The atomic coordinates and structure factors (code 2GZ1, 2GYY, 2GZ2, and 2GZ3) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: ASADH, aspartate-β-semialdehyde dehydrogenase; MR, molecular replacement; DTT, dithiothreitol; ASA, L-aspartate-β-semialdehyde; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid; ITC, isothermal titration calorimetry; r.m.s.d., root mean square deviation.

Examination of Key Intermediates in the Catalytic Cycle of Aspartate-β-semialdehyde Dehydrogenase from a Gram-positive Infectious Bacteria*

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Aspartate-β-semialdehyde dehydrogenase (ASADH) catalyzes a critical branch point transformation in amino acid biosynthesis. The products of the aspartate pathway are essential in microorganisms, and this entire pathway is absent in mammals, making this enzyme an attractive target for antibiotic development. The first structure of an ASADH from a Gram-positive bacterium, *Streptococcus pneumoniae*, has now been determined. The overall structure of the apoenzyme has a similar fold to those of the Gram-negative and archaeal ASADHs but contains some interesting structural variations that can be exploited for inhibitor design. Binding of the coenzyme NADP, as well as a truncated nucleotide analogue, into an alternative conformation from that observed in Gram-negative ASADHs causes an enzyme domain closure that precedes catalysis. The covalent acyl-enzyme intermediate was trapped by soaking the substrate into crystals of the coenzyme complex, and the structure of this elusive intermediate provides detailed insights into the catalytic mechanism.

The dramatic increase in the number of multidrug-resistant microorganisms has provided greater urgency to the search for new antibiotics against novel drug targets. Essential gene products involved in bacterial metabolism represent attractive targets for such compounds; however, exploitation of these targets in drug development has not been achieved. Among the potential metabolic targets, several genes have been identified in the aspartate biosynthetic pathway that are essential for the survival of *Salmonella* during infection (1). The aspartate pathway present in plants, bacteria, and fungi leads to the formation of the following four essential amino acids: threonine, lysine, methionine, and isoleucine (2, 3). This pathway also contains some interesting structural variations that can be exploited for inhibitor design. Binding of the coenzyme NADP, as well as a truncated nucleotide analogue, into an alternative conformation from that observed in Gram-negative ASADHs causes an enzyme domain closure that precedes catalysis. The covalent acyl-enzyme intermediate was trapped by soaking the substrate into crystals of the coenzyme complex, and the structure of this elusive intermediate provides detailed insights into the catalytic mechanism.

Among the genes that belong to this pathway, the *asd* gene that encodes for aspartate-β-semialdehyde dehydrogenase (ASADH) has been identified as essential in both Gram-negative and Gram-positive bacteria such as *Salmonella enterica* (1), *Legionella pneumophila* (6), *Mycobacterium bovis* (7), *Bacillus subtilis* (8), and *Staphylococcus aureus* (9). ASADH catalyzes the production of aspartate-β-semialdehyde, the first branch point intermediate in the aspartate pathway, and the structure of the enzyme has been determined from several infectious Gram-negative bacteria (10–12). The enzymes from these organisms show high sequence and structural homology, whereas a recently determined archaeal ASADH structure (13) contains significant differences in its overall structure and in its coenzyme binding specificity. Insights into the mechanism of ASADH were obtained through the structural characterization of a tetrahedral intermediate in the catalytic cycle of the enzyme from *Hemophilus influenzae* (14); however, the structure of the putative acyl-enzyme derived from this intermediate has remained elusive.

We have now determined the first structure of an ASADH from a Gram-positive bacterial species, *Streptococcus pneumoniae* (*sp*ASADH). The role of coenzyme binding in domain closure was determined by an examination of the apoenzyme and several coenzyme complex structures from this organism. In addition, soaking the substrate ASA into crystals of the NADP complex has led to the trapping and first structural characterization of the acyl-enzyme intermediate.
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EXPERIMENTAL PROCEDURES

Enzyme Expression and Purification—The asd gene encoding ASADH was amplified from a S. pneumoniae genomic DNA template purchased from American Type Culture Collection (ATCC). Nucleotide primers were designed to incorporate an NcoI restriction site that includes the ATG start codon and an XhoI site. Ligation of the PCR fragment into a pET 28a vector results in fusion of the asd gene to a C-terminal hexahistidine tag. This construct was verified by restriction mapping and DNA sequencing (MWG Biotech), and the enzyme was expressed in Escherichia coli BL21 (DE3) cells. Standard growth conditions (37 °C) were employed until an A_{600} of 0.7 was reached, at which point the temperature was reduced to 33 °C, and induction of ASADH expression was accomplished through the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside. Cell growth was continued for 5 h to allow maximum enzyme expression, and cells were then harvested by centrifugation. The soluble cell extract containing spASADH was passed through a TALON® Co^{2+} affinity matrix (BD Biosciences) for specific binding to the engineered hexahistidine tag on the C terminus of spASADH. Eluted spASADH was then dialyzed into a low ionic strength buffer (50 mM potassium phosphate, 1 mM EDTA, 1 mM DTT) for further purification by anion exchange chromatography. Dialysis of the purified enzyme into 20 mM HEPES, pH 7.2, 100 mM NaCl, 0.2 mM EDTA was done prior to storage at −80 °C.

Kinetic Characterization—Initial velocity kinetics of spASADH was determined at room temperature with a SPECTRAmax® 340PC® Microplate Spectrophotometer (Molecular Devices). To measure the activity of spASADH, the reverse reaction was followed, with the increase in the absorbance of NADPH monitored at 340 nm. The reaction mixture consisted of 200 mM phosphate, NADP, and L-aspartate-β-semialdehyde (ASA) to a volume of 50 μl in a flat bottom 96-well plate. The enzyme was added to each well to a final volume of 150 μl to initiate the reaction. The Michaelis constants (K_m) for each substrate were determined by holding two of the three substrates at fixed concentrations, while the other was varied by serial dilutions, ranging from 0.47 to 30 mM for phosphate, 0.03 to 2.0 mM for ASA, and 0.02 to 1.0 mM for NADP. Data were fit to the Michaelis-Menten equation with BASIC versions of the kinetics programs described by Cleland (15).

Isothermal Titration Calorimetry—Isothermal titration calorimetry (ITC) experiments were performed on a VP-ITC titration calorimeter (Microcal Inc., Northampton, MA). Titration of NADP, 2',5'-ADP, and NAD in spASADH were carried out in 25 mM HEPES, pH 8.0, 50 mM ammonium acetate, and 0.1 mM tris(2-carboxyethyl)phosphine at 25 °C. Enzyme concentrations were in the range of 50–100 μM, with the concentration of NADP in the syringe between 1 and 2 mM. Each titration was performed in triplicate by the successive addition of 5-μl aliquots to a final coenzyme concentration of 100 μM. Data analysis was performed using Origin 7.0 as distributed from Microcal. Integrated titration curves were each fit to a one-site binding model and corrected for background heats of injection by subtracting reference data obtained by titrating the ligand into buffer.

Crystallization and Data Collection—Initial crystallization conditions were identified by sparse matrix screening of spASADH at a protein concentration of 10 mg/ml. Both index screens (Hampton Research) and JBScreen Basic (Jena Bioscience) yielded initial crystals for the apoenzyme. The optimized conditions for the apoenzyme crystals consisted of 100 mM MES, pH 6.5, 50 mM magnesium or calcium acetate, and 12–13% polyethylene glycol 3350. Crystals grew rapidly within a few hours, but these crystals did not diffract x-rays beyond 5 Å. The enzyme was then exchanged into 50 mM citrate, pH 6.0, 100 mM ammonium acetate, and 2 mM DTT and crystallized under the same conditions with an additional 10 mM DTT. Treatment of the enzyme in this fashion gave less nucleation and slowed crystal growth to 2–3 days, resulting in a more ordered crystal lattice and higher diffracting crystals. Prior to flash freezing in liquid N_2, the crystals were transferred in three incremental steps into the well solution plus 20% glycerol. An spASADH-NADP complex crystal was obtained by cocrystallizing the enzyme with 2 mM NADP. Single crystals of this complex did not grow under the apo crystallization conditions; instead a new crystal form was identified by grid screening. The NADP complex crystals were grown in 50 mM citrate, pH 6.0, 200 mM ammonium acetate, 10 mM DTT, and 19–20% polyethylene glycol 8000. The spASADH-NADP-ASA complex crystals were obtained by soaking the NADP complex crystals with ASA for 45 min. Each of the NADP complex crystals was cryoprotected in the mother liquor with an additional 20% glycerol. Crystals of the 2',5'-ADP complex were obtained by cocrystallization with 10 mM 2',5'-ADP under similar conditions as the spASADH-NADP complex. Cryo-protection of the spASADH-2',5'-ADP complex crystals was achieved by introducing 20% ethylene glycol into the mother liquor in three incremental steps. All of the diffraction data were collected on a Rigaku High-brilliance FR-E rotating anode x-ray generator with an Raxis IV image plate detector. D³trek was used for integration and scaling of all diffraction data using a σ cutoff of 2. Data collection statistics for the apoenzyme and each of the enzyme complexes are summarized in Table 1.

Structure Determination, Refinement, and Analysis—The structure of apo spASADH was solved by MR using the program Phaser using data between 30 and 2.5 Å resolution with a final Z-score of 24.32. Initial attempts to find a replacement solution did not succeed with any of the ASADH structures that we had determined previously. Instead, homology models of several ASADHs (Protein Data Bank codes 1GL3, 1NWH, 1MB4, and 1YS4) were superimposed, and the nonhomologous regions were trimmed to generate an ensemble of ASADH search models containing only the conserved regions. This ensemble was then used to find the correct orientation of spASADH, because the use of any individual ASADH model had failed to yield a solution. The model of mjASADH (Protein Data Bank code 1YS4) with the highest sequence identity to spASADH was subjected to rigid body refinement followed by simulated annealing refinement in CNS (16) to obtain the initial electron density map. The structure of spASADH was built by a combination of manual building within Xfit (17) and automatic building with arp/Warp (18) to obtain the initial spASADH model, followed by iterative rounds of simulated annealing refinement in CNS. The final model of apo spASADH was refined.
Overall Structure of S. pneumoniae ASADH—The structure of apo spASADH was solved by MR using a combined ensemble of ASADH search models, each with less than 30% sequence identity to spASADH. An accurate structural alignment of the ASADH search templates and truncation of nonconserved regions was critical for the success of the MR protocol because none of the individual structures lead to a suitably complete model. spASADH is assembled into a functional homodimer with a similar overall fold to the previously studied ASADHs (Fig. 1). Each monomer (358 residues) contains 13 β-strands and 8 α-helices that fold into two domains, an N-terminal Rossmann fold (residues 1–127, 330–358) and a C-terminal dimerization domain (residues 128–329). The N-terminal domain consists of five parallel β-strands enclosed by three α-helices. Two additional β-strands decorate the top of the Rossmann fold and are directly involved in coenzyme binding. The C-terminal domain is constructed primarily of six mixed β-strands that create the conserved dimer interface. A helical segment bridging the top of the β-sheet interface contributes additional interactions to further stabilize the ASADH dimer. The active site is centered at the N terminus of an α-helix positioned at the interface between the N- and C-terminal domains (Fig. 1B).

Sequence and Structural Comparison among the ASADH Family—The ASADH family is diverse and can be divided into three branches consisting of the enzymes from Gram-negative bacteria, Gram-positive bacteria, and Archaea/fungi. These branches were initially identified and partitioned through sequence alignments, and with representative high resolution structures now available from each branch (10, 12–14), they have now been compared by structural alignments. spASADH is the first member of the Gram-positive bacterial ASADH branch that has been structurally characterized. spASADH is a good representative of the other Gram-positive bacterial ASADHs with greater than 40% sequence identity to these enzymes, although having less than 25% identity with any of the Gram-negative enzymes. Unexpectedly, this spASADH has slightly lower sequence homology to the Gram-negative enzymes than to the archaefungal branch, with sequence identities from this comparison ranging from 18 to 30%. Alignment of the structures that have been determined from each branch of the ASADH family also shows that spASADH is most similar to the archaenal ASADH from Methanococcus jannaschii (mjASADH). The backbone r.m.s.d. between spASADH and mjASADH is only ~0.7 Å, compared with >1.2-Å deviations from the three Gram-negative bacterial ASADHs that have been structurally characterized (Table 2).

In addition to overall sequence and structural comparisons, there are several conserved insertions and deletions that further differentiate the three ASADH branches. We have shown previously that mjASADH has three conserved insertions totaling 30 residues when aligned with the Gram-negative bacterial ecASADH (13), with each insertion located on the surface of the structure. In addition, a 48-amino acid deletion in mjASADH results in the complete removal of the helical subdomain (Fig. 1C), which makes a significant contribution to the dimer interface in ecASADH. Gram-negative bacterial ASADHs have the largest dimer interface, formed by a conserved hydrophobic β-sheet and complemented by this helical subdomain (Fig. 1A) creating over 3400 Å² of buried surface area. The thermophilic mjASADH structure that is missing this helical subdomain has
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(A) Helical subdomain

(B) Coenzyme binding loop

(C)
Covalent Acyl-enzyme Intermediate in ASADH

TABLE 2
Summary of ASADH structure comparison

| Enzyme form | Sequence identity to spASADH | Buried dimer surface | r.m.s.d. to spASADH dimer | r.m.s.d. to spASADH monomer |
|-------------|-----------------------------|----------------------|----------------------------|-----------------------------|
|             | %                           | Å²                   | r.m.s.d. Å                 | r.m.s.d. Å                  |
| spASADH     | 24                          | 2673                 | 1.33                       | 1.04                        |
| ecASADH     | 22                          | 3834                 | 1.31                       | 1.23                        |
| hiASADH     | 24                          | 3478                 | 2.11                       | 1.24                        |
| vcASADH     | 24                          | 3620                 | 2.23                       | 1.75                        |
| mjASADH     | 27                          | 1943                 | 0.76                       | 0.72                        |

FIGURE 1. Structural comparison of the three ASADH branches. Each of the structures shares a conserved domain organization and exists as a functional homodimer. The largest structural differences manifest themselves in the coenzyme binding loop and in a helical subdomain bridging the dimerization interface. A, representative Gram-negative bacterial ASADH from E. coli (ecASADH, white and green). B, S. pneumoniae ASADH (spASADH, white and purple), the first Gram-positive bacterial ASADH structure. C, the most distant member of the ASADH family from M. jannaschii (mjASADH, white and orange). The coenzyme NADP and the active site amino acids for each ASADH branch are also shown (blue sticks).

| Covalent Acyl-enzyme Intermediate in ASADH |
|-------------------------------------------|
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A, B, C. R. Faehnle and R. E. Viola, unpublished results.

a much smaller dimer interface of less than 2000 Å² (Table 2). This subdomain is present in spASADH, but a 16-residue deletion results in a single α-helix followed by an unstructured loop forming the top portion of the dimer interface (Fig. 1B) with about 2600 Å² of buried surface area.

Structural variations between these branches also occur along a stretch of residues on the surface that contains the binding pocket for NADP. ecASADH is representative of the Gram-negative ASADHs, with a highly flexible coenzyme binding loop in the absence of NADP that becomes ordered only in response to NADP binding (11). In contrast, for the archaeal branch a 13-residue insertion in mjASADH leads to a reorientation of this loop by about 90°C causing it to drape over the coenzyme binding pocket (13). spASADH has the same number of residues in this region as the Gram-negative bacterial enzymes, but two short β-strands (Fig. 1B) replace the loop observed in ecASADH. The structural differences in the coenzyme binding loop have been proposed to account for the observed differences in the bound coenzyme conformation between the Gram-negative and archaeal branches of the ASADH family (13). Because the coenzyme binding region in spASADH is different from those in the other branches, it was unclear which coenzyme binding conformation will be adopted in Gram-positive ASADHs or if there is a bound coenzyme conformation that is unique to this branch.

Catalytic Activity Differences between the ASADH Branches—The Gram-negative bacterial ASADHs are significantly more catalytically efficient than the Gram-positive or archaeal homologues. The catalytic turnover rates for the Gram-negative enzymes range from 600 s⁻¹ for ASADH from E. coli to about 120 s⁻¹ for the Vibrio cholerae enzyme (22). In contrast, the thermophilic mjASADH has a turnover of only 4 s⁻¹, even when measured at 70 °C (13). This newly examined Gram-positive spASADH is also found to be much less efficient than the Gram-negative enzymes, with a turnover of only 2 s⁻¹. These large differences in activity between the ASADH branches are quite unexpected because all of the essential functional amino acids in the active site are conserved throughout the ASADH family. However, the low overall sequence homology throughout the family and the presence of insertions and deletions in critical regions within the structure may help to explain the differences in catalytic efficiency. Although the binding sites for the amino acid substrate are essentially unchanged among the ASADHs, the residues that form critical interactions with NADP in the Gram-negative bacterial ASADHs are not conserved in the Gram-positive bacterial and archaeal ASADHs. In addition, several secondary structural elements that are missing or truncated in these newly examined ASADHs eliminate many of the interactions between the enzyme and NADP that were shown previously to be important for binding recognition in Gram-negative bacterial ASADH. These sequence alterations have important consequences for coenzyme conformation and binding specificity and are likely involved in the observed differences in catalytic efficiency.

We have recently shown that there is a dual NADP/NAD coenzyme specificity in mjASADH (13), whereas the Gram-negative bacterial ASADHs are all highly specific for NADP. We now find that spASADH, as well as several other Gram-positive bacterial ASADHs, including those from S. aureus and Enterococcus faecalis, also exhibit similar dual coenzyme specificity, albeit with strong a preference for NADP. This expanded coenzyme specificity in Gram-positive and archaeal ASADHs can be attributed to a new set of interactions between the enzyme and NADP that take place in the context of an altered coenzyme binding conformation.

NADP Binding Induces an “Open” to “Closed” Transition—in order to investigate the protein movements that are caused by coenzyme binding, we have compared the apo spASADH structure to an enzyme-NADP complex obtained by cocrystallization. Binding of NADP to spASADH induces rotation of the N-terminal coenzyme binding domain toward the C-terminal dimerization domain. This domain closure can best be described by an opened to closed transition that is energetically driven by the interactions that are formed between NADP and amino acid functional groups from both the N- and C-terminal domains of the enzyme. Hydrogen bonding, electrostatic, van der Waals, and hydrophobic interactions each play a role in facilitating NADP binding and in stabilization of the closed catalytically active form of ASADH. The DynDom program (23) was used to characterize the NADP-induced domain closure, by identifying the regions that have moved along with the hinge bending residues capable of facilitating this movement. The moving region includes the majority of the N-terminal coen-

3 C. R. Faehnle and R. E. Viola, unpublished results.
zyme binding domain (Fig. 2), whereas the fixed region contains residues that make up the C-terminal dimerization domain. The binding of NADP causes the moving domain to rotate by 8° around a hinge axis toward the fixed domain. Hinge bending regions are found in both the N- and C-terminal domains (Fig. 2A) and include residues 72–74, 94–98, 106–107, 111–117, 121–125, and 329–336 (marked in green in Fig. 2C). Although a significant displacement of the C-α backbone occurs within the N-terminal domain, the active site of the apoenzyme is already preformed and undergoes only local movements in response to NADP binding.

The essential role of this dynamic motion in the ASADH catalytic mechanism is exemplified by the nature of the highly conserved amino acid residues found in these hinge regions. Two hinges positioned directly on the rotation axis contain glycine residues that join the N- and C-terminal domains. The first hinge contains the sequence 121GXX123, with this glycine residue conserved among most Gram-positive and Gram-negative ASADHs. The second hinge with a consensus 329GAA331 motif is conserved among all the annotated ASADH sequences.

Comparison of the Differences in NADP Binding Conformations—The overall domain movements encountered in the transition from the apoenzyme to the complex with bound NADP in spASADH are similar to what was observed previously for ecASADH (11). Rotation of the N-terminal domain toward the active site in response to NADP binding appears to be a universal mechanism utilized throughout the ASADH family. In each case the active site pocket is nearly unchanged as the enzyme goes from an opened to closed conformation. Despite this conserved overall domain movement, the interactions with and conformations of NADP are substantially different across the various branches of the ASADH family. Consistent with its similar sequence and structural alignments, the NADP conformation in spASADH closely resembles that of NADP bound to the archaeal mjASADH, but in a conformation that is quite distinct from their Gram-negative counterparts. A new binding pocket is found in spASADH for the adenine base, along with a remodeled 2'-phosphate-binding site. Thus, facilitation of the commonly observed domain closure in spASADH is driven by a different set of interactions compared with those that drive the same closure in ecASADH. The adenine binding pocket in spASADH is forged between an α-helix and the coenzyme binding loop of the N-terminal domain. Interactions between the adenine ring and the side chains of Thr-76 and Ser-37, along with a cation-π interaction with Arg-39, serve to anchor the adenine ring in this pocket (Fig. 3A). In the Gram-negative enzymes the adenine ring occupies a completely different binding pocket that also includes a cation-π interaction but now with Arg-10, and a hydrogen bond between the 6-amino group of adenine and the backbone carbonyl of Pro-193 from the other subunit of the dimer (Fig. 3B). Despite this complete reorientation of both the adenine and ribose rings, the 2'-phosphate group of NADP is held in nearly the same position in each enzyme in the ASADH family (Fig. 3), through interactions with hydroxyl groups from two serines and a threonine as well as an arginine guanido group.

Stoichiometry and Thermodynamics of NADP Binding—The direct link between coenzyme binding in one subunit and an
newly determined structure of the spASADH-NADP complex contains a functional dimer in the asymmetric unit, with electron density found in the active sites of each subunit that are consistent with a bound NADP. A molecule of NADP was therefore modeled and refined in each subunit. This discrepancy between the coenzyme complex structure and the observed ITC binding stoichiometry is most likely a result of the high levels (2 mM) of NADP used in the crystallization experiments compared with the much lower coenzyme levels (100 μM) in the ITC titration.

Inspection of the spASADH-NADP structure shows that in one subunit of the dimer Arg-39 is positioned within 3.2 Å of the adenine ring of NADP, whereas Arg-39 in the opposite subunit is >6 Å away, much too far to provide a strong cation-π interaction. Additionally, the coenzyme binding loop that contains Arg-39 is in an opened conformation in this opposite subunit. The overall affinity for NADP in both Gram-negative and Gram-positive bacterial ASADHs is comparable, with K_d values in the 10 μM range (Table 3). However, an analysis of the thermodynamic parameters contributing to the free energy (ΔG) of NADP binding between Gram-negative and Gram-positive ASADHs shows clear differences. NADP binding in Gram-negative bacterial ASADHs is largely driven by a favorable enthalpy change (ΔH), whereas spASADH exhibits a ΔH that is half that observed for either hiASADH or vcASADH (Table 3). The less negative ΔH observed for spASADH is likely the result of weaker interactions between the enzyme and the adenine half of NADP, because the interactions with the nicotinamide mononucleotide portion are conserved between the Gram-negative and Gram-positive ASADHs (Fig. 3). However, this lower ΔH in spASADH is compensated for by a more favorable entropy change (ΔS). The entropic change in spASADH most likely arises from the altered NADP conformation as well as the observed differences in NADP binding stoichiometry. The altered conformation of NADP bound in spASADH gives rise to 30% less solvent accessibility compared with the NADP molecule bound in the structure from vcASADH. These results are consistent with a more efficient sequestering of a hydrophobic moiety (the adenine ring) from solvent being entropically favorable. Perhaps even more important is the favorable conformational entropy change in spASADH, where a single NADP binding per dimer allows the unoccupied subunit to maintain some degree of conformational freedom (Table 3).

**Binding of Coenzyme Analogues—2',5'-ADP, a coenzyme analogue that contains only the adenosine portion of NADP, has been shown to be a good competitive inhibitor of the archaeal mjASADH (K_i = 50 μM), although nearly 50-fold weaker inhibition is observed in the Gram-negative bacterial ASADHs (13). These differences are consistent with the alterations in NADP binding described above between the enzymes in these two branches. A similar pattern of inhibition is observed for 2',5'-ADP interacting with spASADH, thus providing the possibility of targeting the adenosine-binding site of Gram-positive ASADHs with selective inhibitors. Although NADP induces a domain closure upon binding, it is not clear if 2',5'-ADP inhibits the enzyme by binding to the opened form and inducing formation of the closed conformation. To address this question we solved the structure of an spASADH complex...
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TABLE 3
Thermodynamics of coenzyme binding to ASADH

| Coenzyme/enzyme form | N/dimer | $K_d$ (μM) | $ΔG$ (kcal/mol) | $ΔH$ (kcal/mol) | $TΔS$ (kcal/mol) |
|----------------------|---------|------------|----------------|----------------|-----------------|
| NADP                 |         |            |                |                |                 |
| $sp$ASADH            | 1       | 6.0 ± 0.8  | −7.1 ± 0.9     | −6.7 ± 0.3     | 0.40 ± 0.02     |
| hiASADH              | 2       | 17.0 ± 0.3 | −6.6 ± 0.1     | −12.9 ± 0.3    | −6.3 ± 0.1      |
| tcASADH              | 2       | 10.0 ± 0.2 | −6.9 ± 0.1     | −12.7 ± 0.3    | −5.8 ± 0.1      |
| $2',5'$-ADP          |         |            |                |                |                 |
| $sp$ASADH            | 1       | 6.0 ± 1.0  | −7.1 ± 1.6     | −11.1 ± 0.9    | −3.9 ± 0.3      |

FIGURE 4. Structure of $sp$ASADH complexed with $2',5'$-ADP. Only the adenosine portion of NADP is required for an opened to closed conformational change in $sp$ASADH. The critical interaction providing the torque necessary for the NADP-induced domain closure is accomplished by the interaction between the backbone amide of Met-162 and the diphosphate portion of NADP, analogous to the interaction between the backbone amide and the diphosphate of the coenzyme.

The thermodynamics associated with $2',5'$-ADP binding to $sp$ASADH were measured by ITC and reveal a binding constant (6 μM) and a free energy change ($ΔG = −7.1$ kcal/mol) that are identical to those for NADP. However, the binding of $2',5'$-ADP is driven by a more favorable increase in enthalpy ($−11.1$ versus $−6.7$ kcal/mol) relative to NADP and a corresponding negative entropy contribution (Table 3). This was somewhat unexpected given that fewer interactions take place between the enzyme and $2',5'$-ADP because of the absence of the nicotinamide half of the coenzyme. These interactions between the nicotinamide moiety and the enzyme apparently play a negligible role in NADP binding. When NADP binds nearly all of the ordered solvent molecules observed in the apo-structure are displaced, an event that would be favorable entropically as water molecules are released into the bulk solvent, but this would contribute unfavorably to the overall enthalpy as numerous hydrogen bonds are lost between water and the enzyme. The new interactions that are formed between the enzyme and $2',5'$-ADP only the solvent in the adenine binding pocket is displaced. Upon undergoing an opened to closed conformational change, the enzyme must experience more favorable interactions with solvent in the closed conformation than it does with the nicotinamide moiety of the coenzyme. These results are consistent with our observations that both nicotinamide and nicotinamide mononucleotide are neither substrates nor inhibitors of $sp$ASADH.

To further probe the coenzyme-binding site and provide a structural basis to explain the ability of $sp$ASADH to accept either NADP or NAD as a hydride acceptor, we also attempted to cocrystallize this enzyme with NAD. Diffraction data were collected on these crystals, but the electron density map did not indicate the presence of NAD in the binding pocket, and the enzyme was found to be in its opened conformation. These results are consistent with the increased Michaelis constant measured for NAD in $sp$ASADH ($K_m = 3.3$ mM), which is similar to that reported in $mj$ASADH. The absence of NAD in the crystal structure supports the important role of the $2'$-phosphate of NADP in binding in $sp$ASADH. However, this interaction is not absolutely essential, because the enzyme is catalytically competent in the presence of higher levels of NAD. Furthermore,
titration of NAD into spASADH shows a markedly reduced heat change (<0.2 kcal/mol) when compared with either NADP or 2′,5′-ADP, making the direct measurement of a binding constant by ITC impossible.

We have now identified the minimal interactions between the enzyme and NADP that are needed to generate and stabilize the closed conformation in order to support hydride transfer and catalysis. The nicotinamide half of the dinucleotide is not essential either for binding of the coenzyme or for inducing domain closure, with only the 2′-phospho-AMP moiety of NADP required to accomplish this task. However, the interactions between the enzyme and the nicotinamide portion are important to properly orient the nicotinamide ring for hydride transfer. Conserved hydrogen bonds to the amide nitrogen and oxygen atoms of the nicotinamide ring accomplish this task in both the Gram-negative and Gram-positive ASADHs (Fig. 3).

Capture of the Thioacyl Enzyme Intermediate—The physiological reaction catalyzed by ASADH is the reductive dephosphorylation of β-aspartyl phosphate to produce ASA, coupled with the oxidation of NADPH. Our original mechanism proposed for this reaction involves the formation of three different covalent enzyme intermediates during the catalytic cycle. First, nucleophilic attack of the active site cysteine thiolate on the phosphoester of β-aspartyl phosphate results in formation of a tetrahedral thioacetal intermediate. Next, collapse of this thioacetal forms a stable thioacyl enzyme intermediate through the expulsion of phosphate. Finally, transfer of a hydride from NADPH to the thioester would form a tetrahedral hemithioacetal that, upon collapse, releases the product ASA and regenerates the free enzyme (25).

In order to provide additional evidence in support of this catalytic mechanism for spASADH, we designed substrate trapping experiments whereby intermediates in the reaction cycle could potentially be observed through crystallization and x-ray diffraction studies. We had previously trapped the tetrahedral hemithioacetal intermediate by soaking the products ASA and phosphate into apo hiASADH crystals (14). Covalent adducts with ASA were obtained in the absence and presence of bound phosphate that provided detailed insights into the first steps of ASADH catalysis. Here substrate trapping experiments were designed to allow catalysis to proceed beyond these initial stages. This has now been accomplished by soaking the preformed NADP complex with the product ASA. In the previous experiments the absence of the hydride acceptor NADP leads to a trapped tetrahedral intermediate that could not proceed through the catalytic cycle (14). In the spASADH-NADP complex the addition of ASA allows hydride transfer from ASA to NADP to occur to produce NADPH. This would result in the formation of a covalently bound thioacetyl enzyme intermediate (Fig. 5A) that should be stable in the absence of an external nucleophile such as phosphate. In fact, the acyl enzyme had been shown previously to form in solution and was sufficiently stable to be purified by size exclusion chromatography (26). However, our previous attempts to trap the acyl-enzyme intermediate structure were unsuccessful, most likely because of the rapid rate of hydrolysis of the acyl-enzyme in the higher pH range that was used for crystallization.
Covalent Acyl-enzyme Intermediate in ASADH

carbon-oxygen bond length, and loss of a negative charge from the tetrahedral intermediate each contribute to changes in interactions from those that were observed in the tetrahedral enzyme/ASA intermediate in hiASADH. The ASA oxygen distance to the amide nitrogen of Asn-127 decreases by ~0.4 Å in the acyl-enzyme structure, whereas the distance to the water molecule (W1) that occupies the phosphate-binding site is increased by ~0.8 Å.

As is true for other pyridine-linked dehydrogenases, hydride transfer from ASA to NADP is stereochemically controlled, with the hydride removed from the pro-R position of ASA. Prior to this trapping and characterization of the acyl-enzyme intermediate, it had not been established whether the breakdown of the acyl-enzyme occurs by nucleophilic attack of phosphate on the same or the opposite face from which hydride transfer had occurred. The position of the water molecule that occupies the phosphate-binding site in this new acyl-enzyme intermediate structure (Fig. 5B) clearly shows that nucleophilic attack by phosphate (or release of phosphate in the physiological direction) must occur from the opposite face of the covalently bound acyl-enzyme.

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