Structure Analysis of the Staphylococcus aureus UDP-N-acetyl-mannosamine Dehydrogenase Cap5O Involved in Capsular Polysaccharide Biosynthesis* § §

Received for publication, December 23, 2010, and in revised form, March 6, 2011. Published, JBC Papers in Press, March 23, 2011, DOI 10.1074/jbc.M110.216002

Jakub Gruszczynski1,1, Aurore Fleurie1,1, Vanesa Olives-Illana1,2,3, Emmanuelle Béchet4, Isabelle Zanella-Cleon1, Solange Moréra1, Philippe Meyer1, Guillaume Pompidor1, Richard Kahn3, Christophe Grangeasse1,2, and Sylvie Nessler4,4

From the 1Laboratoire d’Enzymologie et Biochimie Structurales, Centre de Recherche de Gif, CNRS, 91198 Gif sur Yvette, France, the 2Institut de Biologie et Chimie des Protéines, UMR 5086 (CNRS, Université Lyon 1), 7 Passage du Vercors, 69367 Lyon, France, and the 3Institut de Biologie Structurale J.-P. Ebel, UMR 5075 (CNRS, CEA, UJF), 41 Rue Jules Horowitz, 38027 Grenoble, France

Bacterial UDP-sugar dehydrogenases are part of the biosynthesis pathway of extracellular polysaccharides. These compounds act as important virulence factors by protecting the cell from opsonophagocytosis and complement-mediated killing. In Staphylococcus aureus, the protein Cap5O catalyzes the oxidation of UDP-N-acetyl-mannosamine to UDP-N-acetyl-mannosaminuronic acid. Cap5O is crucial for the production of serotype 5 capsular polysaccharide that prevents the interaction of bacteria with both phagocytic and nonphagocytic eukaryotic cells. However, details of its catalytic mechanism remain unknown. We thus crystallized Cap5O and solved the first structure of an UDP-N-acetyl-mannosamine dehydrogenase. This study revealed that the catalytic cysteine makes a disulfide bond that has never been observed in other structurally characterized members of the NDP-sugar dehydrogenase family. Biochemical and mutagenesis experiments demonstrated that the formation of this disulfide bridge regulates the activity of Cap5O. We also identified two arginine residues essential for Cap5O activity. Previous data suggested that Cap5O is activated by tyrosine phosphorylation, so we characterized the phosphorylation site and examined the underlying regulatory mechanism.

Staphylococcus aureus is an opportunistic bacterial pathogen responsible for a diverse spectrum of human and animal diseases ranging from soft-tissue infections to life-threatening bacteremia, endocarditis, and abscesses within multiple organs (1). Among the multiple factors responsible for the pathogenesis of staphylococcal infections, capsular polysaccharides (CPSS)§ have been shown to possess antiphagocytic properties allowing the bacterium to persist in the blood and tissues of infected hosts (2, 3). CPSSs are produced by most S. aureus isolates from human and serotypes 5 (CP5) and 8 (CP8) strains are the most prevalent (3). Both CP5 and CP8 are heteropolymers composed of N-acetyl-fucosamine and N-acetyl-d-mannosamine. They differ only in the linkages between sugars and acetylation sites (4). The cap5 and cap8 loci are allelic and comprise a ~17.5-kb region of the chromosome. Each locus contains 16 genes, cap5A (cap8A) through cap5P (cap8P) (5). Twelve out of these sixteen genes are nearly identical in both gene clusters. The deletion of the conserved cap5O gene product has been shown to inhibit CP5 synthesis, thus enhancing adherence of bacteria to endothelial cells (6).

Cap5O possesses an UDP-N-acetyl-mannosamine dehydrogenase (UDP-ManNAcDH) activity catalyzing the oxidation of UDP-N-acetyl-mannosamine (UDP-ManNAc) to UDP-N-acetyl-mannosaminuronic acid (UDP-ManNAcA), the donor of ManNAcA residues in CP5 (7, 8). Cap5O belongs to the UDP-glucose/GDP-mannose dehydrogenase family, a group of enzymes that catalyze the NAD+−dependent 2-fold oxidation of an alcohol to an acid. Structure-function analysis has been performed with the UDP-glucose dehydrogenase (UDP-GlcDH) from Streptococcus pyogenes (PDB entries 1DLI and 1DLJ) (9) and with the GDP-mannose dehydrogenase (GDP-ManDH) from Pseudomonas aeruginosa (PDB entries 1MV8, 1MFZ, and 1MUU) (10). Both enzymes display a dimeric quaternary structure. Each monomer is characterized by the presence of three regions: a conserved N-terminal NAD-binding domain, a central α-helical region involved in dimerization and a C-terminal substrate-binding domain. The N-terminal NAD-binding domain consists of a typical Rossmann fold that is also found in a degenerated form in the less conserved C-terminal NDP-sugar-binding domain. It is linked via an additional αβαββ-motif extending the central β-sheet to the central dimerization domain, which consists of a bundle of 4 α-helices.

* This work was supported in part by grants from the CNRS, the University of Lyon, and the Agence National de la Recherche (ANR-05-MIIM-031, ANR-07-JCJC0125, and ANR-08-BLAN-0143), the Association pour la Recherche sur le Cancer, and the “Direction Générale des Armées” (to E. B.), “Région Rhône-Alpes cluster 10” (to A. F.), and “Région Ile de France” (to J. G.).

§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S6.

1 Both authors contributed equally to this work.

2 Present address: INSERM Cibles Therapeutiques, Institut de Génétique Moléculaire, Hôpital St Louis, Paris, France.

3 To whom correspondence may be addressed. Tel.: 33-4-72-72-26-88; Fax: 33-4-72-72-26-01; E-mail: cgrangeasse@ibcp.fr.

4 To whom correspondence may be addressed. Tel.: 33-1-69-82-34-59; Fax: 33-1-69-82-31-29; E-mail: nessler@lebs.cnrs-gif.fr.

5 The abbreviations used are: CPS, capsular polysaccharide; UDP-ManNAcDH, UDP-N-acetyl-mannosamine dehydrogenase; UDP-ManNAc, UDP-N-acetyl-mannosamine; UDP-ManNAcA, UDP-N-acetyl-mannosaminuronic acid; SAD, single-wavelength anomalous diffraction; rmsd, root mean-square deviation.
The active site is formed by a cleft at the interface between the N- and C-terminal domains. Interestingly, UDP-GlcDH and GDP-MaN DH display a different domain organization, and form domain-swapped dimers (supplemental Fig. S1).

These dehydrogenases share the same active site topology and the same enzymatic mechanism (10, 11). The reaction consists on a 4-electron oxidation using two equivalents of NAD$^+$ to convert NDP-sugars into their acidic form. Initial oxidation of the alcohol C6 hydroxyl proceeds by transfer of a hydride to NAD$^+$ and produces a sequestered aldehyde intermediate. Nucleophilic attack of the aldehyde by an active site thiol induces the formation of a tetrahedral thiocarbanion. The latter is oxidized to a thioester intermediate by transfer of the remaining hydride to a second molecule of NAD$^+$. In the final step of the reaction, the thioester is irreversibly hydrolyzed to give the acid. A conserved nucleophilic cysteine is involved in thioester formation (Cys-260 and Cys-268 in UDP-GlcDH and GDP-ManDH, respectively). This catalytic mechanism is supposed to be common to all members of the family. This hypothesis is based on the conservation of several residues proposed to be involved in catalysis. Lys-263/271 (UDP-GlcDH/GDP-ManDH) interacts with the catalytic thiol thus increasing its nucleophilic character and stabilizing the active thiolate anion. Lys-204/Lys-210 acts as the catalytic base assisting hydride transfer. Asp-264/272, Thr-118/124, Asn-208/214, and Glu-141/157 activate water molecules and participate in the tetrahedral intermediate formation or hydride transfer (12). Residues responsible for sugar specificity have not yet been identified and sequence signatures specific of each subfamily remain to be characterized.

Enzymatic assays performed with Cap5O demonstrate that its biochemical properties are similar to those described for other members of the family (7). Sequence alignment (Fig. 1) shows that Cap5O residue Cys-258 corresponds to the nucleophilic cysteine involved in thioester formation. It also shows that the remaining catalytic residues are conserved, except for Lys-263/271, which is replaced by a valine. On the other hand, it has been demonstrated that Cap5O dehydrogenase activity is positively controlled by tyrosine phosphorylation (13), as also reported for the UDP-GlcDHs from Bacillus subtilis (14) and Escherichia coli (15). The phosphorylated tyrosine of these two UDP-GlcDHs has recently been identified as Tyr-70 and Tyr-71 in B. subtilis and E. coli, respectively (16, 17). However, no equivalent of this phosphorylated tyrosine is found in Cap5O (Fig. 1), suggesting that this UDP-ManNAcDH might have an alternative mechanism of activation by phosphorylation. The phosphorylation site of Cap5O has not yet been identified.

To address the structural basis for the specificity and regulatory mechanism of S. aureus Cap5O, we have determined its crystal structure and performed functional experiments. This study allowed us to highlight residues potentially involved in the specific recognition of the sugar. We have also identified the phosphorylation site of Cap5O and assessed the influence of tyrosine phosphorylation on its activity. This structural analysis also revealed the existence of an unexpected disulfide bond. We therefore propose an original regulatory process based on both tyrosine phosphorylation and reversible reduction of a disulfide bond involving the catalytic residue Cys-258.

**EXPERIMENTAL PROCEDURES**

**Plasmids Construction and Site-directed Mutagenesis**—Plasmids pQE30-Cap5O and pQE30-A1B2 described in Refs. 13 and 18 were used to produce the S. aureus His-tagged protein Cap5O and the His-tagged tyrosine-kinase Cap5A1B2, respectively. Site-directed mutagenesis of Cap5O was carried out by PCR amplification using specific primers and DNA fragments were cloned into the pQE30 vector (supplemental Table S1). The S. aureus UDP-N-acetylglucosamine 2-epimerase Cap5P was PCR-amplified using specific primers and S. aureus Reynolds strain genomic DNA as template. The DNA fragment synthesized was digested by enzymes BamHI and HindIII and then ligated into the pQE30 vector previously opened with the same enzymes. The resulting recombinant plasmid was termed pQE30-Cap5P. The nucleotide sequences of all synthesized and mutated genes were checked to ensure error-free amplification.

**Protein Expression and Purification**—For crystallization, the full-length N-terminally His$_6$-tagged UDP-N-acetylmannosamine dehydrogenase from S. aureus (Cap5O) was expressed in E. coli strain BL21 STAR (DE3) (Invitrogen) in 2TY medium. Expression was induced at OD600 of 1.0 by addition of 0.5 mM IPTG. After 3 h of induction at 37 °C, cells were harvested by centrifugation and resuspended in the lysis buffer (25 mM Tris-HCl pH 7.8, 300 mM NaCl, 10% glycerol) supplemented with 300 μl of Protease Inhibitor Mixture (Sigma-Aldrich). Cells were flash-frozen in liquid nitrogen and stored at −80 °C until further processing. The bacterial pellet was thawed on ice, resuspended in fresh lysis buffer containing 0.5 mg/ml of lysozyme (Euromedex) and 1 mg/ml of DNase I (Roche) and lysed by sonication. After centrifugation at 50,000 × g for 45 min, the supernatant was applied onto a Ni-NTA column and extensively washed with buffer A (25 mM Tris-HCl, pH 7.8, 300 mM NaCl, 10 mM imidazole, 50 mM β-mercaptoethanol). Samples were eluted with buffer A containing 300 mM imidazole. Peak fractions were concentrated and purified by gel filtration on a Superdex S200 26/60 equilibrated with buffer B (20 mM Tris-HCl, pH 7.8, 100 mM NaCl). Fractions containing pure protein were pooled together and stored at 4 °C. For kinase and dehydrogenase assays, Cap5O mutants were purified using the same procedure. Analysis by circular dichroism spectroscopy has shown that the introduced mutations do not affect the secondary structure of the proteins (supplemental Fig. S2). Cap5P and Cap5A1B2 were overexpressed and purified as described above for Cap5O but in the absence of reducing agent.

**Crystallization and Data Collection**—Freshly purified Cap5O was concentrated by ultrafiltration just prior to crystallization, and the protein concentration was determined spectrophotometrically using a calculated extinction coefficient ε(280 nm) of 0.39 (cm/mg/liter)$^{-1}$. Commercially available crystallization solutions were screened using a Cartesian Robot (Genomics Solutions) and the sitting-drop/vapor diffusion method. Initial crystallization hits were reproduced and optimized manually using the hanging-drop method. Crystals were obtained with the protein at a concentration of 50 mg/ml in the
presence of 10 mM NAD\(^+\) equilibrated against a crystallization solution containing 0.1 M Tris-HCl, pH 7.5, 0.2 M sodium acetate, and 24% (w/v) polyethylene glycol 4,000. A mative data set was collected at 2.8 Å resolution on beamline ID14–3 at the ESRF (Grenoble, France). Derivative crystals were obtained by co-crystallizing Cap5O in the presence of [Eu(DPA)]\(^{3+}\) (19). Crystals were obtained with 15 mg/ml Cap5O in the presence of 5 mM NAD\(^+\), 5 mM MgCl\(_2\), and 50 mM Na\(_2\)[Eu(DPA)]\(^{3+}\), equilibrated against a crystallization solution containing 0.1 M Tris-HCl, pH 8.0, 0.2 M sodium acetate, and 32% (w/v) polyethylene glycol 4,000. For data collection, crystals were cryo-protected by sequential soaking in crystallization solutions supplemented with 10 and 20% (w/v) of glycerol. Diffraction data were collected on beamline ID14–4 at the ESRF (Grenoble, France). The wavelength was set at 1.2525 Å, which is not optimal (Euro- pium LIII absorption edge = 1.7771 Å) but allowed us to measure an anomalous signal strong enough for SAD phasing.

Structure Solution and Refinement—Collected data were integrated with MOSFLM (20) and scaled using SCALA from the CCP4 suite of programs (21). For the europium derivative data, de novo phasing was achieved by the single-wavelength anomalous diffraction method (SAD) using PHENIX (22). An initial model covering about 60% of the sequence was automatically built using the same program suite. Subsequent model building was done manually in COOT (23). The refinement procedure was carried out with BUSTER (24). The resulting model of the Cap5O derivative was used as a search model to solve the structure of the native protein by molecular replacement using the program PHASER (25). Table 1 summarizes the solving the structure of the native protein by molecular replace- ment using the program PHASER (25). Table 1 summarizes the

Sample Preparation and Mass Spectrometry Analysis—The purified Cap5O–C92S mutant was subjected to in vitro phosphorylation with non-radioactive ATP, as described in the phosphorylation assays section, prior to analysis on SDS-PAGE. In-gel digestion was performed as described by Shevchenko et al. (26) with minor modifications. The samples were reduced with 60 μl of 10 mM dithiothreitol in 50 mM NH\(_4\)HCO\(_3\) for 15 min at 50 °C. Alkylation was performed with 60 μl of 100 mM iodoacetamide in 50 mM NH\(_4\)HCO\(_3\) for 15 min at room temperature in the dark. The gel pieces were dried using 0.4 ml of CH\(_3\)CN. For proteolytic digestion, the protein-containing gel pieces were treated with 40 μl of trypsin solution, 0.02 μg/μl in 50 mM NH\(_4\)HCO\(_3\) (sequence grade, Promega, France) for 45 min at 50 °C. A second extraction step was performed using 30 μl of a H\(_2\)O/CH\(_3\)CN/HCOOH (60:36:4; v/v/v) mixture for 30 min at 30 °C, and all extracts were finally pooled and dried in a vacuum concentrator and redissolved in 0.3% trifluoroacetic acid (20 μl). Mass spectrometry experi- ments were performed with a LTQ Velos (ThermoScientific, France) instrument in the positive ion mode. The ion source was equipped with a picoTip emitter as nanospray needle (FS360–75–30–CE-5-C10.5, NewObjective) operating at 1.5 kV. Typically, two scan events were used: 1) m/z 400–2000 survey scan MS with enhanced resolution; 2) data-dependent scans MS/MS on the twenty most intense ions from event 1. The spectra were recorded using dynamic exclusion of previously analyzed ions for 0.6 min. The MS/MS normalized collision energy was set to 35 V. Reversed-phase LC was performed with an Ultimate 3000 nano-LC system (Dionex, France). Chromatographic separation of the peptides was achieved by reversed phase HPLC on a C\(_{18}\) PepMap micro-precolumn (5 μm; 0.3 mm × 5 mm) for desalting and a C\(_{18}\) PepMap nano- column (3 μm; 75 μm × 150 mm) with a gradient elution at a flow rate of 300 nl/min. Eluent A was a mixture of H\(_2\)O/ CH\(_3\)CN/formic acid (95:5:0.1; v/v/v). Eluent B was a mixture of H\(_2\)O/CH\(_3\)CN/formic acid (20:80:0.1; v/v/v). The gradient pro- gram was used from 0% B to 50% B over 60 min and 100% B for 10 min.

Phosphorylation Assays—In vitro phosphorylation assays were carried out using the active chimera Cap5A1B2 obtained by fusing the cytoplasmic S. aureus Cap5B2 tyrosine kinase to the C-terminal cytoplasmic peptide of the membrane activator Cap5A1 (27). 2 μg of the purified wild-type or mutant Cap5O proteins were incubated with 0.2 μg of Cap5A1B2 in 25 mM Tris-HCl, pH 7.5, 1 mM DTT, 5 mM MgCl\(_2\), 1 mM EDTA, and 25 μM ATP with 200 mCi/ml [γ\(^32\)P]ATP. The efficiency of phos- phorylation was examined in a time-dependent assay at 37 °C. One master mixture was prepared for each reaction, and aliquots were collected at 1, 2, 5, and 10 min. The samples were analyzed by SDS-PAGE electrophoresis. The gels were then soaked in 16% TCA for 10 min at 90 °C. The radioactive-labeled proteins were visualized by autoradiography using direct film exposure.

Dehydrogenase Assays—Cap5O was tested for UDP-N-acetyl-mannosamine dehydrogenase activity using a spectrophotometric assay adapted from (13) and (7). Briefly, the absorbance at 340 nm, resulting from NADH formation, was measured on a Tecan UV-visible spectrophotometer each 30 s for 30 min in a thermostated 96-well plate at 37 °C. All the reagents except NAD\(^+\) were mixed and placed at 37 °C. Reac- tions were monitored upon NAD\(^+\) addition. The reaction mix- tures contained 20 μg of Cap5O (wild type or mutated), 3 μg of Cap5P, 0.5 mM UDP-N-acetyl-glucosamine, 50 mM Tris-HCl, pH 8.5, and 1.5 mM NAD\(^+\) in a final volume of 200 μl. For measuring Cap5O activity in reducing conditions, 0.5 mM DTT was added to the mixture. Control reactions were made in the presence of NADH.
Structure of the Staphylococcal UDP-ManNAcDH

TABLE 1

Diffraction statistics for the S. aureus Cap5O crystals

| Parameters | Parameters | [Eu(DPA)]_3^{3-} | Native |
|------------|------------|------------------|--------|
| Data collection | Space group | P2_1,2_1 | P2_1,2_1 |
| | Cell parameters a, b, c (Å) | 40.22, 131.50, 158.82 | 40.22, 131.50, 158.82 |
| | Cell angles α, β, γ (Å) | 90.0, 90.0, 90.0 | 90.0, 90.0, 90.0 |
| | Resolution (Å) | 29.63-2.50 (2.59-2.50) | 29.12-2.80 (2.94-2.80) |
| | Observed reflections | 198,886 | 148,928 |
| | Unique reflections | 30,007 | 22,319 |
| | Completeness (%) | 99.7 (98.3) | 98.4 (89.8) |
| | Mean R diff (Å) | 14.4 (2.2) | 12.1 (1.9) |
| | Refinement statistics | Protein atoms | 6465 | 6496 |
| | | Ligand atoms | 162 | 88 |
| | | Solvent atoms | 120 | 43 |
| | | R_all (%) | 20.1 (21.8) | 21.7 (22.34) |
| | | Rfree (%) | 24.6 (26.9) | 26.3 (26.41) |
| | | Mean B value (Å²) | 57.66 | 70.03 |
| | | Rmsd bond length (Å) | 0.014 | 0.013 |
| | | Rmsd bond angles (°) | 1.7 | 1.7 |

* Numbers in parentheses represent values in the highest resolution shell.

** R_all = \sum l I(h) - \langle I(h) \rangle / \sum l I(h) where I(h) is the intensity value of the i-th measurement and \langle I(h) \rangle is the corresponding mean value of I(h) for all I measurements.

*** Rfree is the same as R_all but calculated with a 5% subset of all reflections that was never used in crystallographic refinement.

absence of Cap5O, Cap5P, or NAD+. Cap5O activity was also determined after phosphorylation by the active tyrosine kinase chimera Cap5A1B2. For this purpose, 20 μg of Cap5O (wild type or mutated) were first incubated for 30 min at 37 °C with 5 μg of Cap5A1B2 in 25 mM Tris-HCl pH 8.5, 5 mM MgCl2, 50 mM NaCl, and 200 μM ATP. Then, 3 μg of Cap5P, 0.5 mM UDP-N-acetyl-glucosamine, 50 mM Tris-HCl pH 8.5, and 1.5 mM NAD+ were added and absorbance at 340 nm was read as previously described. Control assays without ATP, NAD+, or UDP-ManNAc were run in parallel.

RESULTS

Cap5O/NAD+/[Eu(DPA)]_3^{3-} Structure—Cap5O shares low sequence identity with UDP-GlcDH (PDB code 1DLI) (9) and P. aeruginosa GDP-ManDH (PDB code 1MY8) (10) (20 and 23% sequence identity, respectively). We thus solved the structure of S. aureus Cap5O at 2.5Å resolution by SAD phasing using as heavy atom an europium-dipicolinate dianion complex [Eu(DPA)]_3^{3-} that has recently proven to exhibit cross-linking properties (19) (Table 1). The asymmetric unit contains a dimer consisting of two Cap5O subunits. The N-terminal residues 1–148 display a Rossmann fold with a central 6-stranded parallel β-sheet motif extending the central α7/α8–α9 motif with two antiparallel strands (residues 149–196). The central dimerization domain (residues 197–301) consists of a bundle of 4 α-helices from each subunit: two long helices (α9 and α12) and two smaller helices (α10 and α11). The C-terminal sugar-binding domain (residues 302–420) displays a degenerated Rossmann fold with a central 5-stranded parallel β-sheet (strands order βββββ/ββBBB) covered by helices α13 and η3 on one side and by helices α14 and η2 on the other side (Fig. 2). Interestingly, both subunits are oxidized and display a Cys-92 to Cys-258 intramolecular disulfide bridge (Fig. 3A).

Cap5O/NAD+ Structure—We have also solved the structure of Cap5O in the absence of [Eu(DPA)]_3^{3-}, using the Cap5O-dipicolinate lanthanide complex as a search model for molecular replacement (Table 1). Both Cap5O dimers are similar upon superimposition with the protein from S. pyogenes (PDB code 1DLI) (9) shows a rmsd distance of 3.2Å over 658 residues. Cap5O monomers contain the three distinct domains characteristic of UDP-glucose/GDP-mannose dehydrogenase family. The N-terminal domain corresponding to the NAD-binding domain (residues 1–148) displays a typical Rossmann fold with a central 6-stranded parallel β-sheet (strands order ββββββ/BBBββββ) covered by the three helices α1, α2, α3 on one side and by the helices α4–η1, α5, and α6 on the other side. It is completed by an additional α7/α8–α9–βH motif extending the central β-sheet at the βF extremity with two antiparallel strands (residues 149–196). The central dimerization domain (residues 197–301) consists of a bundle of 4 α-helices from each subunit: two long helices (α9 and α12) and two smaller helices (α10 and α11). The C-terminal sugar-binding domain (residues 302–420) displays a degenerated Rossmann fold with a central 5-stranded parallel β-sheet (strands order βββββ/ββBBB) covered by helices α13 and η3 on one side and by helices α14 and η2 on the other side (Fig. 2). Interestingly, both subunits are oxidized and display a Cys-92 to Cys-258 intramolecular disulfide bridge (Fig. 3A).

FIGURE 2. Overall structure of S. aureus Cap5O. Schematic representation of the Cap5O dimer. One subunit is shown in beige. The second subunit is colored by domain. The N-terminal NAD-binding domain (residues 1–150) is colored in cyan. It is completed by an additional subdomain (residues 151–196) colored in blue. The central dimerization domain (residues 197–301) colored in green. The C-terminal substrate binding domain (residues 302–420) is colored in magenta. The bound NAD cofactor is shown as dots colored by atom type.

FIGURE 3. (A) Overall structure of S. aureus Cap5O/NAD+. Schematic representation of the Cap5O/NAD+ dimer. One subunit is shown in beige. The second subunit is colored by domain. The N-terminal NAD-binding domain (residues 1–150) is colored in cyan. It is completed by an additional subdomain (residues 151–196) colored in blue. The central dimerization domain (residues 197–301) colored in green. The C-terminal substrate binding domain (residues 302–420) is colored in magenta. The bound NAD cofactor is shown as dots colored by atom type.
reduced, and the two cysteines are 11 Å apart (Fig. 3B). Formation of the disulfide bond between Cys-92 and Cys-258 induces a small movement of the N- and C-terminal domains toward each other (Fig. 3C). More importantly, the main difference between the reduced and the oxidized conformations concerns the loop between residues 258 and 258. When the loop first residue Cys-258 is not implicated in the S-S bridge with Cys-92, it acquires a helical conformation; helix α11. The Cys-92/Cys-258 disulfide bond sequesters the catalytic Cys-258 residue directly involved in the catalytic mechanism via thioester formation. The oxidized form thus corresponds to an inactive conformation of the enzyme. These observations suggest that oxydo/reduction of the catalytic Cys-258 could control Cap5O activity.

Role of the Cys-92/Cys-258 Disulfide Bridge—To characterize the putative regulatory role of the disulfide bridge, we evaluated the effect of a reducing agent on the activity of Cap5O. UDP-ManNAc, the substrate of Cap5O, is not commercially available. Therefore we have used a coupled assay based on Cap5P, an UDP-N-acetylgalactosamine 2-epimerase synthesizing UDP-ManNAc from UDP-N-acetylgalactosamine (UDP-GlcNAc). Cap5O activity was assessed in the presence and in the absence of DTT. As previously reported (7, 8), addition of the reducing agent in the reaction mixture clearly increased the activity of the enzyme (Fig. 4). To verify that the Cys-92/Cys-258 disulfide bond observed in the crystal structures was indeed responsible for this effect, we tested the activity of the Cap5O-C92S mutant supposed to mimic the active reduced form of the enzyme. Surprisingly, the C92S mutation has a strongly inhibitory effect on Cap5O activity (Fig. 4), suggesting an additional role for Cys-92 than just sequestering Cys-258. This second cysteine is however outside the active site and no evident catalytic role could be
extended conformation similar to that seen in many NAD-protein complexes. It is deeply buried (more than 90% of its total surface area) in a pocket of the N-terminal dinucleotide-binding domain with an interface area of about 575 Å². The ribose of the adenosine moiety forms a hydrogen bond with the side chain of the conserved Asp-30, and the second ribose interacts with the conserved residues Thr-82 and Thr-119. The two phosphates display typical interactions with main chain atoms of the glycine-rich phosphate binding-loop (residues 7–12) and with the guanidinium group of the conserved Arg-322. The nicotinamide ring pointing toward the C-terminal UDP-ManNAc binding domain is poorly defined in the electron density map of all subunits. This has already been reported for other NAD-protein complexes in the absence of substrate and may be due to multiple rotational conformers of the nicotinamide ring (10).

**UDP-sugar Binding Analysis**—In the complex with [Eu(DPA)]₃³⁻, the latter binds in a deep cleft between the two domains of Cap5O, with an interface area of about 560 Å². As previously reported for these lanthanide complexes (19), strong interactions are observed between the carboxyl groups of the three dipicolinate dianions (DPA) and the arginine side chains. In the complex with Cap5O, these residues are Arg-152 from loop βF-α7 of the N-terminal domain, Arg-211 from the long dimerization helix α9 and Arg-244 from the loop α10-α11 of the neighboring subunit (Fig. 5B). Arg-244 is well conserved among NDP-sugarDHs and has already been shown to interact with the substrate of UDP-GlcDH (PDB entry 1DLI) (9) and GDP-ManDH (PDB entry 1MV8) (10). Interestingly, Cap5O residues Arg-152 and Arg-211 have no equivalent in UDP-GlcDH or GDP-ManDH while they are conserved in the homologs of Cap5O annotated UDP-N-acetyl-d-mannosamine DHs (Fig. 1). It is therefore tempting to hypothesize that these two basic residues are particularly important for substrate binding and more especially for N-acetyl specific recognition. Superimposition of the Cap5O/NAD⁺/[Eu(DPA)]₃³⁻ complex on the structure of *S. pyogenes* UDP-GlcDH in complex with UDP-glucuronate (PDB code 1DLI) (Fig. 6A), shows that [Eu(DPA)]₃³⁻ superimposes on the sugar moiety of the substrate (Fig. 6B), thus supporting the hypothesis that Arg-152 and Arg-211 could be responsible for specific sugar recognition. To verify this hypothesis, we mutated these two arginines into the corresponding UDP-GlcDH residues (Cap5O-R152F-R111L) and compared the activity of this double Cap5O mutant with the wild-type protein using the Cap5P-coupled enzymatic assay described above. The double mutant was not able to oxidize UDP-ManNAc (Fig. 4). However, it did not gain the ability to oxidize UDP-Glc either (data not shown). These results confirm the essential role of the two arginines but more experiments, such as testing the oxidation of UDP-Man, are needed to prove their involvement in the recognition of the acetyl group or in the recognition of the specific sugar epimerization state. We also cannot rule out a possible involvement in catalysis instead of specific substrate recognition.

**Cap5O Tyrosine Phosphorylation**—Cap5O has been previously shown to be activated upon tyrosine phosphorylation (13) catalyzed by the *S. aureus* chimera Cap5A1B2 (See “Experimental Procedures,” Phosphorylation Assays section) (18). In
Using an antiphosphotyrosine antibody (supplemental Fig. S5), occurs, due to relaxed specificity of the kinase. Interestingly, phosphorylation suggests that nonspecific phosphorylation identified as the most likely phosphorylation site. Residual tyrosine mutants (data not shown). Therefore, Tyr-89 was reduced compared with wild-type Cap5O and to the four other mutants (data not shown). The signal of Cap5O-Y89F, however, was always strongly phosphorylated signal, all mutants were phosphorylated as efficiently as the wild-type protein (Fig. 7A). Complete loss of the Cap5O-Y89F phosphorylation signal was never achieved. The assay was also performed with the wild-type Cap5B2 tyrosine kinase in presence of membranes enriched with its full-length transmembrane activator Cap5A1 (27). Different ratios of kinase/substrate and different incubation times were used. The signal of Cap5O-Y89F however, was always strongly reduced compared with wild-type Cap5O and to the four other tyrosine mutants (data not shown). Therefore, Tyr-89 was identified as the most likely phosphorylation site. Residual phosphorylation suggests that nonspecific phosphorylation occurred, due to relaxed specificity of the kinase. Interestingly, Cap5O-Y89F did not display any phosphorylation signal when using an antiphosphotyrosine antibody (supplemental Fig. S5), a less sensitive detection method than radioactive labeling. This suggests that nonspecific phosphorylation only happened at a very low level.

**Validation of the Phosphorylation Site**—Interestingly, the Cap5O-C92S mutant displayed a more intense phosphorylation signal than the wild-type enzyme (Fig. 7A), suggesting that this mutation increases the accessibility of the phosphorlatable tyrosine. The mutant Cap5O-C92S was thus incubated with non-radioactive ATP in the presence of Cap5A1B2 and subjected to mass spectrometry analysis after tryptic digestion to confirm the phosphorylation site. The sequence coverage of the protein was 96.5%, and phosphorylation occurred only on peptide (V66-R90) with an 80 Da mass increment from 2758.38 to 2838.38 Da (monoisotopic mass). The MS/MS spectrum of the corresponding triply charged ion at m/z 943.8 unambiguously confirmed the presence of the phosphate group on Tyr-89 (Fig. 7B), a residue carried by the same loop (β4-αd) as Cys-92. This result is confirmed by the analysis of the doubly charged ion spectrum (supplemental Fig. S6). The absence of mass increments lower or higher than 80 Da excludes the eventualty of any other post-translational modifications of Cap5O.

**Effect of Tyr-89 Phosphorylation on Cap5O Activity**—To measure the effect of Tyr-89 phosphorylation on Cap5O activity, we incubated Cap5O with Cap5A1B2 in the presence of ATP prior to perform the enzymatic assays. A strong increase of Cap5O activity was observed compared with the control performed in the absence of ATP (Fig. 4). We then verified that the basal activity of Cap5O is not affected by the Y89F mutation but that the activating effect of Cap5A1B2 is lost (Fig. 4). Taken together, these experiments thus clearly demonstrate that Cap5O activity is activated by Cap5A1B2-dependent phosphorylation of residue Tyr-89. The Cap5O/NAD⁺ and the Cap5O/NAD⁺/[Eu(DPA)₃]³⁻ structures have been deposited in the Protein Data Bank under code 3OJL and 3OJO, respectively.

**DISCUSSION**

This study demonstrates that *S. aureus* UDP-ManNAcDH Cap5O belongs to the NDP-sugar dehydrogenase family and that it displays the same dimeric conformation as UDP-GlcDHs. The catalytic cysteine Cys-258, the acid/base catalyst Lys-204 and residues Asp-262, Thr-119, Asn-208, and Glu-151 (Cap5O numbering) participating in tetrahedral intermediate formation or hydride transfer are conserved. This suggests that Cap5O most probably possesses the same NAD-dependent 4-electrons oxidation mechanism as the other members of the family. On the other hand, we propose that specific recognition of the sugar moiety of the substrate involves two essential arginine residues, Arg-152 and Arg-211, only found in the sequences of Cap5O closest homologues that are therefore likely to be dehydrogenases specific for NDP-ManNAc.

The presence in the crystal structure of a disulfide bridge between Cys-92 and the catalytic Cys-258 suggests that Cap5O dehydrogenase activity could be regulated by a redox-switch mechanism. Our functional analysis demonstrating that Cap5O requires reducing conditions for full dehydrogenase activity supports this hypothesis. Formation of the Cys-92/Cys-258 disulfide bridge sequesters the essential catalytic cysteine,
stabilizing an inactive conformation where helix α11 carrying Cys-258 is destroyed and replaced by a flexible loop. A blast search against the UniProtKB database revealed that Cys-92 is conserved among several Cap5O homologues, suggesting that a similar redox regulation mechanism could be used by other NDP-sugar dehydrogenases. As observed in protein tyrosine phosphatases of the Cdc25 family, the formation of a disulfide bond between the catalytic residue and an adjacent back-door cysteine would hinder the reactive catalytic cysteine to oxidize into an irreversibly inactivated sulfinic acid (30–32).

In addition to this redox-switch, our results show that Cap5O activity is also regulated by tyrosine phosphorylation and we identified Tyr-89 as the phosphorylatable residue. A similar phosphorylation-dependent regulatory mechanism has been reported for two UDP-GlcDHs from E. coli (15, 17) and B. subtilis (14, 16), suggesting that tyrosine phosphorylation of UDP-sugarDHs seems to represent a conserved regulatory mechanism for several bacteria to control extracellular polysaccharide production. However, the phosphorylation site of these UDP-GlcDHs (Tyr-70 and Tyr-71, in E. coli and B. subtilis, respectively) is located in a very distinct region of the protein compared with Cap5O Tyr-89, suggesting different phosphorylation-dependent activation mechanisms. This latter possibility is not surprising if we consider the poor conservation of phosphorylation sites among bacterial orthologs (33). The analysis of the phosphorylation-mediated activation mechanism of E. coli and B. subtilis UDP-GlcDH is highly speculative (16) mostly due to the absence of structural data. In Cap5O, Tyr-89 is located on the same loop (β0-α4) as Cys-92, which forms the disulfide bond with the catalytic Cys-258. Because Tyr-89 points toward the flexible (α11-α12) loop, we can suggest that once phosphorylated, Tyr89-P could participate in the stabilization of helix α11 and thus in the correct positioning of the catalytic residue Cys-258.

In Cap5O, both redox and phosphorylation-dependent regulatory mechanisms thus seem intimately linked. Under normal growth conditions, the bacterial cytoplasm is a reduced environment. Therefore, the described redox-switch might be
required for Cap5O activity when the bacterium is exposed to oxidizing condition. Such conditions are encountered by several pathogens during host infection, for example during polymorphonuclear leukocytes phagocytosis that induces production of reactive oxygen species (34). Interestingly, it was shown that genes involved in capsule synthesis, including Cap5O, the tyrosine-kinase Cap5B2 and its transmembrane activator Cap5A1, are up-regulated during *S. aureus* neutrophil phagocytosis (34). In such an oxidizing environment, the thioredoxin system, that is the major disulfide reductant in the bacterial cytosol, is also activated (35) and it would contribute together with the tyrosine kinase Cap5B2 to trigger Cap5O activity to allow optimal capsule production. Obviously, this last hypothesis is highly speculative but it could represent an appealing work model to explain the biological function of the Cap5O redox-switch.

Acknowledgments—We acknowledge the use of beamline ID14 at the European Synchrotron Radiation Facility and the Imagif crystallization platform. We thank M. Becchi and R. Montserret (Common Center for Microanalysis of Proteins, Institut de Biologie et Chimie des Proteines, Lyon, France) for excellent expertise and technical assistance in mass spectrometry analysis and circular dichroism, respectively. We also thank Dr. E. Issakidis-Bourguet (Institut de Biologie des Plantes, University Paris-Sud 11, Orsay, France) for constructive discussion about redox regulation mechanisms and Dr. R. Grenha (Laboratoire d’Enzymologie et Biochimie Structurales, Gif-sur-Yvette, France) for careful reading of the manuscript.

REFERENCES

1. Watts, A., Ke, D., Wang, Q., Pillay, A., Nicholson-Weller, A., and Lee, J. C. (2005) *Infect. Immun.* 73, 3502–3511
2. Thakker, M., Park, J. S., Carey, V., and Lee, J. C. (1998) *Infect. Immun.* 66, 5183–5189
3. O’Riordan, K., and Lee, J. C. (2004) *Clin. Microbiol. Rev.* 17, 218–234
4. Jones, C. (2005) *Carbohydr. Res.* 340, 1097–1106
5. Sau, S., Bhasin, N., Wann, E. R., Lee, J. C., Foster, T. J., and Lee, C. Y. (1997) *Microbiology* 143, 2395–2405
6. Pohlmann-Dietze, P., Ulrich, M., Kiser, K. B., Döring, G., Lee, J. C., Fournier, J. M., Botzenhart, K., and Wolz, C. (2000) *Infect. Immun.* 68, 4865–4871
7. Portolés, M., Kiser, K. B., Bhasin, N., Chan, K. H., and Lee, J. C. (2001) *Infect. Immun.* 69, 917–923
8. Kawamura, T., Ishimoto, N., and Ito, E. (1979) *J. Biol. Chem.* 254, 8457–8465
9. Campbell, R. E., Mosimann, S. C., van De Rijn, I., Tanner, M. E., and Strynadka, N. C. (2000) *Biochemistry* 39, 7012–7023
10. Snook, C. F., Tipton, P. A., and Beamer, L. J. (2003) *Biochemistry* 42, 4658–4668
11. Campbell, R. E., and Tanner, M. E. (1999) *J. Org. Chem.* 64, 9487–9492
12. Ge, X., Penney, L. C., van de Rijn, I., and Tanner, M. E. (2004) *Eur. J. Biochem.* 271, 14–22
13. Soulat, D., Grangeasse, C., Vagany, E., Cozzone, A. J., and Duclos, B. (2007) *J. Mol. Microbiol. Biotechnol.* 13, 45–54
14. Mijakovic, I., Poncet, S., Boel, G., Mazé, A., Gillet, S., Jamet, E., Decottignies, P., Grangeasse, C., Doublot, P., Le Maréchal, P., and Deutscher, J. (2003) *EMBO J.* 22, 4709–4718
15. Grangeasse, C., Obadia, B., Mijakovic, I., Deutscher, J., Cozzone, A. J., and Doublot, P. (2003) *J. Biol. Chem.* 278, 39323–39329
16. Petranovic, D., Grangeasse, C., Macek, B., Abdillatif, M., Gueguen-Chaignon, V., Nessler, S., Deutscher, J., and Mijakovic, I. (2009) *J. Mol. Microbiol. Biotechnol.* 17, 83–89
17. Lacour, S., Bechet, E., Cozzone, A. J., Mijakovic, I., and Grangeasse, C. (2008) *PLoS One* 3, e3053
18. Soulat, D., Jault, J. M., Duclos, B., Geourjon, C., Cozzone, A. J., and Grangeasse, C. (2006) *J. Biol. Chem.* 281, 14048–14056
19. Pompidor, G., Daléau, A., Vicat, J., Toupet, L., Giraud, N., Kahn, R., and Maury, O. (2008) *Angew. Chem. Int. Ed. Engl.* 47, 3388–3391
20. Powell, H. R. (1999) *Acta Crystallogr. D. Biol. Crystallogr.* 55, 1690–1695
21. Collaboration Computational Project, N. (1994) *Acta Crystallogr. D. Biol. Crystallogr.* 50, 760–763
22. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, V. B., Davis, I. W., Echols, N., Headd, J. I., 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
23. Emsley, P., and Cowtan, K. (2004) *Acta Crystallogr. D. Biol. Crystallogr.* 60, 2126–2132
24. Blanc, E., Rosvers, P., Vonrhein, C., Flensburg, C., Lea, S. M., and Bricogne, G. (2004) *Acta Crystallogr. D. Biol. Crystallogr.* 60, 2210–2221
25. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) *J. Appl. Crystallogr.* 40, 658–674
26. Schwenger, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal Chem.* 68, 850–858
27. Olivares-Illana, V., Meyer, P., Bechet, E., Gueguen-Chaignon, V., Soulat, D., Lazerg-Riquier, S., Mijakovic, I., Deutscher, J., Cozzone, A. J., Laprèvotte, O., Morera, S., Gueguen-Chaignon, V., and Nessler, S. (2008) *PLoS Biol.* 6, e143
28. Rehder, D. S., and Borges, C. R. (2010) *Biochemistry* 49, 7748–7755
29. Kettenhofen, N. J., and Wood, M. J. (2010) *Chem. Res. Toxicol.* 23, 1633–1646
30. Sohn, J., and Rudolph, J. (2003) *Biochemistry* 42, 10060–10070
31. Chen, C. Y., Willard, D., and Rudolph, J. (2009) *Biochemistry* 48, 1399–1409
32. Tsai, S. J., Sen, U., Zhao, L., Greenleaf, W. B., Dasgupta, J., Fiorillo, E., Orru, V., Bottini, N., and Chen, X. S. (2009) *Biochemistry* 48, 4838–4845
33. Soufi, B., Jers, C., Hansen, M. E., Petranovic, D., and Mijakovic, I. (2008) *Biochim. Biophys. Acta* 1784, 186–192
34. Voyich, J. M., Braughton, K. R., Sturdevant, D. E., Whitney, A. R., Säid-Salim, B., Porcella, S. F., Long, R. D., Dorward, D. W., Gardner, D. J., Kreiswirth, B. N., Musser, J. M., and DeLeo, F. R. (2005) *J. Immunol.* 175, 3907–3919
35. Uziel, O., Borovok, I., Schreiber, R., Cohen, G., and Aharonowitz, Y. (2004) *J. Bacteriol.* 186, 326–334
36. Gouet, P., Courcelle, E., Stuart, D. I., and Métoz, F. (1999) *Bioinformatics* 15, 305–308