fitting to the Michaelis-Menten equation using the
program Origin 7.5. Three independent assays were performed to calculate the means ± S.D. for all $K_m$ and $k_{cat}$ values.

RESULTS

Overall Structure of Spr1479/SapH—We initially found crystals of full-length Spr1479 (SapH-FL) but could not optimize them to a high diffraction quality. Limited proteolysis combined with LC-MS enabled us to define a relatively stable region of residues 1–262 (termed SapH). We overexpressed this region to calculate the means ± S.D. for all $K_m$ and $k_{cat}$ values.

TABLE 1
Crystal parameters, data collection, and structure refinement

|                     | SapH-KI | SapH | SapH-PO$_4$ | SapH-AMP |
|---------------------|---------|------|-------------|----------|
| Space group         | P2$_1$2$_1$2$_1$ | P2$_1$2$_1$2$_1$ | P2$_1$2$_1$2$_1$ | P2$_1$2$_1$2$_1$ |
| Unit cell (Å)       | 57.98, 75.11, 149.51; 90.00$^\circ$ | 56.56, 75.10, 148.80; 90.00$^\circ$ | 58.88, 74.94, 149.33; 90.00$^\circ$ | 57.65, 74.94, 149.13; 90.00$^\circ$ |
| Resolution (Å)      | 50.00–2.90 (3.00–2.90)$^a$ | 30.00–1.90 (1.97–1.90) | 30.00–2.30 (2.38–2.30) | 50.00–2.20 (2.24–2.20) |
| Unique reflections   | 15,110 (1456) | 50,589 (4756) | 28,781 (2920) | 33,388 (1572) |
| Completeness (%)     | 99.9 (99.4) | 99.4 (94.9) | 95.0 (98.5) | 99.3 (95.8) |
| (I/σ(I))            | 29.7 (15.6) | 21.8 (3.8) | 22.2 (3.6) | 26.1 (4.3) |
| Average redundancy   | 9.0 (17.2) | 8.2 (39.5) | 7.4 (51.7) | 6.9 (28.4) |
| Structure refinement | 11.4 (9.8) | 6.7 (5.6) | 7.0 (6.8) | 6.7 (5.8) |

$^a$ The values in parentheses refer to statistics in the highest bin.  
$^b$ The mean residues are the observed and calculated structure factor amplitudes, respectively. 
$^c$ The value of $R_{int}$ is the measure of the observed reflection and $R_{calc}$ is the calculated structure factor amplitudes, respectively. 
$^d$ $R_{int}$ was calculated with 5% of the data excluded from the refinement. 
$^e$ r.m.s.d. from ideal values. 
$^f$ Categories were defined by MOLPROBITY.

program Origin 7.5. Three independent assays were performed to calculate the means ± S.D. for all $K_m$ and $k_{cat}$ values.

RESULTS

Overall Structure of Spr1479/SapH—We initially found crystals of full-length Spr1479 (SapH-FL) but could not optimize them to a high diffraction quality. Limited proteolysis combined with LC-MS enabled us to define a relatively stable region of residues 1–262 (termed SapH). We overexpressed this region and subjected it to crystallization, and the resulting crystals were diffraction to a resolution of 1.90 Å.

An asymmetric unit contains a dimer of SapH with an interface area of ~1700 Å$^2$. Met-3–Leu-260 in subunit A and Met-3–His-252 in subunit B are well fitted in the final model. The two subunits are very similar, with an overall root mean square deviation (r.m.s.d.) of 0.15 Å over 210 Ca atoms (Fig. 1A). Size-exclusion chromatography also confirmed the existence of SapH-FL and SapH as a dimer in solution (data not shown). The two five-stranded β-sheets of subunit A are aligned antiparallel to their counterparts in subunit B, forming two continuous 10-stranded mixed β-sheets, which are perpendicular to the dimer-related non-crystallographic 2-fold axis (Fig. 1A). Each subunit adopts a compact core structure with a four-layer αβαβα-sandwich fold: two five-stranded β-sheets sandwiched by helices α1 and α2 on one side and helix α6 on the opposite side (Fig. 1B). One β-sheet is composed of strands β4–β8, whereas the facing β-sheet consists of strands β1–β3, β9, and β10 (Fig. 1B). Beyond the core structure, SapH has a cap of five α-helices (α3–α5, α7, and α8) packing against the active site. Among these five α-helices, α3–α5 (Asn-66–His-109) form one side wall of the active site, whereas the other two α-helices α7 and α8 (Val-225–Asn-249) at the C terminus might function as a scaffold. A Dali search using the five-helix subdomain revealed no hit of a similar domain. Compared with the core domain, the secondary structural elements of this additional subdomain appear to be more variable in length and structure, which may be responsible for the versatile substrate specificities among the MPP superfamily members.

The core structures of subunits A and B are packed against each other to form a dimer interface, which consists mainly of the backbones of strands β7 and β10 from both subunits. Specifically, the interface is composed of hydrogen bonds between Val-214 Oα and Arg-220 Nε, Met-216 Nα and Phe-218 Oα, Met-216 Oα and Phe-218 Nα, Phe-218 Nα and Met-216’ Nα, Phe-218 Oα and Met-216’ Nα, and Arg-220 Nα and Val-214’ Oα from strand β10 and between Leu-170 Nα and Leu-170’ Oα and Leu-170 Oα and Leu-170’ Nα from strand β7 (the residues in subunit B are labeled with a prime) (Fig. 1C). In addition to these backbone hydrogen bonds, the carboxyl group of Asp-209 (Asp-209’) forms two hydrogen bonds with Nη1 and Nη2 of Arg-200’ (Arg-200), further stabilizing the dimer.

Binuclear Metals at the Active Site—During the model building and refinement process, two electron density peaks were outstanding in the $F_o - F_c$ Fourier difference map (at the 30σ level), indicating the presence of metals. The brown color of concentrated protein in solution indicated the presence of iron. Atomic absorption spectroscopy showed that iron and manganese were present at molarity ratios to the protein of 0.7 and 0.8, respectively. X-ray diffraction experiments at various wavelengths were done to confirm the chemical identity of these metals. We collected data at a wavelength of 1.74 Å and found two strong anomalous peaks of 31.0 and 30.7 in the structure model-phased anomalous Fourier difference maps. This result excluded the presence of zinc, copper, nickel, and cobalt, which have a theoretical absorption K-edge below 1.61 Å. To further
SapH can be closely superimposed onto that of *M. tuberculosis* cyclic nucleotide phosphodiesterase Rv0805 (Fig. 2B) (37), despite the fact that SapH and Rv0805 have a sequence identity of only 17% and markedly different structures, with an r.m.s.d. of 3.3 Å over 149 Ca atoms. The highly conserved αβα-sandwich core structure and binuclear active site strongly implied that SapH might act as a phosphodiesterase, phosphatase, or Ap₅A hydrolase.

**Phosphodiesterase Activity**—We first tested the enzymatic activity of SapH for generic phosphomonoesterase or phosphodiesterase substrates (pNPP or bis-pNPP) in the presence of 0.2 mM Fe³⁺ and 0.2 mM Mn²⁺. No activity for pNPP was detected (data not shown). By contrast, both SapH-FL and SapH showed considerable activity for bis-pNPP. The *Kₘ* and *κ_{cat}* values were 1.76 ± 0.02 mM and 5.70 ± 0.12 s⁻¹, respectively, for SapH-FL and 2.46 ± 0.15 mM and 5.51 ± 0.22 s⁻¹, respectively, for SapH (supplemental Table S1). These results suggested that SapH is a phosphodiesterase.

We further systematically screened all of the reported physiological substrates of metallophosphodiesterase. These substrates fall into three groups: cyclic nucleotides, nucleic acids, and phospholipids. We tested the following representative substrates of phospholipases: 2′,3′-cAMP (Sigma), 3′,5′-cAMP (Sigma), double- or single-stranded DNA and RNA, and the generic substrate p-nitrophenylphosphoryl-choline (Sigma). However, SapH-FL showed no catalytic activity for any of the above substrates. This indicates that SapH-FL most likely performs a novel phosphodiesterase activity for a unique physiological substrate, or the activity of SapH-FL for the above-listed substrates needs the assistance of an unknown partner. Similar cases of phosphodiesterases with unknown physiological substrate have also been reported previously (32, 33).

The metal-coordinating residues have a crucial role in SapH activity. Mutation of Asp-39, His-13, or His-128 to Ala completely abolished the phosphodiesterase activity. Activity was elevated by the addition of 0.2 mM Fe³⁺ and/or Mn²⁺ and somewhat inhibited by the addition of Mg²⁺, Ni²⁺, Ca²⁺, Co²⁺, Zn²⁺, or Fe²⁺ individually (supplemental Fig. S1). Moreover, the addition of 5 mM EDTA did not change the activity (supplemental Fig. S1), indicating that the co-purified metals have a very high affinity for SapH.
Nucleotide/Derivative Hydrolase Activity—In addition to phosphodiesterases, the Dali search returned two hits of ApaH-related symmetric Ap4A hydrolases from T. brucei and S. flexneri (r.m.s. d. of 3.1 Å over 177 Ca atoms and 3.0 Å over 184 Ca atoms and sequence identities of 21 and 20%, respectively). Thus, we tested the hydrolase activity of SapH-FL for Ap4A, as well as Ap3A and Ap5A (Sigma). The results showed that SapH hydrolyzed all three molecules asymmetrically, producing ADP and AMP from Ap3A, ATP and AMP from Ap4A, and AMP from Ap5A. The $k_{cat}$ values of SapH-FL for the three substrates are comparable, whereas the $K_m$ values for Ap3A, Ap4A, and Ap5A (Sigma) are 0.28 mM for Ap3A, 2.09 mM for Ap4A, and 1.04 mM for Ap5A (Table 2). It is worth noting that the $K_m$ value for Ap3A is comparable with that of T4 bacteriophage NudE (0.41 mM) and E. coli Orf186 (0.15 mM), both of which are Nudix proteins (38, 39).

However, the molarity ratio of the product AMP is obviously higher than that of ATP in the reaction with Ap4A, suggesting that ATP might be further hydrolyzed to AMP. Assays showed that both SapH-FL and SapH had relatively low activity of hydrolyzing ATP to AMP (supplemental Fig. S2). This gave us the idea that SapH-FL should have a broad spectrum of substrate specificity. In consequence, several nucleotides and derivatives (Sigma), including ADP, ATP, ADP-ribose, NAD(H), and NADP(H), were tested. SapH-FL could somewhat hydrolyze ATP, NAD(H), and ADP-ribose but not ADP or NADP(H). The relative activities are 24% (ATP), 13% (ADP-ribose), 10% (NADH), and 2% (NADP(H)) of that of the activity for Ap3A (supplemental Table S2). Furthermore, nucleotides GTP, CTP, and UTP were also tested. SapH-FL could hydrolyze GTP and CTP at a comparable rate to ATP, whereas little activity for UTP was detected (Table 2 and supplemental Table S2). Despite the relatively lower activity of SapH-FL for these substrates, the $K_m$ value for ATP is ~2.37 mM, which is in the range of intracellular ATP concentrations (1–5 mM) (40). Thus, SapH might function as an ATP pyrophosphatase in vivo.

AMP-binding Pocket—The electrostatic potential surface of SapH revealed an extended pocket of two clefts perpendicular to each other, with binuclear metals at the corner. To reveal the substrate-binding mode, we attempted to prepare crystals of SapH in the presence of inorganic phosphate, AMP, and ATP by either crystal soaking or co-crystallization. We also tried crystallizing the inactive W67H mutant of SapH in complex with Ap3A. At the end, we obtained only the phosphate- and AMP-complexed structures (termed SapH-PO4 and SapH-AMP, respectively) by soaking SapH crystals with 500 mM NaH2PO4 or 30 mM AMP. In SapH-PO4, the inorganic phosphate bridges the two metals at the active site and makes hydrogen bonds with the metal-coordinating residues Asn-66, His-252, and His-146 (Fig. 3A). The binding mode is very similar to that of other enzymes in the MPP superfamily, suggesting that SapH also adopts the common catalytic mechanism (9, 12, 41).

In SapH-AMP, one molecule of AMP is deeply buried in the active-site pocket, with the adenine moiety in an anti-conformation and the ribose ring in a C4’ exo-conformation (Fig. 3B). The adenine and ribose ring are frozen in the inner cleft between loop 10 (between β5 and α6) and loop 14 (between β8 and η1) via hydrogen bonds and hydrophobic interactions. The O4’ of ribose forms a hydrogen bond with Arg-137 η2, whereas O3’ and O2’ form two hydrogen bonds with His-252 Nδ1. The adenine is stabilized in an anti-conformation via a...
\( \pi-\pi \) stacking interaction against Phe-189 and a hydrogen bond with His-141 N\( \delta \). The phosphate group is bound to the active site in the same manner as the inorganic phosphate in SapH (Fig. 3B).

To reveal the Ap\( _3 \)A-binding mode, we docked Ap\( _3 \)A onto SapH using the RosettaDock program (42). Restraints were used to fix the AMP moiety of Ap\( _3 \)A in a position similar to that in the complex of SapH-AMP. In the model, the inner and outer clefts accommodate the AMP (the first adenine nucleotide and P\(^1\)-phosphate group) and ADP (the second adenine nucleotide and P\(^2\)- and P\(^3\)-phosphate groups) moieties of Ap\( _3 \)A, respectively (Fig. 3C). The AMP moiety in the inner cleft could be well superimposed with that of SapH-AMP. The ADP moiety is stabilized in the outer cleft via two hydrogen bonds between the P\(^2\)-phosphate group and Arg-137. The adenosine moiety is stabilized by \( \pi-\pi \) stacking against Trp-135 and two hydrogen bonds with Asn-134 O\( \gamma \) and Ser-70 O\( \gamma \), respectively (Fig. 3C). Multiple-sequence alignment revealed that Ser-70, Asn-134, Trp-135, and Arg-137 at the outer cleft are highly conserved in streptococci. It is worth noticing that the active-site residues Asn-66, Trp-67, and Ser-70 are all from helix \( \alpha \)3 of the five-helix cap.

The phosphate in SapH-PO\(_4\) and SapH-AMP could be closely superimposed with the P\(^3\)-phosphate group of the SapH-Ap\( _3 \)A model. Moreover, this phosphate could be superimposed with that of protein analogs such as dAMP in nuclease.

**FIGURE 3.** AMP-binding pocket of SapH. A, inorganic phosphate-binding site in SapH-PO\(_4\). The residues are shown as sticks. The hydrogen bonds are shown as dashed lines. B, AMP-binding site. The binding pocket is between loops 10 and 14. The binding residues are shown as cyan sticks and AMP as green sticks. The metals and Wat1 are rendered as spheres. C, docking model of Ap\( _3 \)A (yellow for its C atoms) in the active-site pocket.
Mre11 from *P. furiosus* (9) and AMP in cyclic nucleotide phosphodiesterase Rv0805 (6), despite the binding modes being very different. These results suggest that the metal-bound phosphate group is under attack from the planar water molecule, driven by the binuclear metals. As for SapH, the inner cleft is complementary to an AMP (but not ADP or ATP) moiety, leaving the P¹-phosphate group at the elbow exposed to the nucleophilic water molecule. Furthermore, protein fluorescence spectrometry assays revealed that the addition of GTP and CTP to the apo-form SapH could trigger a comparable decrease in flu-

**FIGURE 4. Multiple-sequence alignment.** *A*, the binuclear active site is conserved in Gram-positive bacteria. *B*, the AMP-binding residues are conserved in streptococci. The metal-coordinating residues are indicated by blue triangles, whereas the AMP- and Ap₃A-binding residues are indicated by red triangles.

| Enzyme | bis-pNPP | ATP/Ap₃A/Ap₄A |
|--------|----------|----------------|
|        | $K_m$    | $k_{cat}$    | $k_{cat}/K_m$ |
|        | $s^{-1}$ | $s^{-1}$     | $s^{-1}$ |
| W67A   | 0.53 ± 0.05 | 1.08 ± 0.24 | 2.06 ± 0.13 |
| W67H   | 11.79 ± 1.15 | 2.56 ± 0.20 | 2.22 ± 0.01 |

*ND, not detectable.*
Streptococcal Nucleotide and Phosphodiester Hydrolase SapH

DISCUSSION

SapH Is a Novel Member of the MPP Superfamily—To date, all nucleotide/derivative hydrolases of known structure belong to the Nudix hydrolase superfamily (43). However, the core structure of SapH adopts an αβα-sandwich fold rather than an αβα-sandwich fold with a Nudix signature motif (17, 19, 43). The αβα-sandwich fold and binuclear active site of SapH are highly conserved in members of the MPP superfamily, which share a low sequence identity (<20%) with SapH. Among these MPP superfamily members, only Bacillus subtilis PrpE (44) and E. coli ApaH (13) were found to hydrolyze ApA_4. However, PrpE is a Tyr-specific phosphatase and an asymmetric ApA_4 hydrolase of unknown three-dimensional structure. E. coli ApaH was proved to be a symmetric ApA_4 hydrolase. The structure of its 100% sequence-identical homolog from S. flexneri (Protein Data Bank code 2DFJ) also has an αβα-sandwich fold with two layers of five-stranded β-sheets sandwiched by α-helices on the two sides, respectively (36). Superposition of SapH onto S. flexneri ApaH revealed an r.m.s.d. of 3.0 Å over 184 Ca atoms. The core αβα-sandwich structure of SapH is quite similar to that of ApaH. Among the helices of the additional subdomain, three helices (α3–α5) of SapH exhibit structural similarity to the counterparts of ApaH, despite that helix α4 (Pro-B4—Glu-99) of SapH is 10 residues longer (supplemental Fig. S3). By contrast, the other two α-helices (α7 and α8) at the C terminus of SapH are not found in ApaH. Alternatively, ApaH has an insertion of three additional α-helices (α7–α9, Asp-128–Arg-182) located at the opposite side of the active site (supplemental Fig. S3). Moreover, the active site of ApaH is coordinated by two Mn^{2+} ions. Therefore, SapH represents the first structure of a novel nucleotide/derivative hydrolase in the MPP superfamily.

SapH Is a Bifunctional Enzyme Conserved Exclusively in Streptococci—In addition to its phosphodiesterase activity, SapH demonstrates nucleotide/derivative hydrolase activity for ATP and ApA_4. This dual function of SapH might be a gain of function due to subtle substitutions at the active site that were proposed to explain the diverse substrate specificity of the MPP superfamily. For instance, the metal and substrate specificities of Clostridium thermocellum polynucleotide kinase/phosphatase can be dramatically altered by substitution of the active-site residues (45–49). Based on the analysis of the phosphate-binding residue His-98 in Rv0805, mutation of the corresponding Cys-74 to His in E. coli phosphodiesterase YfcE results in a gained 2′,3′-cAMP phosphodiesterase activity (48). In SapH, the corresponding residue Trp-67 also forms a hydrogen bond with the phosphate in SapH-PO_4 and SapH-AMP. Compared with wild-type SapH-FL, the W67A mutant has a lower K_m and k_cat and comparable activity (k_cat/K_m) for bis-pNPP. By contrast, the W67H mutation increases the K_m for bis-pNPP by ~7-fold, resulting in activity that is about one-fifteenth of that of the wild-type protein (Table 3). However, both the W67A and W67H mutants have no detectable activity for ATP, ApA_4, or ApA_4 (Table 3). These results indicate that Trp-67 of SapH-FL is indispensable for hydrolysis of ATP, ApA_4, and ApA_4.

To find the evolutionary hints of the bifunctionality of SapH, we performed a multiple-sequence alignment. The binuclear active-site residues Asp-11, His-13, Asp-39, Asn-66, His-128, His-164, and His-252 are highly conserved in Gram-positive bacteria (Fig. 4A). However, in addition to Trp-67, the other AMP-binding residues Arg-137, His-141, Phe-189, and His-252 are conserved exclusively in streptococci (Fig. 4B). Thus, we propose that the emergence of Trp-67, together with substitutions of residues at the substrate-binding pocket, resulted in a gain of nucleotide/derivative hydrolase function for streptococcal SapH, in addition to its phosphodiesterase activity.

REFERENCES

1. Hausdorff, W. P., Bryant, J., Paradiso, P. R., and Siber, G. R. (2000) Clin. Infect. Dis. 30, 100–121
2. Hoskins, J., Albom, W. E., Jr., Arnold, J., Blaszcak, L. C., Burgett, S., DeHoff, B. S., Estrem, S. T., Fritz, L., Fu, D. J., Fuller, W., Geringer, C., Gilmour, R., Glass, J. S., Khoja, H., Kraft, A. R., Lagace, R. E., LeBlanc, D. J., Lee, L. N., Leffkowitz, E. J., Lu, J., Matsushima, P., McAhren, S. M., McHenry, M., McLeaster, K., Mundy, C. W., Nicas, T. I., Norris, F. H., O’Gara, M., Peery, R. B., Robertson, G. T., Rockey, P., Sun, P. M., Winkler, M. E., Yang, T. Y., Young-Bellido, M., Zhao, G., Zook, C. A., Bultz, R. H., Jaskunas, S. R., Rostecek, P. R., Jr., Skrakut, P. L., and Glass, J. I. (2001) J. Bacteriol. 183, 5709–5717
3. Tettelin, H., Nelson, K. E., Paulsen, I. T., Eisen, J. A., Read, T. D., Peterson, S., Heidelberg, J., DeBoy, R. T., Haft, D. H., Dodson, R. J., Durkin, A. S., Gwinn, M., Kolonay, J. F., Nelson, W. C., Peterson, J. D., Umayan, L. A., White, O., Salzberg, S. L., Lewis, M. R., Rudane, D., Holztappel, E., Khouri, H., Wolf, A. M., Utterback, T. R., Hansen, C. L., McDonald, L. A., Feldblyum, T. V., Anguissoli, S., Dickinson, T., Hickey, E. K., Holt, I. E., Loftus, B. J., Yang, F., Smith, H. O., Venter, J. C., Dougherty, B. A., Morrison, D. A., Hollingshead, S. K., and Fraser, C. M. (2001) Science 293, 498–506
4. Wizemann, T. M., Heinrichs, J. J., Adamou, J. E., Erwin, A. L., Koenig, S., Choi, G. H., Barash, S. C., Rosen, C. A., Masure, H. R., Tuomanen, E., Gayle, A., Brewah, Y. A., Walsh, B., Barren, P., Lathigra, R., Hanson, M., Langermann, S., Johnson, S., and Koenig, S. (2001) Infect. Immun. 69, 1593–1598
5. Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L., and Simon, D. (1998) Infect. Immun. 66, 5620–5629
6. Podobnik, M., Tyagi, R., Matange, N., Dermal, U., Gupta, A. K., Mattox, R., Seshadri, K., and Visveswariah, S. S. (2009) J. Biol. Chem. 284, 32846–32857
7. Bateman, A., Birney, E., Cerutti, L., Durbin, R., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M., and Sonnhammer, E. L. (2002) Nucleic Acids Res. 30, 276–280
8. Voegtli, W. C., White, D. J., Reiter, N. J., Rusnak, F., and Rosenzweig, A. C. (2000) Biochemistry 39, 15365–15374
9. Hopfner, K. P., Karcher, A., Craig, L., Woo, T. T., Carney, J. P., and Tainer, J. A. (2001) Cell 105, 473–485
10. Klubunde, T., Sträter, N., Fröhlich, H., Witzel, H., and Krebs, B. (1996) J. Mol. Biol. 259, 737–748
11. Knöfel, T., and Sträter, N. (2001) J. Mol. Biol. 309, 239–254
12. Schenk, G., Gahan, L. R., Carrington, L. E., Mitic, N., Valizadeh, M., Ham-
StrFcococcal Nucleotide and Phosphodiester Hydrolase SapH

ilton, S. E. de Jersey, J., and Guddat, L. W. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 273–278

13. Guranowski, A., Jakubowski, H., and Holler, E. (1983) J. Biol. Chem. 258, 14784–14789

14. Guranowski, A. (2000) Pharmacol. Ther. 87, 117–139

15. Jezyk, A., Kanajia, S. P., Nishida, Y., Nakagawa, N., Praveen, S., Shinkai, A., Kuramitsu, S., Yokoyama, S., and Sekar, K. (2010) Acta Crystallogr. D Biol. Crystallogr. 66, 116–124

16. Swarbrick, J. D., Baxtannay, T., Mikel, D., Zhang, X. R., Blackburn, G. M., Gayler, K. R., and Gooley, P. R. (2000) J. Mol. Biol. 302, 1165–1177

17. Swarbrick, J. D., Buysa, S., Gunawardana, D., Gayler, K. R., McLennan, A. G., and Gooley, P. R. (2005) J. Biol. Chem. 280, 8471–8481

18. Bailey, S., Sedelnikova, S. E., Blackburn, G. M., Abdelghany, H. M., Baker, P. J., McLennan, A. G., and Rafferty, J. B. (2002) Structure 10, 589–600

19. Iwai, T., Kuramitsu, S., and Masui, R. (2004) J. Biol. Chem. 279, 21732–21739

20. Bessman, M. J., Frick, D. N., and O’Handley, S. F. (1996) J. Biol. Chem. 271, 25059–25062

21. Gabelli, S. B., Blanchet, M. A., Bessman, M. J., and Amzel, L. M. (2001) Nat. Struct. Biol. 8, 467–472

22. Otwinowskiz, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326

23. Brodersen, D. E., de La Fortelle, E., Vonrhein, C., Bricogne, G., Nyborg, I., and Kjeldgaard, M. (2000) Acta Crystallogr. D Biol. Crystallogr. 56, 431–441

24. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

25. Vagin, A., and Teplyakov, A. (2010) Acta Crystallogr. D Biol. Crystallogr. 66, 22–25

26. Collaborative Computational Project No. 4. (1994) Acta Crystallogr. D Biol. Crystallogr. 50, 760–763

27. Murshudov., G. N., Vagin, A. A., and Dodson., E. J. (1997) Acta Crystallogr. D Biol. Crystallogr. 53, 240–255

28. Emsley, P., and Cowtan, K. (2004) Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

29. Davis, I. W., Leaver-Fay, A., Chen, V. B., Block, J. N., Kapral, G. J., Wang, X., Murray, L. W., Arendall, W. B., 3rd, Snoeyink, J., Richardson, J. S., and Richardson, D. C. (2007) Nucleic Acids Res. 35, W375–W383

30. Laskowski, R., Macarthur, M., Moss, D., and Thornton, J. (1993) J. Appl. Crystallogr. 26, 283–291

31. Delano, W. L. (2002) The PyMOL Molecular Graphics System, DeLano Scientific LLC, San Carlos, CA

32. Miller, D. J., Shuvalova, L., Evdokimova, E., Savchenko, A., Yakunin, A. F., and Anderson, W. F. (2007) Protein Sci. 16, 1338–1348

33. Chen, S., Yakunin, A. F., Kuznetsova, E., Buss, D., Pufan, R., Proudfoot, M., Kim, R., and Kim, S. H. (2004) J. Biol. Chem. 279, 31854–31862

34. Wang, D., Guo, M., Liang, Z., Fan, J., Zhu, Z., Zang, J., Zhu, Z., Li, X., Teng, M., Niu, L., Dong, Y., and Liu, P. (2005) J. Biol. Chem. 280, 22962–22967

35. Kissinger, C. R., Parge, H. E., Knighton, D. R., Lewis, C. T., Pelletier, L. A., Tempczyk, A., Kalish, V. J., Tucker, K. D., Showalter, R. E., and Moomaw, E. W. (1995) Nature 378, 641–644

36. Wang, Q. H., Hu, W. X., Gao, W., and Bi, R. C. (2006) Proteins 65, 1032–1035

37. Shenoy, A. R., Capuder, M., Draskovic, P., Lamba, D., Visweswariah, S. S., and Podobnik, M. (2007) J. Mol. Biol. 365, 211–225

38. O’Handley, S. F., Frick, D. N., Dunn, C. A., and Bessman, M. J. (1998) J. Biol. Chem. 273, 3192–3197

39. Xu, W., Gauss, P., Shen, J., Dunn, C. A., and Bessman, M. J. (2002) J. Biol. Chem. 277, 23181–23185

40. Traut, T. W. (1994) Mol. Cell. Biochem. 140, 1–22

41. Bellinzoni, M., Wehenkel, A., Shepard, W., and Alzari, P. M. (2007) Structure 15, 863–872

42. Gray, J. J., Moughon, S., Wang, C., Schueler-Furman, O., Kuhlman, B., Rohl, C. A., and Baker, D. (2003) J. Mol. Biol. 331, 281–299

43. Mildvan, A. S., Xie, Z., Azurmendi, H. F., Saraswat, V., Legler, P. M., Massiah, M. A., Gabelli, S. B., Blanchet, M. A., Kang, L. W., and Amzel, L. M. (2005) Arch. Biochem. Biophys. 433, 129–143

44. Iwanicki, A., Herman-Antosiewicz, A., Pierechod, M., Séror, S. J., and Mildvan, A. S. (2007) Acta Crystallogr. D Biol. Crystallogr. 63, 116–124

45. Martins, A., and Shuman, S. (2005) RNA 11, 1271–1280

46. Keppetipola, N., and Shuman, S. (2006) RNA 12, 73–82

47. Keppetipola, N., and Shuman, S. (2007) Nucleic Acids Res. 35, 7721–7732

48. Keppetipola, N., and Shuman, S. (2008) J. Biol. Chem. 283, 30942–30949