The human pathogen *Staphylococcus aureus* does not utilize the glutathione thiol/disulfide redox system employed by eukaryotes and many bacteria. Instead, this organism produces CoA as its major low molecular weight thiol. We report the identification and purification of the disulfide reductase component of this thiol/disulfide redox system. Coenzyme A disulfide reductase (CoADR) catalyzes the specific reduction of CoA disulfide by NADPH. CoADR has a pH optimum of 7.5–8.0 and is a dimer of identical subunits of M_r 49,000 each. The visible absorbance spectrum is indicative of a flavoprotein with a λ_max = 452 nm. The liberated flavin from thermally denatured enzyme was identified as flavin adenine dinucleotide. Steady-state kinetic analysis revealed that CoADR catalyzes the reduction of CoA disulfide by NADPH at pH 7.8 with a K_m for NADPH of 2 μM and for CoA disulfide of 11 μM. In addition to CoA disulfide, CoADR reduces 4,4'-diposphopantethine but has no measurable ability to reduce oxidized glutathione, cystine, pantethine, or H_2O_2. CoADR demonstrates a sequential kinetic mechanism and employs a single active site cysteine residue that forms a stable mixed disulfide with CoA during catalysis. These data suggest that *S. aureus* employs a thiol/disulfide redox system based on CoA-CoA-disulfide and CoADR, an unorthodox new member of the pyridine nucleotide-disulfide reductase superfamily.

*Staphylococcus aureus* is a Gram-positive opportunistic pathogen that is a major cause of nosocomial infections. *S. aureus* was the first organism identified as having resistance to penicillin and has since acquired resistance to most clinically approved antibiotics (1). Methicillin-resistant strains of *S. aureus* is a Gram-positive opportunistic pathogen that is a major cause of nosocomial infections. *S. aureus* was the first organism identified as having resistance to penicillin and has since acquired resistance to most clinically approved antibiotics (1). Methicillin-resistant strains of *S. aureus* (MRSA)\(^1\) are multiply drug-resistant and pose serious challenges to modern medicine and surgery. Current therapy for MRSA infections relies upon intravenous treatment with vancomycin. There is concern that resistance to vancomycin, which has emerged in *Enterococcus faecalis*, may transfer to MRSA, resulting in infections for which there may be no effective therapy (2). Efforts are now being focused to identify new cellular targets and to develop new chemotherapeutics effective against MRSA. We are investigating the unique thiol metabolism of *S. aureus* and other Gram-positive bacteria in hopes of facilitating this process.

Aerobic organisms maintain high levels of low molecular weight thiols that in combination with specific disulfide reductases support the reducing intracellular environment and the thiol/disulfide distribution of other thiol-containing molecules. Glutathione (GSH, γ-glutamyl-cysteinyl-glycine) is perhaps the best studied low molecular weight thiol. It protects these organisms from oxygen toxicity by functioning as a slowly autoxidizing reserve of cysteine and as a cofactor in the detoxification of peroxides, epoxides, and other products resulting from reaction with oxygen. It is also a cofactor in the reduction of disulfides and ribonucleotides and in the isomerization of protein disulfides (3–6). Glutathione reductase (GR, EC 1.6.4.2) catalyzes the NADPH-dependent reduction of intracellular oxidized glutathione (GSSG) and thereby maintains the high intracellular GSH/GSSG ratio (GSH/GSSG > 100). Together, GSH, GSGG, and GSR make up the primary thiol/disulfide redox system of aerobic eukaryotes and Gram-negative bacteria (4, 5, 7, 8).

Many organisms do not produce GSH but instead employ alternative low molecular weight thiols that participate in unique thiol/disulfide redox systems (5, 7–12). It was recently shown that *S. aureus*, like many of the Gram-positive bacteria, produces no GSH but rather produces millimolar levels of reduced CoA as its predominant thiol (Fig. 1) (13). CoA, which is required as a cofactor in many acyl transfer reactions, likely has additional functions in *S. aureus* analogous to GSH. As a first step toward understanding this novel thiol metabolism, we set out to isolate and characterize the activity responsible for maintaining CoA in its reduced form. Other organisms that utilize alternative thiols produce an enzyme analogous to GSR whose preferred substrate is the disulfide of the predominant thiol in the cell (14–16). All such enzymes investigated belong to a widespread family of pyridine nucleotide-disulfide oxidoreductases that includes GSR, lipooamide dehydrogenase, and mercuric reductase. Most of these enzymes are homodimeric flavoproteins with M_r of ~100,000 that utilize a conserved active site disulfide bond to effect catalysis. We describe here the identification, purification, and characterization of a CoA disulfide reductase (CoADR) from *S. aureus* that...
catalyzes the NADPH-dependent reduction of CoA disulfide (Equation 1).

\[
\text{CoASSCoa + NADPH + H}^+ = 2\text{CoASH} + \text{NADP}^+ \quad \text{(Eq. 1)}
\]

In this and the following paper we highlight the similarities and the striking differences between CoADR and other members of the disulfide reductase superfamily.

**EXPERIMENTAL PROCEDURES**

**Materials—**Reduced NADPH, riboflavin, flavin adenine mononucleotide, flavin adenine dinucleotide (FAD), coenzyme A disulfide, glutathionyl-coenzyme A mixed disulfide, 3'-dephospho-coenzyme A, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), lysostaphin, bichinchoninic acid solution, 4% copper sulfate pentahydrate solution, 2,5'-ADP-Sepha-
rose, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (Missis-
sauga, ON, Canada). 3'-dephospho-CoA was oxidized to the disulfide by incubation at room temperature overnight in Tris-HCl buffer (20 mM), pH 9.0, containing copper (5 mM). EDTA was added to the solution (to 10 mM) to chelate the copper. 4-Phosphopantetheine was formed by incubation of CoA with nucleotide pyrophosphatase. The thiol was oxidized as described above, and the disulfide was purified by HPLC. The purity of the disulfide was confirmed by HPLC and by thiold analysis upon reaction with monobromobimane (mBBr) (described below). All other chemi-
cals were of reagent grade or better and were used without further purification.

All protein chromatography was performed on a fast phase liquid chromatography system equipped with UV and conductivity flow cells, a MonoQ 5/5 (anion exchange) column, a C 10/10 column containing 2,5'-ADP-Sepharose (5 ml), and a Superose 6HR 10/30 (gel filtration) column (Pharmacia Biotech Inc.). SDS-polyacrylamide gel electrophore-
sis (SDS-PAGE) was performed on a Mini Protean (Bio-Rad, Burling-
one, ON, Canada) using a Tris-glycine buffer as described (17).

**Purification of CoADR from S. aureus—**An overnight culture of 3,5,000 ml of S. aureus was centrifuged (5,000 × g for 10 min), resuspended in 3 ml of Tris-HCl buffer (20 mM), pH 8.0, containing lysostaphin (5 μg/ml), and incubated at 37 °C for 30 min until the suspension became viscous. Glass beads (1 g; 0.2 mm) and PMSF (to 1 mM) were added, and the mixture was vortexed for 2 min and then centrifuged (15,000 × g for 10 min) to remove the insoluble cellular debris. The resulting viscous lysate was dialyzed exhaustively (3,600 M cutoff) against Tris-HCl buffer (20 mM), pH 8.0, containing EDTA (1 mM). The dialysate (40 ml) was then assayed for the pyridine nucleotide (1 mM) and disulfide (1 mM)-dependent reduction of DTNB (1 mM). A typical assay was 800 μl.

**Identification of a Coenzyme A Disulfide Reductase from S. au-
reus—**An overnight culture of S. aureus was grown in tryptophic soy broth (10 ml) at 37 °C and was used as an inoculum (0.4 ml) for each of ten 2-liter flasks containing trypti-

case soy broth (1 liter). These cells were shaken (180 rpm) for 12 h at 37 °C before being harvested by centrifugation (7,000 × g for 15 min). All subsequent handling of the sample prior to chromatography was carried out at 4 °C. The cell pellet was resuspended in a minimum volume of Tris-HCl buffer (20 mM), pH 8.0, containing PMSF (1 mM) and lysostaphin (5.0 mg), incubated at 37 °C with agitation for 1 h (or until viscous), passed twice through a French pressure cell operating at 1,500 lbf/in², and then centrifuged (15,000 × g for 20 min) to remove insoluble cellular debris. The supernatant was brought to 50% saturation with (NH₄)₂SO₄, stirred for 15 min, and centrifuged (15,000 × g, 10 min). The resulting supernatant was brought to 80% saturation with (NH₄)₂SO₄ and centrifuged, and the pellet containing the CoADR ac-
tivity was dissolved in a minimum volume of TE buffer containing PMSF (1 mM). The resulting solution was dialyzed exhaustively (3,500 M cutoff) against TE buffer containing PMSF (1 mM).

All chromatography was carried out at room temperature on a Pharmacia fast phase liquid chromatography. The dialyzed (NH4)2SO4 fraction (10 mM/mmol) was loaded (100 ml/min) to a 2′′ 3′′ADP-Sepharose affinity column equilibrated with Tris-HCl buffer (20 mM), pH 8.0. The column was washed (25 ml) and then eluted with a linear gradient (35 ml) of NaCl (0–4 M) in the same buffer. The fractions (1 ml) exhibiting CoADR activity were pooled, concentrated and dialyzed in Tris-HCl buffer (20 mM), pH 9.0, twice, to reduce conductivity, and applied (1.0 ml/min) to a Mono Q HR 5/5 column equilibrated with the same buffer. The column was washed (5 ml) and then eluted with a linear gradient (25 ml) of NaCl (0.3–0.6 M) in Tris-HCl buffer (20 mM), pH 9.0. The purity of fractions showing CoADR activity was determined by SDS-PAGE (5% stacking gel, 12% running gel) and silver staining.

**Determination of Native Molecular Weight**—Purified CoADR (25 μg) was applied to a Superose 6 HR 10/30 gel exclusion column (0.5 ml/min) equilibrated with Tris-HCl buffer (20 mM), pH 8.0, containing NaCl (1 M) and then eluted isocratically in the same buffer. Fractions containing CoADR were identified by UV absorbance and by measuring enzyme activity. The native molecular weight of CoADR was calculated by comparison of its elution volume to that of protein standards as described (21).

**Identification of Flavin Cofactor**—The visible absorption spectrum of purified CoADR is indicative of a flavoprotein. To identify the bound flavin cofactor, CoADR (5 μg or 100 μmol in 0.1 ml) was heated to 98 °C for 10 min and then centrifuged (15,000 × g, 10 min) to remove the denatured protein. The supernatant was then separated by reverse phase HPLC as described previously (15). The elution of the released cofactor was then compared with that of riboflavin, flavin adenine dinucleotide, and FAD (100 pmol each).

**Quantitation of Active Site Thiols**—A solution of oxidized CoADR (9.5 μM) in Tris-HCl buffer (20 mM), pH 8.0, containing EDTA (1 mM) was incubated with NADPH (0.2 mM) for 10 min at ambient temperature before being diluted (1:1) with the same buffer containing urea (8 M) and DTNB (0.2 mM). The absorbance at 412 nm was then measured and compared with that of a similar reaction in which CoADR was not incubated with NADPH. The number of thiols liberated per FAD (ε280 = 11,300 M−1 cm−1) was then calculated. To determine the identity of the two thiols detected, oxidized CoADR (9.5 μM) in 100 μl of Tris-HCl buffer (20 mM), pH 8.0, containing NADPH (20 μM) and CoA disulfide (200 μM), 3′-dephospho-CoA disulfide (200 μM), or CoASSG (200 μM) was incubated (incubation 1) at 37 °C for 10 min, and then the reaction was stopped through a Centricon 30 (30,000 M cutoff) to separate CoADR from the low molecular weight reaction products. The extensively washed CoADR fraction was resuspended in 100 μl of Tris-HCl buffer (20 mM), pH 8.0, containing EDTA (1 mM) and NADPH (0.2 mM) and incubated for 10 min at 37 °C (incubation 2). The reaction product was either 1) filtered as above with the thiol content of the high and low molecular weight fractions determined by DTNB assay or 2) reacted directly with NEM and/or mBBr and then separated on HPLC as described above to identify any low molecular weight thiols present. The results were then compared with similar reactions in which oxidized CoADR was incubated alone or in the presence of either CoA disulfide or NADPH but not both.

**Kinetic Characterization of the Substrate Specificity and Kinetic Mechanism of CoADR**—Kinetic measurements were performed in a 1-cm pathlength quartz cuvette maintained at 37 °C. Each assay (0.3 ml) was carried out in Tris-HCl buffer (50 mM), pH 7.8, containing NaCl (50 mM), CoA (2–10 mM; subunit concentration determined using the calculated extinction coefficient for CoA Ε260 = 12,800 M−1 cm−1), NADPH (2–200 μM), and CoA disulfide (2–200 μM), 3′-dephospho-CoA disulfide (10–500 μM), 4,4′-diphosphopantetheine (2 μM to 2 mM), pantetheine (10 μM to 100 μM), glutathione disulfide (10 μM to 100 μM), cystine (10 μM to 100 μM), CoA-glutathione mixed disulfide (10 μM to 1 M), or H2O2 (10 μM to 10 mM) (EDTA 10 μM) was present in the stock solution of 3′dephospho-CoA disulfide and was thus present in the enzyme assays for this substrate up to 0.5 μM. We have found that EDTA up to 10 mM has no effect on the observed kinetics of the reaction catalyzed by CoADR). Enzyme and NADPH were combined in buffer anaerobically (4 °C), and the reaction was initiated by the addition of the disulfide substrate or H2O2 (reduction of each substrate was monitored at ≥7 different substrate concentrations). The activity of CoADR was monitored at 340 nm as the decrease in absorbance resulting from the oxidation of NADPH. To study the kinetic mechanism of CoADR a two-substrate kinetic analysis was performed under identical conditions except for the concentrations of enzyme and substrate used.
A comparison of enzyme activity from an *S. aureus* extract incubated with various disulfide substrates and pyridine nucleotide cofactors was performed to determine whether any specific disulfide reductases were present. The extract catalyzed the reduction of DTNB slowly in the presence of either NADH or NADPH. However, in the presence of both NADPH and CoA disulfide this reduction was 3-fold greater than that observed for NADPH alone and 8-fold greater than that observed for NADH alone. No disulfide enhanced the rate of NADH-dependent reduction of DTNB, nor did GSSG, cysteine, or pantethine enhance the NADPH-dependent reduction of DTNB. These results suggested that *S. aureus* produces a CoADR that catalyzes specifically the reduction of CoA disulfide by NADPH and does not produce detectable glutathione reductase.

The purification of CoADR from *S. aureus* is described in Table I. The crude extract was first subjected to a 50–80% ammonium sulfate precipitation. This fraction was dialyzed and applied to a 2′,5′-ADP-Sepharose affinity column, which provided a ~250-fold purification. CoADR from the ADP column was contaminated by three other proteins that were efficiently removed by MonoQ anion exchange chromatography. CoADR eluted as two separate peaks of activity. The second peak (shown by the arrow in Fig. 4B) had a higher specific activity than the first and was the only fraction retained for further study. The difference between the first and second peak is unknown but could be due to modification(s) (such as loss of an FAD or deamidation) incurred during the purification process. However, no efforts have been made to investigate these speculations.

**Physical Characterization**—The known pyridine nucleotide-disulfide oxidoreductases are multimeric, most being homomers, and have a FAD noncovalently bound to each subunit (14, 16, 24, 25). Purified CoADR migrates as a single polypeptide of ~50,000 apparent molecular weight according to SDS-PAGE (Fig. 4C). The native molecular weight of CoADR was estimated by its relative elution through a Superose 6 HR 10/30 gel filtration column. A plot of the parameter, *K*, versus the log of the molecular weight of various protein standards is shown in Fig. 4D. The parameter, *K*, is defined as (*Vp − Vc*)/Vp, where *Vp* is the elution volume, *Vc* is the column void volume, and *Vc* is the volume of the stationary phase (21). Native CoADR elutes between chicken ovalbumin (44 kDa) and bovine gamma globulin (158 kDa) from the Superose gel filtration column, and the K value calculated for CoADR (0.49) can be extrapolated from the standard plot to a *M* of approximately 90,000. This suggests that native CoADR is a homodimer.

The visible absorbance spectrum of purified CoADR is typical of that of a flavoenzyme (Fig. 5A) and shows a λmax at 375 and 452 nm, having a shoulder at 478 nm. The flavin bound to CoADR could be released from the enzyme by thermal denaturation and was identified as FAD by reverse phase HPLC chromatography (Fig. 5B). In addition to the peak corresponding to FAD, an additional peak running at 24 min was observed. This peak is likely a degradation product of FAD because it was observed for FAD run alone (data not shown) and is present in the control chromatogram (Fig. 5A). Quantitation of the flavin released from CoADR revealed that 90 ± 10 pmol or 0.9 ± 0.1 FAD molecules/subunit CoADR were released. An extinction coefficient for a native CoADR subunit in Tris-HCl buffer (20 mM), pH 9.0, at 452 nm was estimated as 12,800 m−1 cm−1 from the extinction coefficient for FAD (ε450 = 11,300 m−1 cm−1) using the following equation.

\[
e(\text{CoADR})_{450} = [A(\text{native CoADR})_{450}(11,300 \text{ m}^{-1} \text{ cm}^{-1})]/[A(\text{liberated FAD})_{450} \text{ (Eq. 2)}]
\]

**Thiol Titration of Active Site**—The deduced amino acid sequence of CoADR contains two cysteine residues, and only one of these (Cys63) lies in the putative active site region (36). To determine whether CoADR utilizes a thiol-disulfide exchange mechanism, a thiol titration of the active site of the enzyme was performed. CoADR in its oxidized state and reduced with NADPH was denatured in the presence of DTNB. The denature oxidized enzyme had 0.9 ± 0.1 reactive thiol/subunit, whereas denatured CoADR that had been reduced with NADPH had 3.2 ± 0.2 reactive thiols/subunit. This suggests that CoADR in its oxidized state has one reduced cysteine and that upon reduction with NADPH a disulfide bond was reduced. However, the 3.2 thiols/subunit measured also suggested that one of the thiols produced upon reduction with NADPH was not an enzymic cysteine (because CoADR only has two cysteines/subunit) (36).

CoADR and NADH peroxidase from *E. faecalis* both have a single cysteine in their active site, thus CoADR might form a mixed disulfide intermediate with the substrate thiol similar to the sulfenic acid formed during reduction of hydroquinone peroxide.

![FIG. 3. Cu⁺⁺ catalyzed autooxidation of cysteine, glutathione, and coenzyme A. Each thiol (1 mM) was incubated with CuCl₂ (1 μM). At various times samples were removed, and total free thiol was measured by reaction with DTNB.](http://www.jbc.org/content/mm/by/2017/09/14/)

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total units</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble extract</td>
<td>3870</td>
<td>2560</td>
<td>1.51</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>50–80% ammonium sulfate</td>
<td>2200</td>
<td>246</td>
<td>8.7</td>
<td>5.8</td>
<td>57</td>
</tr>
<tr>
<td>Dialysate 2′–5′ ADP-Sepharose</td>
<td>1800</td>
<td>0.84</td>
<td>2143</td>
<td>1420</td>
<td>47</td>
</tr>
<tr>
<td>MonoQ</td>
<td>960</td>
<td>0.21</td>
<td>4570</td>
<td>3030</td>
<td>25</td>
</tr>
</tbody>
</table>
by NADH peroxidase. To address this question, CoADR was incubated in the presence of limiting NADPH and an excess of disulfide (incubation 1) and then separated from the low molecular weight reaction products by filtration. The resulting oxidized enzyme was then reduced with an excess of NADPH (incubation 2), and the reaction products were separated into low and high molecular weight fractions and characterized for their thiol content. Both the low and high molecular weight fractions contained thiol, 0.8 ± 0.1 mol of thiol/subunit of enzyme in the low molecular weight fraction (Table II) and 2.2 ± 0.2 mol thiol/subunit enzyme in the high molecular weight fraction. This suggested that the disulfide bond reduced by NADPH had both a high and a low molecular weight component. Enzyme treated only with NADPH during incubation 1, washed extensively, treated again with NADPH during incubation 2, and then size fractionated produced only one thiol/subunit in the high molecular weight fraction and no thiols in the low molecular weight fraction, providing further evidence for a low molecular weight component to the reduced disulfide. A thiol analysis of the low molecular weight thiol carried out by mBBr modification and separation on HPLC (as described earlier) identified the low molecular weight thiol as reduced disulfide substrate (CoA, 3'-dephospho-CoA, and CoA in the reduction of CoA disulfide, 3'-dephospho-CoA disulfide, and CoASSG). No substrate-derived thiols were detected in the high molecular weight fraction. Enzyme alone or treated with CoA disulfide during incubation 1 had results similar to that found for the enzyme treated with NADPH and CoA disulfide, suggesting that the enzyme is isolated from cells as the CoA mixed disulfide. Together, these data suggest that CoADR in the oxidized state forms a stable mixed disulfide with half of the disulfide substrate and that this mixed disulfide is reduced upon treatment with NADPH. In addition, these data suggest the mixed disulfide is preferentially formed with that half of the substrate resembling CoA because no GSH was detected in the CoASSG sample.

**Kinetic Characterization of CoADR—**CoADR is highly specific for its physiological substrates, CoA disulfide and NADPH, and at the optimum pH 7.8 is saturated by micromolar concentrations of each. The $K_m$ for NADPH, at a saturating CoA disulfide concentration, was 2 $\mu$M, and the $K_m$ for CoA disulfide, at a saturating NADPH concentration, was 11 $\mu$M. The results of the kinetic analysis of the CoADR catalyzed reduction of various disulfide substrates by NADPH are shown in Table III. Although CoADR reduces CoA disulfide preferentially, it also reduces 3'-dephospho-CoA disulfide ($K_m$ = 40 $\mu$M), 4,4'-diphosphopantethine ($K_m$ = 80 $\mu$M), and CoASSG ($K_m$ = 1100 $\mu$M). Activity of CoADR upon pantethine, cystine, GSSG, or peroxide was below the detectability of the assay ($D_A 340 \# 0.0001 \text{min}^{-1}$) and thus has an upper limit of 0.1%, the activity observed for CoA disulfide. A two-substrate kinetic analysis of the NADPH-dependent reduction of CoA disulfide and 3'-dephospho-CoA disulfide by CoADR suggests that the reaction proceeds by a sequential rather than a simple ping-pong kinetic mechanism (Fig. 6).

**DISCUSSION**

Although cysteine is ubiquitous, it rarely is the predominant cellular thiol of aerobic organisms. High intracellular levels of cysteine apparently are toxic because rapid metal catalyzed

---

**FIG. 4.** Purification of CoADR from *S. aureus*. A, chromatogram for the elution of CoADR from a 2',5'-ADP-Sepharose column. B, chromatogram for the elution of CoADR from the MonoQ column. The arrows mark where the CoADR activity eluted. C, silver-stained SDS-PAGE analysis of different fractions containing CoADR activity: I, 50–80% (NH$_4$)$_2$SO$_4$ fraction; II, 2',5'-ADP active eluate; and III, MonoQ active eluate. D, the native molecular weight of CoADR was determined by gel filtration chromatography and a plot of the parameter K versus the log of the molecular weight of various protein standards. The parameter, K, is defined as $(V_e - V_0)/V_s$, where $V_e$ is the elution volume, $V_0$ is the column void volume, and $V_s$ is the volume of the stationary phase (21).
and a stable intracellular redox environment. It was shown that *S. aureus* does not produce GSH but instead produces CoA as its predominant cellular thiol (Fig. 2) (13). Like GSH, CoA is stable to metal catalyzed oxidation (Fig. 3), is found at millimolar levels in the cell and is maintained in its reduced form (CoASH/CoASSCoA ≥ 450). This thiol/disulfide ratio is likely an underestimate because the calculated disulfide content would be contaminated by cellular thioesters, which liberate thiol upon treatment with dithiothreitol (28). CoA thus represents a stable intracellular thiol/disulfide redox buffer in *S. aureus*. The current understanding of cysteine biosynthesis, however, does not provide an efficient route for conversion of CoA to cysteine. If indeed aerobes store cysteine in a form that is resistant to oxidation, the manner by which *S. aureus* stores cysteine is not obvious.

The thiol/disulfide ratio of thiols in equilibrium with air is \(-10^{-16}\), yet aerobes maintain a cytoplasmic thiol/disulfide ratio of \(10^2-10^3\) (29). *S. aureus* maintains this ratio, at least in part, using the enzyme CoADR, which catalyzes specifically the NADPH-dependent reduction of CoA disulfide. CoADR is purified over 3000-fold from *S. aureus* by 2',5'-ADP affinity and anion exchange chromatography, resulting in a 25% recovery of the active enzyme (Table I). Chromatography with 2',5'-ADP-Sepharose, which mimics the adenyldiphosphate moiety of NADPH, is the most effective step in the purification of CoADR, and the concentration of NaCl (\(-2\) M) necessary to elute CoADR from this column reflects the high affinity CoADR has for this cofactor (\(K_a\) (NADPH) = 2 \(\mu\)M). Native CoADR has an apparent \(M_r\) of 90,000 ± 10,000 according to gel filtration chromatography (Fig. 4D) and runs as a single 49-kDa band on an SDS-PAGE gel (Fig. 4C). These data are consistent with a subunit \(M_r\) of 49,200 calculated from the deduced primary structure of CoADR (36) and suggest that CoADR is a homodimer. The absorption spectrum (Fig. 5A) and HPLC analysis (Fig. 5B) show CoADR to be a flavoprotein that noncovalently binds a single FAD to each 49-kDa subunit.

The known pyridine nucleotide-disulfide oxidoreductases utilize a pair of active site cysteine residues to catalyze a thiol/disulfide exchange reaction with the disulfide substrate (23, 24). In human erythrocyte GSR, Cys58, the interchange cysteine, reduces the substrate disulfide resulting in the formation of a transient mixed disulfide intermediate. Cys58', the charge transfer cysteine, is adjacent to the enzyme bound FAD and 

---

**Fig. 5.** A, the absorption spectrum of purified CoADR. CoADR shows absorbance peaks typical of flavoproteins (\(\lambda_{\text{max}}\) at 276, 375, and 452 nm). **B**, identification of the flavin cofactor of CoADR by reverse phase HPLC. a, flavin standards: riboflavin, flavin adenine mononucleotide (FMN), and FAD. b, the flavin released from thermally denatured CoADR.

---

**Table II**

*Analysis of active site thiols of CoADR*

CoADR was oxidized by pretreatment (incubation 1) with a 10:1 molar ratio of disulfide:NADPH at 37 °C for 10 min. Oxidized CoADR was then filtered from the low molecular weight reaction products, washed extensively, and then reduced with NADPH (200 \(\mu\)M) (incubation 2). The reaction products were either 1) filtered as above with the thiol content (SH/subunit) of the high and low molecular weight fraction determined by DTNB assay or 2) reacted directly with NEM and/or mBBr and then separated on HPLC as described under “Experimental Procedures” to identify any low molecular weight thiols (RSH) present.

<table>
<thead>
<tr>
<th>Pretreatment (incubation 1)</th>
<th>Reduction with NADPH (200 (\mu)M) (incubation 2)</th>
<th>High molecular weight fraction(^a)</th>
<th>Low molecular weight fraction(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SH/subunit</td>
<td>RSH</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>–</td>
<td>0.9 ± 0.1</td>
<td>(≤0.1 ± 0.1)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.2 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>CoA disulfide</td>
<td>–</td>
<td>0.8 ± 0.1</td>
<td>(≤0.1 ± 0.1)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.9 ± 0.2</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>CoA disulfide:NADPH (10:1)</td>
<td>–</td>
<td>2.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.8 ± 0.2</td>
<td>(≤0.1 ± 0.1)</td>
</tr>
<tr>
<td>3'-dephospho-CoA disulfide:NADPH (10:1)</td>
<td>–</td>
<td>2.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.8 ± 0.2</td>
<td>(≤0.1 ± 0.1)</td>
</tr>
<tr>
<td>CoASSG:NADPH (10:1)</td>
<td>–</td>
<td>2.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.8 ± 0.2</td>
<td>(≤0.1 ± 0.1)</td>
</tr>
</tbody>
</table>

\(^a\) A – in the column indicates the enzyme was not treated during the incubation.

\(^b\) A – in the column indicates no low molecular weight thiols were detected.
oxidase and NADH peroxidase from *E. faecalis* (30). These enzymes, also members of the pyridine nucleotide-disulfide oxidoreductase superfamily, utilize this single catalytic cysteine to form a stable sulfinic acid (Cys-SOH) during the reduction of O₂ and H₂O₂. We have shown that the NADPH reduction of oxidized CoADR results in the production of two thioles/enzyme subunit, of which half originates from an enzymic cysteine residue (presumably Cys⁴⁴) and half originate from the disulfide substrate (Table II). These results suggest that reduction of disulfides by CoADR occurs by a thiol-disulfide exchange reaction but involves only a single cysteine residue that forms a stable mixed disulfide (Enz-SSR) analogous to the sulfinic acid formed by NADH peroxidase. A proposed catalytic mechanism for CoADR is shown in Equation 2.

\[
\text{E-SH} + (\text{CoAS})_2 \rightarrow \text{E-S-S-CoA} + \text{CoASH} \quad (\text{Eq. 3})
\]

\[
\text{E-S-S-CoA} + \text{NADPH} + \text{H}^+ \rightarrow \text{E-SH} + \text{NADP}^+ + \text{CoASH} \quad (\text{Eq. 4})
\]

In the reduced enzyme Cys⁴⁴ is a free thiol. Upon encounter with CoA disulfide the Cys⁴⁴ thiolate anion attacks one sulfur atom of the substrate, displacing a molecule of CoA and forming a Cys⁴⁴-CoA mixed disulfide. This oxidized state is stable and is the enzyme form isolated from cells (reduction of this sample with NADPH releases CoA; Table II). NADPH binds the oxidized enzyme and reduces the mixed disulfide via the FAD cofactor, regenerating the reduced enzyme and another molecule of CoA. However, the sequential kinetics observed from a two-substrate kinetic analysis (Fig. 6) suggests that CoADR does not utilize a simple two-site ping-pong mechanism. The specific sequence of binding events and the flow of electrons cannot be addressed from the present data but will have to await future stopped flow kinetic studies.

CoADR is a very specific for NADPH (K₅ = 2 μM) and CoA disulfide (K₅ = 11 μM), suggesting that these compounds are natural substrates for this enzyme. A kinetic survey of disulfide substrates (Table III) demonstrates the specificity of CoADR and the contribution of the 3'-phosphoryl, the adenyl, and the 4-phosphoryl groups to the binding and turnover of CoA disulfide. This analysis shows that CoADR recognizes only substrates containing the 4-phosphopantetheine moiety and is most proficient in the reduction of CoA disulfide. A comparison of the kinetic constants for 3'-dephospho-CoA disulfide reveals that loss of the 3' phosphate moieties from CoA disulfide results in a 10-fold increase in K₅ with no detectable change in k₅. A similar comparison for 4,4'-diphosphopantethine and 3'-dephospho-CoA disulfide demonstrates that loss of the adenyl moiety results in a 2−3-fold decrease in k₅ and little change in K₅. The 4 and/or 4' phosphate moieties are essential for substrate binding and turnover because reduction of pantethine by CoADR is not detectable. CoADR does reduce CoASSG, although poorly (K₅ = 1.4 mM), but does not reduce GSSG, cystine, O₂, or H₂O₂ (despite the homology to *E. faecalis* NAD oxidase and peroxidase; Ref. 36). It should be noted that upon reduction of CoASSG CoADR selectively forms a mixed disulfide with CoA (Table II). Thus, the active site is designed to form a mixed disulfide with the more tightly bound portion of the substrate.

Although CoADR apparently belongs to a new separate subfamily of disulfide reductases (36), CoADR is physically and functionally similar to the pyridine nucleotide-disulfide oxidoreductase superfamily. Indeed, all of the low molecular weight disulfide reductases appear to belong to this family. Enzymes studied include trypanothione reductase (14, 21), bis-gulutamyl-cysteine reductase (15), and 4,4'-diphosphopantetheine reductase (16). Although each appears to catalyze the same chemical reaction, the NAD(P)H reduction of a disulfide bond, each enzyme is specific for the disulfide of the predominant thiol in the cell. CoADR is related functionally to the *Bacillus megaterium* 4,4'-diphosphopantetheine reductase (4PPR). Although both CoADR and 4PPR each prefer substrates having the 4'-phosphopantethenyl moiety, these enzymes are quite different. 4PPR has a K₅ approximately 10-fold higher for CoA disulfide (7.6 mM) than for its preferred substrate 4,4'-diphosphopantetheine (0.67 mM) (16), which itself is 2 orders of magnitude higher than the K₅ for turnover of CoA disulfide by CoADR. 4PPR is also 20% larger. It is interesting that *B. megaterium* produces an enzyme of such specificity, because *B. megaterium* likely has a thiol content similar to that of *B. subtilis*, which like *S. aureus* produces CoA as its predominant thiol. The *B. megaterium* enzyme was identified and isolated from spores and may have evolved to function in this dormant state. If so, there may be an additional enzyme such as CoADR that is expressed in vegetative cells.

GSH plays many different roles in cellular metabolism. How these functions occur in organisms lacking GSH represents an enigma in the field of thiol biology. In organisms like *S. aureus*, which produce CoA as a predominant thiol, many GSH-medi-
targets. Indeed, successes in this area have been reported in the design of inhibitors to trypanothione reductase (32–35). The disparate disulfide specificities, structures (36), and catalytic mechanisms of CoADR and GSR suggest that CoADR could be a useful target for developing new drugs. The study of bacterial mechanisms of CoADR and GSR suggest that CoADR could be a useful target for developing new drugs. The study of bacterial thiol metabolism will likely unveil many such promising targets.

Acknowledgments—S. B. delCardayre thanks the members of the Davies lab for useful discussions and insight regarding this work.

REFERENCES

26. Deleted in proof
Coenzyme A Disulfide Reductase, the Primary Low Molecular Weight Disulfide Reductase from Staphylococcus aureus: PURIFICATION AND CHARACTERIZATION OF THE NATIVE ENZYME

Stephen B. delCardayré, Kevin P. Stock, Gerald L. Newton, Robert C. Fahey and Julian E. Davies

doi: 10.1074/jbc.273.10.5744

Access the most updated version of this article at http://www.jbc.org/content/273/10/5744

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 28 references, 13 of which can be accessed free at http://www.jbc.org/content/273/10/5744.full.html#ref-list-1