JOURNAL OF BACTERIOLOGY, Feb. 1993, p. 623–629
0021-9193/93/030623-07$02.00/0

Vol. 175, No. 3

Characterization of a Broad-Range Disulfide Reductase from *Streptomyces clavuligerus* and Its Possible Role in β-Lactam Antibiotic Biosynthesis

YAIR AHARONOWITZ,* YOSSEF AV-GAY, RACHEL SCHREIBER, AND GERALD COHEN

Department of Molecular Microbiology and Biotechnology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, 69978, Israel

Received 23 July 1992/Accepted 10 November 1992

Streptomyces clavuligerus **is a potent producer of penicillin and cephalosporin antibiotics. A key step in the biosynthesis of these β-lactam compounds is the cyclization of the thiol tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N by the enzyme isopenicillin N synthase (IPNS). However, bis-ACV, the oxidized disulfide form of the tripeptide, is not a substrate for IPNS. We show here that *S. clavuligerus* possesses an NADPH-dependent disulfide reductase of broad substrate specificity that efficiently catalyzes the reduction of disulfide bonds in bis-ACV and in other low-molecular-weight disulfide containing compounds and proteins. The disulfide reductase comprises two protein components, a 70-kDa reductase consisting of two identical subunits, and a 12-kDa heat-stable protein reductant. The structural and functional properties of the disulfide reductase resemble those of the thioredoxin class of oxidoreductases. When the disulfide reductase system is coupled with IPNS, it quantitatively converts bis-ACV to isopenicillin N. These findings suggest that the disulfide reductase may play a role in the biosynthesis of penicillins and cephalosporins in streptomycetes. We also show here that *S. clavuligerus* lacks glutathione reductase and have previously reported that *Streptomyces* species do not contain glutathione. This disulfide reductase may therefore be important in determining the thiol-disulfide redox balance in streptomycetes.**

Penicillin and cephalosporin antibiotics are produced by a wide variety of microorganisms including some filamentous fungi, numerous gram-positive *Streptomyces* species, and a few unicellular gram-negative bacteria (4). The initial steps in the biosynthesis of these β-lactam antibiotics involve the formation of a linear tripeptide, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), and its subsequent conversion to isopenicillin N by the enzyme isopenicillin N synthase (IPNS) (1). The latter reaction has been extensively studied and exhibits an absolute requirement for ferrous ions, an electron donor, usually provided as ascorbate, and molecular oxygen (16). ACV can exist in two states, either as the reduced monomeric form which possesses a free cysteine thiol or as the oxidized disulfide (bis-ACV) dimer (19). Significantly, only the reduced form of ACV is cyclized to isopenicillin N by IPNS. Therefore, in vitro IPNS reactions are carried out in the presence of an excess of a synthetic reducing agent such as dithiothreitol (DTT). In view of these findings, we have asked whether microorganisms that produce penicillin and cephalosporin antibiotics possess an enzymatic system that reduces the disulfide form of ACV (bis-ACV) to its thiol form.

Several NADPH-dependent flavoprotein oxidoreductases are known to carry out reversible thiol-disulfide reactions (21). Within the cell they function to poise the thiol-disulfide redox balance toward the reduced state. One possible bis-ACV disulfide-reducing system is glutathione reductase, a widely distributed enzyme consisting of two identical 52-kDa subunits that contain a pair of redox active cysteines (18). The major role of glutathione reductase is to maintain glutathione, γ-glutamyl-L-cysteinyl-glycine, in its reduced thiol state. Although glutathione and ACV are both cysteine-containing tripeptides and structurally resemble one another, it is unlikely that glutathione reductase is able to effectively reduce bis-ACV. This is because glutathione reductase exhibits narrow substrate specificity (18). Another possible bis-ACV disulfide-reducing system is thioredoxin-thioredoxin reductase. Thioredoxin is a ubiquitous heat-stable 12-kDa protein that has the properties of a general disulfide reductant (10). It reduces disulfides in low-molecular-weight substrates and in proteins through reversible oxidation of two vicinal cysteine residues. Thioredoxin is specifically reduced by thioredoxin reductase, an enzyme that shares similar catalytic and structural properties with glutathione reductase (17). The microbial thioredoxin reductases contain two identical 35-kDa subunits. Thus, whereas glutathione reductase and thioredoxin-thioredoxin reductase are functionally related enzymatic systems, they may readily be distinguished from one another by physical and biochemical means. Recently we showed that β-lactam-producing (and nonproducing) *Streptomyces* species do not contain glutathione and presumably therefore lack glutathione reductase (3). The purpose of the present paper is to describe the characterization of a disulfide reductase from a β-lactam-producing *Streptomyces* species that accepts bis-ACV as a substrate and that may play a role in the biosynthesis of penicillin.

MATERIALS AND METHODS

Materials. Chromatography resins, DEAE-Sephacel, and 2′5′-ADP–Sepharose were supplied by Pharmacia. Ultragel AcA54 was from LKB. Dithiothreitol (DTT), NADPH, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and bovine insulin were purchased from Sigma. ACV was a gift of Gist-brocades, Delft, Holland.

Enzymes. Thioredoxin from *Spirulina platensis* was ob-

* Corresponding author. Electronic mail address: f59yair@ccsg.tau.ac.il.

tained from Sigma. *Escherichia coli* thioredoxin was obtained from Calbiochem, and the thioredoxin reductase was partially purified as described in this work for *Streptomyces clavuligerus*. Glutathione reductases used in this work were all purchased from Sigma.

Bacteria and growth conditions. *S. clavuligerus* DSM 738 (ATCC 27064; NRRL 3585) was cultivated at 30°C on tryptic soy broth (Biolife) containing 10 g of soluble starch per liter (TSBS). TSBS was added in 50-ml volumes in 250-ml flasks, inoculated with spores, and incubated for 48 h at 250 rpm. This seed culture was transferred at 2% (vol/vol) into fresh TSBS (1-liter amounts in 4-liter flasks) and incubated for a further 36 to 48 h at 150 rpm. Cultures were harvested by centrifugation and washed with 50 mM Tris · HCl buffer (pH 8.0) containing 0.1 mM EDTA. Packed cells were stored at −20°C until used. *Micrococcus luteus* ATCC 9341 (12) was used as the indicator organism for the detection of isopenicillin N in bioassays (see below).

Enzyme assays. (i) Disulfide reductase activity. The *S. clavuligerus* disulfide reductase contains two protein components denoted LMW and HMW for low and high molecular weight, respectively. The activity of the system was measured by three different procedures. Method 1 is based on the DTNB assay described by Holmgren (6). Activity was determined by measuring the increase in A_{412} at 25°C during the initial 5 min of the reaction. The reaction mixture contained, in a final volume of 1 ml, 50 mM Tris · HCl (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, and 0.02 mM DTNB (dissolved in 95% ethanol). Depending on which component was to be assayed, various amounts of the LMW or HMW protein were added to the mixture, and the reaction was started by addition of an excess of the complementary component. Control reactions in which one of the two protein components was omitted, or NADPH was omitted, showed no significant increase in absorbance. Reductase activity was calculated as micromoles of NADPH oxidized per minute according to the relation $\Delta A_{412}/13.6 \times 2$ (15). By reducing the reaction volume to 0.2 ml, the assay could be adapted for use with microtiter plates, and the increase in absorbance was monitored by using an automatic microtiter plate reader. Method 2 is similar to method 1, except that DTNB was replaced with different disulfide compounds at a final concentration of 1.0 mM, and the reaction was followed by the decrease in the A_{340}. Reductase activity was calculated as micromoles of NADPH oxidized per minute. Method 3 was used to assay the reduction of protein disulfides with insulin as the substrate (9). Enzymatic reduction of insulin disulfides by the LMW protein, with DTT as the reductant, was monitored for 1 h by measuring the increase in turbidity of the reaction mixture at 650 nm. The mixture contained, in a final volume of 1.0 ml, 0.5 mM bovine insulin, 100 mM potassium phosphate buffer (pH 7.5), 0.1 mM EDTA, 1 mM DTT, and the LMW protein. Control experiments in which DTT or the LMW protein was omitted were carried out in parallel. In other experiments, DTT was substituted by the HMW protein plus NADPH and the reaction was monitored by the change in A_{340} or by titrating the appearance of free thiols with DTNB.

(ii) IPNS activity. The IPNS assay (11) was carried out as follows. Conversion of ACV to isopenicillin N was carried out in reaction mixtures containing, in a final volume of 0.08 ml, 0.345 mM oxidized form of ACV (bis-ACV), 2.8 mM sodium ascorbate, 45 μM $FeSO_4$, 50 mM Tris · HCl (pH 7.2), 1 mM NADPH, 0.1 mM EDTA, purified IPNS, and the LMW and HMW components of the disulfide reductase. In some experiments crude extracts of *S. clavuligerus* were substituted for purified IPNS and disulfide reductase. Reaction mixtures were incubated for 30 to 60 min at room temperature, and the reaction was terminated by the addition of 0.08 ml of methanol. The amount of isopenicillin N produced was determined by an agar diffusion bioassay with *M. luteus* ATCC 9341 as the indicator strain (12). One unit of activity was defined as the amount which produces a zone of inhibition equivalent to 1 μg of cephalosporin C with the *M. luteus* indicator strain.

Purification of the *S. clavuligerus* disulfide reductase. (i) Preparation of cell extracts. Cultures of *S. clavuligerus* were grown for 48 h on TSBS, harvested by centrifugation, and washed with 50 mM Tris · HCl buffer (pH 8.0). Washed cells or frozen packed cells were suspended in 4 volumes of 50 mM Tris · HCl buffer (pH 8.0)–0.1 mM EDTA (TE buffer). Samples of cells held at 4°C were disrupted in a Branson sonifier and centrifuged for 15 min at 14,000 × *g*.

(ii) Streptomycin sulfate-ammonium sulfate precipitation. Streptomycin sulfate was added to the cell extract with gentle stirring at 4°C to a final concentration of 1% (wt/vol). After 15 min the suspension was centrifuged for 15 min at 14,000 × *g*, and the pellet was discarded. Solid ammonium sulfate was then added to the clear supernatant to 50% saturation, and the mixture was stirred for 20 min at 4°C and centrifuged at 14,000 × *g* for 20 min. The supernatant was collected and adjusted to 80% saturation with solid ammonium sulfate and again stirred for 20 min at 4°C and centrifuged. The resulting material that precipitated between 50 and 80% saturation was saved. The pellet was resuspended in a small volume of TE buffer and applied to an AcA54 column (2.6 by 35 cm) previously equilibrated with TE buffer. The column was developed with TE buffer at a flow rate of 0.5 ml/min, and the fractions were assayed for disulfide reductase activity with DTNB as the substrate. Although disulfide reductase activity was readily detected in the resuspended ammonium sulfate pellet, it was absent in the individual fractions eluted from the gel filtration column. Activity could be recovered only by combining certain fractions containing an LMW protein component (<15 kDa) with certain fractions containing an HMW component (>60 kDa). The two components were further purified as described below, and their activities were detected by assaying in the presence of the complementary component.

(iii) Purification of the HMW protein. Gel filtration fractions containing the HMW protein were combined and applied to a DEAE-Sephacel (Pharmacia) column with a bed volume of 10 ml that had been preequilibrated with TE buffer. The column was developed with a linear gradient of 0 to 0.5 M NaCl in TE buffer. Fractions were tested for DTNB reduction in the presence of the LMW protein. The fractions containing activity in the DTNB assay were pooled and subsequently applied to a 2′5′-ADP–Sepharose column (1 by 10 cm) that had been preequilibrated in TE buffer. The HMW protein was eluted with an NaCl gradient of 0.2 to 0.8 M. Active fractions were pooled, desalted, and kept frozen. The yield of activity at this stage was 20%, and a purification of about 1,100-fold was achieved.

(iv) Purification of the LMW protein. Gel filtration fractions containing the LMW component were combined and applied to a DEAE-Sephacel (Pharmacia) column with a bed volume of 10 ml that had been preequilibrated with TE buffer. The column was eluted with a linear gradient of 0 to 0.3 M NaCl in TE buffer. Fractions were tested for DTNB reduction in the presence of the HMW protein and NADPH. Active fractions were combined and dialyzed with tubing having a 3,000-molecular-weight cutoff. A further ion-ex-

TABLE 1. Disulfide reductase activity in *S. clavuligerus* crude extracts and in different glutathione reductases[a]

Source	Activity (nmol of NADPH oxidized/min) toward:		
	GSSG	DTNB	Bis-ACV
S. clavuligerus crude extract	4.81	17.00	11.30
Spinach GR[b]	5.00	0.73	0.23
Baker's yeast GR	5.00	1.14	0.46
Wheat germ GR	4.91	0.14	0.44
Bovine intestine GR	4.65	0.66	0.11

[a] Specific activities (units per milligram of protein) for the enzymes used were as follows: *S. clavuligerus*, 0.002; spinach, 43.1; baker's yeast, 100.7; wheat germ, 0.057; bovine intestine, 36.0. The amount of enzyme added to each reaction mixture was 0.005 U.

[b] GR, glutathione reductase.

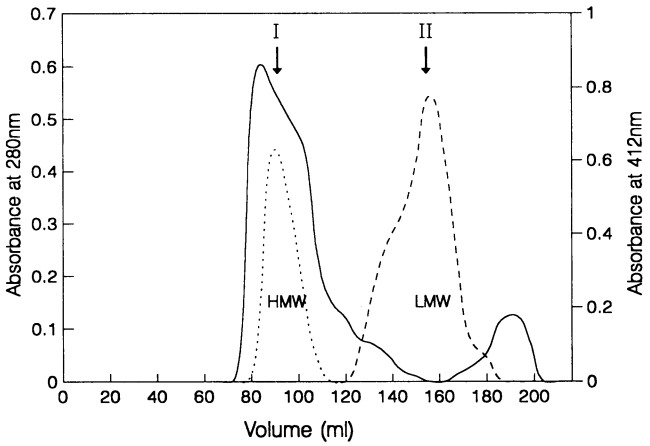

FIG. 1. Ultragel AcA54 chromatography of the ammonium sulfate precipitate of *S. clavuligerus*. The solid line represents A_{280}. Enzyme activity was assayed by method 1 (see text). For the detection of the HMW component (dotted line), 25 to 50 μl of the fraction containing the LMW material (fraction II eluted at 150 ml) was included in the reaction mixture. For the detection of the LMW component (dashed line), 25 to 50 μl of the fraction containing the HMW material (fraction I eluted at 90 ml) was included in the reaction mixture.

change step with CM-Sepharose (bed volume, 10 ml) as the gel matrix and a gradient of 15 to 100 mM sodium acetate buffer (pH 4.9) for elution was sometimes included. The yield of activity at this step was 10%, and a purification of 900-fold was achieved. Purification of the LMW and HMW proteins was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (14). Proteins were detected on gels by staining with Coomassie blue R250. The molecular weights of the native proteins were determined by chromatography on a Superose 12 HR 10/30 column (Pharmacia) with standards with molecular weight of 6,000 to 66,000.

RESULTS

The *S. clavuligerus* disulfide reductase is composed of LMW and HMW components. Crude extracts of *S. clavuligerus* were tested for NADPH-dependent disulfide reductase activity toward oxidized glutathione (GSSG), DTNB, and bis-ACV. Table 1 shows that *S. clavuligerus* contains a disulfide reductase of broad substrate specificity. Reductase activity in the extract was high with each of the substrates. By comparison, commercial preparations of glutathione reductase efficiently reduced GSSG but were much less active toward DTNB and bis-ACV. The low level of activity of the commercial enzymes for the last two substrates may be due to their contamination with other disulfide reductases. Preliminary attempts to purify the disulfide reductase activity in the crude cell extracts according to size were initially unsuccessful. Reductase activity could not be detected in any of the fractions obtained after chromatography of the extracts on Sephadex G-75 and similar gels. In these experiments GSSG was used as the substrate. Subsequently, we observed that activity could be detected when a fraction eluting at a position in the chromatogram corresponding to a protein of greater than 30 kDa was combined with a fraction eluting at a position corresponding to a protein of less than 15 kDa. Figure 1 shows the protein elution profile of an ammonium sulfate fraction of the crude extract following chromatography on Ultragel AcA54. Also shown are fractions which, when combined, result in reconstitution of disulfide reductase activity. The presence of both fractions is essential for NADPH-dependent disulfide reductase activity toward GSSG, DTNB, and bis-ACV. The above observations indicate that *S. clavuligerus* possesses a disulfide reductase composed of two nonidentical proteins. It establishes that this enzymatic system is fundamentally different from that of glutathione reductase.

Purification and characterization of the two protein components. Cell extracts of *S. clavuligerus* were fractionated as described above to obtain partially purified preparations of the LMW and HMW protein components. In all further purification steps, the elution profile of the component to be purified was monitored during chromatography by including in the assay a fraction containing the complementary component and monitoring the NADPH-dependent reduction of DTNB spectrophotometrically.

HMW protein. Table 2 summarizes the steps used in purification of the HMW component of the disulfide reductase. A purification of some 1,000-fold was achieved, with an overall yield of approximately 20%. Following ammonium sulfate fractionation and column chromatography on Ultragel AcA54, DEAE-Sephacel, and 2′5′-ADP–Sepharose, a single band was observed by electrophoresis in denaturing gels (Fig. 2) corresponding in size to a protein of 34.5 kDa. The HMW protein was estimated from densitometric scanning of the gel to be more than 95% pure. The molecular weight of the native HMW protein, as determined by gel filtration on Superose 12 (Pharmacia), was found to be 70,000 ± 2,500. Therefore, there are two subunits in the HMW protein.

LMW protein. Table 2 summarizes the steps involved in the purification of the LMW component of the disulfide reductase. An overall purification of some 500-fold was obtained with a yield of about 27%. Analysis by electrophoresis in denaturing gels showed the LMW protein to be greater than 90% pure and to have a molecular weight of about 12,500 (Fig. 2). The size of the native protein determined by gel filtration on Superose 12 was indistinguishable from that of the denatured protein. The LMW protein could also be purified by an alternative procedure in which the initial steps of cell disruption and ammonium sulfate fractionation were substituted with acetone precipitation, performed directly on the suspended mycelium, followed by

TABLE 2. Purification of the HMW and LMW proteins of the *S. clavuligerus* ACV disulfide reductase[a]

Purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
HMW protein					
50–80% $(NH_4)_2SO_4$ precipitate	221	0.967	0.004	100	1
AcA54	43	0.887	0.021	92	5
DEAE-Sephacel	4.6	0.407	0.088	42	22
2′5′-ADP–Sepharose	0.046	0.208	4.520	21	1,130
LMW protein					
50–80% $(NH_4)_2SO_4$ precipitate	186	0.695	0.004	100	1
AcA54	4.41	0.354	0.080	50	20
DEAE-Sephacel	0.10	0.188	1.900	27	475

[a] Assays were carried out as described in Materials and Methods (enzyme assays, method 1). The assay of the HMW component during the purification used its cross-reactivity with the LMW component, which was included in all assays at 5 μg/ml (DEAE-Sephacel step). Similarly, partially purified HMW protein (DEAE-Sephacel step) was used at 100 μg/ml to assay the activity of the LMW component.

two ion-exchange chromatography steps. The LMW preparation made in this way was extremely pure and was obtained in good yield. However, the procedure suffered from the disadvantage that the HMW protein could not be recovered from the acetone-treated mycelium.

Substrate specificity of the *S. clavuligerus* disulfide reductase. Various substrates containing disulfide bonds were tested with the purified *S. clavuligerus* NADPH-dependent disulfide reductase, including bis-ACV, GSSG, oxidized coenzyme A (CoA), and insulin. Table 3 shows that the rate of disulfide reduction varied greatly for different substrates. Cystine, bis-Cys-Gly, and oxidized CoA were relatively poor substrates. In contrast, bis-ACV, GSSG, and insulin were reduced at a much higher rate. To determine the K_m of the HMW protein for the LMW protein as substrate we used DTNB or GSSG as the electron acceptor. At saturating concentrations of NADPH and electron acceptors, the HMW protein had an apparent K_m of 0.2 μM for the LMW component (Table 3). An apparent K_m of 14.2 μM was determined for the HMW protein for NADPH in conditions

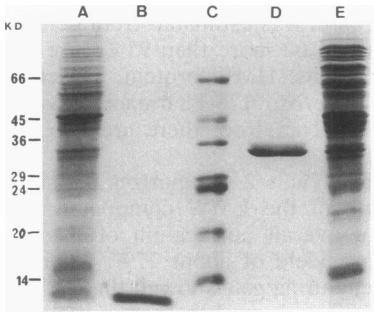

FIG. 2. Analysis of the HMW and LMW components of the *S. clavuligerus* disulfide reductase system by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Lanes: A, 26.5 μg of the AcA54 pooled fraction containing the LMW component; B, 12.5 μg of the CM-Sepharose purification step of the LMW protein; C, molecular mass markers (sizes in kilodaltons); D, 6.4 μg of HMW protein of the 2′5′-ADP–Sepharose step; E, 24.2 μg of the AcA54 pooled fraction containing the HMW component.

TABLE 3. Substrate specificity of the *S. clavuligerus* ACV disulfide reductase

Substrate	Activity[a]	K_m
For HMW[b]		
NADPH		14.20 μM
LMW		0.20 μM
For LMW[c]		
DTNB		2.20 μM
GSSG	0.18	0.31 mM
Bis-ACV	0.92	0.21 mM
CoASSCoA	0.06	0.22 mM
Bis-Cys-Gly	0.06	1.80 mM
Cystine	0.03	5.90 mM
Insulin[d]	0.55	0.33 mM

[a] Activity is reported as nanomoles of NADPH oxidized per minute at 0.1 mM substrate.

[b] Assay mixtures contained 50 mM Tris · HCl buffer (pH 8.0), 1 mM EDTA, and 0.02 mM DTNB in a final volume of 1 ml. Purified HMW protein (0.1 μg of the 2′5′-ADP–Sepharose step) was added to the reaction mixture, and NADPH was added in a concentration range from 0.5 to 200 μM. LMW protein (DEAE-Sephacel step) was added in a concentration range from 2.5 to 200 μM. The increase in A_{412} (for DTNB as the electron acceptor) and decrease in A_{340} (for GSSG as the electron acceptor) were monitored, and activity was calculated as described in Materials and Methods. The K_m for the NADPH was determined by using GSSG as electron acceptor, and the K_m for the LMW protein was separately determined by using both GSSG and DTNB as the electron acceptor with the same result.

[c] The incubation mixture contained 50 mM Tris · HCl buffer (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, 0.1 μg of the HMW component (2′5′-ADP–Sepharose step), and 2.4 μg of the LMW component (DEAE-Sephacel step). Substrates were added in a concentration range from 0.15 to 10 μM for DTNB, 0.1 to 1.6 mM for GSSG, 0.1 to 2.0 mM for bis-Cys-Gly, 0.1 to 0.8 mM for CoASSCoA, 0.125 to 2.0 mM for cystine, and 0.06 to 0.5 mM for bis-ACV. The increase in A_{412} was monitored for DTNB as the substrate, and the decrease in A_{340} was monitored for other substrates.

[d] The incubation mixture contained 50 mM Tris · HCl buffer (pH 8.0), 1 mM EDTA, 0.2 mM NADPH, 20 μg of the LMW component (DEAE step), and 60 μg of the HMW component (DEAE-Sephacel step). Insulin was added in a concentration range of 0.02 to 0.3 mM, and the decrease in A_{340} was monitored.

under which the LMW protein was present in excess and the disulfide substrate was GSSG. In all cases disulfide reductase activity was strictly dependent on the presence of NADPH. No activity was found when NADH was substituted for NADPH.

Reduction of insulin disulfides. Two convenient assays to demonstrate the ability of the LMW protein to reduce disulfide bonds in proteins are based on the thioredoxin assay developed by Holmgren (8, 9). They employ insulin as the disulfide-containing substrate and DTT (9) or thioredoxin reductase and NADPH as the source of reducing power (8). Figure 3A shows the effect of the LMW protein on dissociation of the two polypeptide chains of insulin. The increase in turbidity of the reaction mixture is due to precipitation of the free insulin B chain. DTT by itself reduces insulin disulfides. However, in the presence of the LMW protein the rate of reduction was considerably enhanced. Quantitative analysis of the reduction of insulin disulfides by the complete *S. clavuligerus* reductase system is shown in Table 4. The free thiols released were assayed with DTNB. A high rate of insulin disulfide reduction was observed when all components were present. When the LMW, HMW, or NADPH components were omitted, the extent of insulin disulfide reduction dropped by more than 20-fold. Figure 3B shows that the free thiols formed following incubation of insulin with the complete disulfide reductase system are associated with the separate polypeptide chains.

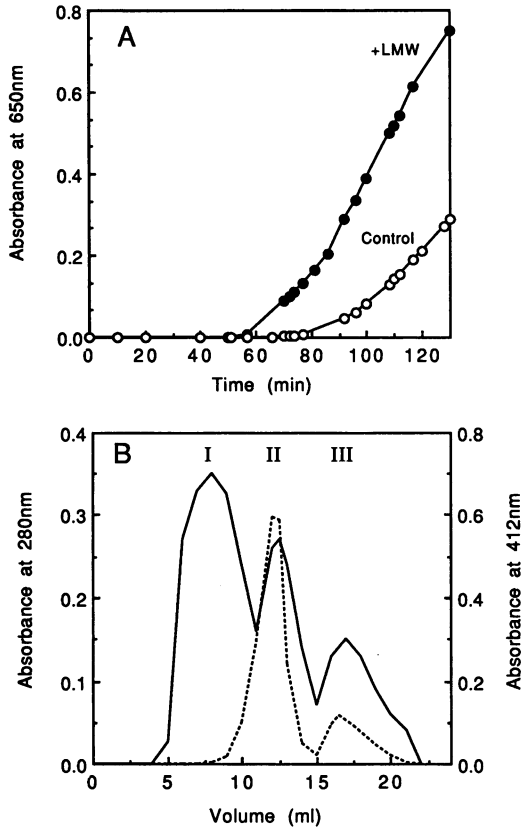

FIG. 3. (A) Reduction of insulin catalyzed by the LMW protein and DTT. One milliliter of incubation mixture contained 0.1 M potassium phosphate buffer, 2 mM EDTA, 0.5 mM porcine insulin, 0.33 mM DTT, and 19.6 μg of LMW protein when added. The A_{650} (precipitation of reduced insulin) is plotted against time. (B) Separation of the A and B chains of enzymatically reduced insulin, and titration of the free thiols formed with DTNB. Insulin was incubated overnight with the *S. clavuligerus* disulfide reductase system and NADPH and then loaded onto a G-15 column. Fractions eluted from the column were treated with DTNB, and the A_{412} was measured (dashed line). I, native insulin; II, insulin B chain; III, insulin A chain.

Ability of the *S. clavuligerus* HMW and LMW proteins to complement *E. coli* thioredoxin and thioredoxin reductase. A commercial *E. coli* thioredoxin and a partially purified preparation of *E. coli* thioredoxin reductase were tested for their ability to replace, respectively, the LMW and HMW components of the *S. clavuligerus* disulfide reductase system. K_m and K_{cat} values for the reaction are presented in Table 5. In the presence of NADPH, *E. coli* thioredoxin was complemented by the *S. clavuligerus* HMW protein. A similar result was obtained with thioredoxin from *Spirulina platensis*. On the other hand, *E. coli* thioredoxin reductase was ineffective in complementing the *S. clavuligerus* LMW protein. These results and those presented above indicate that the LMW and HMW proteins make up a reductase system that resembles that of thioredoxin. Other evidence that supports this view is the fact that the LMW component (DEAE-Sephacel step) is a heat-stable protein: it retained full activity after incubation at 60°C for 15 min and 85% activity after incubation at 75°C for the same time; complete inactivation resulted only after incubation at 95°C for more than 30 min. Furthermore, the spectrum of the pure HMW component (2'5'-ADP–Sepharose step) showed distinct absorption maxima at 266, 373, and 457 nm and a shoulder at 474 nm, characteristic of the thioredoxin reductase flavoprotein (21). The ratios A_{268}/A_{457}, A_{280}/A_{457}, and A_{373}/A_{457} were, respectively, 8.52, 6.32, and 1.17. Because of the small amount of HMW protein obtained, the nature of the flavin was not identified. Both proteins were inactivated by thiol-blocking agents (data not shown).

TABLE 4. Reduction of disulfide bonds in insulin by the *S. clavuligerus* ACV reductase system

Content	NADPH oxidation rate[a] ($\Delta A_{340} \times 10^2$/min)	Reduced disulfides (nmol/min)	Rate of precipitation (A_{650}/h)
Complete reductase system	15.6	25.0	0.60
Minus NADPH	0.5	0.8	0.02
Minus HMW	0.7	1.1	0.03
Minus LMW	0.7	1.1	0.02

[a] The amount of reduced disulfide bonds was determined after treatment of the reaction products with DTNB. A change in A_{340} of 0.0062 is equivalent to 1 nmol of S-S bonds reduced. This relation was used to calculate the equivalent NADPH oxidation rate (15).

TABLE 5. Reduction of different thioredoxin substrates with the *S. clavuligerus* HMW reductase and the *E. coli* thioredoxin reductase[a]

Source of reductase	Source of thioredoxin	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m[b]
S. clavuligerus	*S. clavuligerus*	0.38	374	984
S. clavuligerus	*E. coli*	9.20	1,160	126
S. clavuligerus	*S. platensis*	41.80	324	8
E. coli	*E. coli*	5.2	3,267	628
E. coli	*S. clavuligerus*	NA[c]	NA	

[a] Assays were carried out as described in Materials and Methods (enzyme assays, method 1). Activity was calculated as micromoles of NADPH oxidized per minute according to $\Delta A_{412}/13.6 \times 2$ (15). The *S. clavuligerus* HMW reductase and *E. coli* thioredoxin reductase were of the 2'5'-ADP–Sepharose step. Concentrations were varied over 3 to 30 nM for the *S. clavuligerus* HMW reductase and 1.2 to 5 nM for the *E. coli* thioredoxin reductase. Concentrations of the thioredoxins were varied over 38 to 154 nM for *S. clavuligerus*, 56 to 340 nM for *Spirulina platensis*, and 182 to 1,820 nM for *E. coli*.

[b] The ratio K_{cat}/K_m is a measure of the catalytic efficiency of the reaction.

[c] NA, no activity.

In vitro biosynthesis of isopenicillin N from bis-ACV is driven by the *S. clavuligerus* disulfide reductase. The free thiol form of ACV is believed to be the immediate substrate for IPNS in the formation of isopenicillin N. Oxidized bis-ACV is not a substrate for IPNS. To determine whether bis-ACV can be converted to isopenicillin N by coupling the disulfide reductase with IPNS, we added bis-ACV to crude extracts of *S. clavuligerus* (containing IPNS) and measured isopenicillin N in a bioassay. Table 6 shows that when either DTT or NADPH was present in the reaction mixture, bis-ACV was efficiently converted to isopenicillin N. The dependence of the NADPH-driven reaction on the HMW and LMW components of the disulfide-reducing system is shown in Table 6. Isopenicillin N was formed only when both proteins were present and NADPH was the source of the reducing power. In the absence of any one of the components, isopenicillin N synthesis occurred only if the synthetic reductant DTT was provided. Figure 4 schematically depicts a role for the disulfide reductase in the in vitro conversion of bis-ACV to isopenicillin N.

TABLE 6. Conversion of bis-ACV to isopenicillin N in crude extracts of *S. clavuligerus* containing IPNS and with purified IPNS

Bis-ACV reductant	Amt of isopenicillin N (units)[a]
Crude extract	
Crude extract	2.0
+ DTT	19.8
+ NADPH	15.2
Purified IPNS	
+ DTT	23.0
+ HMW, LMW, NADPH	23.0
+ HMW, LMW	0.9
+ HMW, NADPH	1.1
+ LMW, NADPH	0.9

[a] Isopenicillin N was measured in a bioassay.

DISCUSSION

The biosynthesis of all penicillins and cephalosporins begins with the formation of ACV and its conversion to isopenicillin N. The work reported in this paper was prompted by earlier observations that IPNS is able to catalyze the latter reaction in vitro if ACV is in its thiol state but not if it is in the oxidized disulfide state, bis-ACV (16). We reasoned that β-lactam-producing *Streptomyces* species might contain an activity that reduces bis-ACV to its monomeric thiol form and that might therefore promote β-lactam biosynthesis. We show here that *S. clavuligerus*, a potent producer of β-lactam antibiotics, possesses an NADPH-dependent disulfide reductase of broad substrate specificity. Its activity was greatest toward DTNB, bis-ACV, GSSG, and CoASSCoA and much lower toward disulfides of cysteine and the dipeptide cysteinyl-glycine. Also, it effectively reduces disulfides in high-molecular-weight substrates such as insulin.

These and other properties (see below) of the *S. clavuligerus* disulfide reductase are incompatible with its being glutathione reductase. Thus, DTNB, CoASSCoA, and cystine are not substrates or only very poor substrates for glutathione reductase (18). Also, all attempts to detect glutathione reductase activity in cell extracts of *S. clavuligerus* were unsuccessful. This result is consistent with recent findings that streptomycetes lack glutathione (3). Further analysis of the *S. clavuligerus* disulfide reductase shows that it resembles the thioredoxin class of oxidoreductases. Thus, it is composed of two polypeptides that catalyze the transfer of electrons from NADPH to disulfides in small molecules and proteins. The HMW protein is a flavoprotein reductase. Like the *E. coli* and yeast thioredoxin reductases, it is a dimeric enzyme with subunits of 35 kDa (10). Mammalian thioredoxin reductases are enzymes with subunits of 58-kDa enzymes. The LMW protein is a heat-stable 12-kDa protein that is reduced by the HMW protein and can function, like thioredoxins, as a general disulfide reductant. Because *S. clavuligerus* lacks glutathione and glutathione reductase, we can rule out the possibility that the reductase belongs to a second class of disulfide reductases, the glutaredoxins (7).

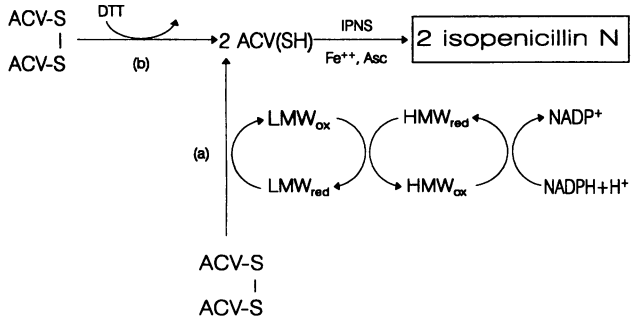

FIG. 4. Proposed mechanism for the coupled enzymatic conversion of bis-ACV to isopenicillin N by *S. clavuligerus* disulfide reductase and IPNS: (a) enzymatic reduction of bis-ACV; (b) chemical reduction of bis-ACV.

An additional insight into the nature of the *S. clavuligerus* disulfide reductase comes from experiments in which the HMW protein was able to partially complement thioredoxin (Table 5). For example, the *S. clavuligerus* reductase (HMW protein) exhibited high activity for the LMW protein as substrate and was also effective in complementing commercially available thioredoxins from *E. coli* and *Spirulina platensis*. In contrast, the *S. clavuligerus* LMW protein very weakly complemented thioredoxin reductase from *E. coli* under conditions in which the *E. coli* thioredoxin was extremely active for its cognate reductase (21). Currently, we are cloning the genes coding for the LMW and HMW proteins. Comparison of their amino acid sequences with the corresponding sequences of thioredoxin and thioredoxin reductase should establish definitively the structural relatedness of the *S. clavuligerus* disulfide reductase system to that of thioredoxin. Willing et al. (22) reported the presence in the gram-positive *Brevibacterium ammoniagenes* of a thioredoxin system and showed that reduced thioredoxin was a hydrogen donor for ribonucleotide reduction. An NADPH-dependent thioredoxin reductase was also detected in cell extracts, but the enzyme was not purified (22). Kollarova et al. (13) reported that a thioredoxin-like protein in *Streptomyces aureofaciens* could serve as a hydrogen donor for its own ribonucleotide reductase.

In the second part of this paper we demonstrate that bis-ACV is converted in vitro to isopenicillin N by the concerted action of disulfide reductase and IPNS (Fig. 4). We propose that disulfide reductase may play a role in maintaining the intracellular level of reduced ACV in β-lactam-producing streptomycetes, thereby supporting the rate of synthesis of isopenicillin N. Because glutathione and its associated enzymes are thought to play an important role in determining the intracellular thiol disulfide redox balance (5), it is plausible that streptomycetes employ an alternative disulfide reductase system. In *Bacillus* species, which also lack glutathione, CoA disulfide reductase has been postulated to play this role (20). We note that bis-ACV disulfide reductase may potentially also be involved in promoting β-lactam synthesis by modulating the thiol-disulfide state of cysteine residues of ACV synthetase and IPNS that are required for activity. In support of this idea, we have found that the *S. clavuligerus* disulfide reductase reversibly modulates the activity of IPNS and ACVS (2). Thus, ACV synthetase and IPNS, the two common enzymes of the β-lactam biosynthetic pathway, and ACV, their respective product and substrate, may be targets for the thiol-disulfide redox systems of the cell.

The results reported in this paper indicate that the major disulfide reductase in *S. clavuligerus* resembles that of thioredoxin. We plan to analyze the in vivo role of this system for β-lactam production by gene disruption experi-

ments. A further objective is to verify whether this reductase is generally present in streptomycetes and whether its activity is regulated during cell growth and differentiation. Preliminary experiments show that *Streptomyces lividans*, a non-β-lactam producer, contains a related disulfide reductase. Studies of this kind should clarify whether this disulfide reductase system has a general role in cellular metabolism as well as a particular role in β-lactam biosynthesis.

ACKNOWLEDGMENTS

We thank Anat Argaman and Michaela Yanco for technical assistance.

This research was supported by grant 88-00441 from the United States-Israel Binational Science Foundation, Jerusalem, Israel.

REFERENCES

1. **Aharonowitz, Y., G. Cohen, and J. F. Martin.** 1992. Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. Annu. Rev. Microbiol. **46:**461–495.
2. **Av-Gay, Y., G. Cohen, and Y. Aharonowitz.** 1991. Modulation of enzymatic activity by a thioredoxin like disulfide reductase in *Streptomyces clavuligerus*, abstr. P2-022. Abstr. Eighth Int. Symp. Biol. Actinomycete 1991. Madison, Wis.
3. **Cohen, A., G. I. Newton, R. Fahey, and Y. Aharonowitz.** 1991. Thioredoxin like disulfide reductase system in *Streptomyces*, abstr. P2-023. Abstr. Eighth Int. Symp. Biol. Actinomycete 1991. Madison, Wis.
4. **Cohen, G., D. Shiffman, M. Mevarech, and Y. Aharonowitz.** 1990. Microbial isopenicillin N synthase genes: structure, function, diversity and evolution. Trends Biotechnol. **8:**105–111.
5. **Fahey, R. C., and A. R. Sundquist.** 1991. Evolution of glutathione metabolism. Adv. Enzymol. Relat. Areas Mol. Biol. **64:**1–53.
6. **Holmgren, A.** 1977. Bovine thioredoxin system: purification of thioredoxin reductase from calf liver and thymus and studies of its function in disulfide reduction. J. Biol. Chem. **252:**4600–4606.
7. **Holmgren, A.** 1979. Glutathione-dependent synthesis of deoxyribonucleotides: characterization of the enzymatic mechanism of *Escherichia coli* glutaredoxin. J. Biol. Chem. **254:**3672–3678.
8. **Holmgren, A.** 1979. Reduction of disulfides by thioredoxin: exceptional reactivity of insulin and suggested functions of thioredoxin in mechanism of hormone action. J. Biol. Chem. **254:**9113–9119.
9. **Holmgren, A.** 1979. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. **254:**9627–9632.
10. **Holmgren, A.** 1985. Thioredoxin. Annu. Rev. Biochem. **54:**237–271.
11. **Jensen, S. E., B. K. Leskiw, L. C. Vining, Y. Aharonowitz, and D. W. S. Westlake.** 1986. Purification of isopenicillin N synthase from *Streptomyces clavuligerus*. Can. J. Microbiol. **32:**953–958.
12. **Jensen, S. E., D. W. S. Westlake, and S. Wolfe.** 1982. Cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to penicillins by cell-free extracts of *Streptomyces clavuligerus*. J. Antibiot. **35:**483–490.
13. **Kollarova, M., E. Kasova, K. Szuttorova, H. Follmann, and J. Zelinka.** 1988. Thioredoxin from *Streptomyces aureofaciens*, p. 37–41. *In* J. Zelinka and J. Balan (ed.), Metabolism and enzymology of nucleic acids including gene manipulations. Plenum Press, New York.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Luthman, M., and A. Holmgren.** 1982. Rat liver thioredoxin and thioredoxin reductase: purification and characterization. Biochemistry **21:**6628–6633.
16. **Perry, D., E. P. Abraham, and J. E. Baldwin.** 1988. Factors affecting the isopenicillin N synthetase reaction. Biochem. J. **255:**345–351.
17. **Russell, M., and P. Model.** 1988. Sequence of thioredoxin reductase from *Escherichia coli*: relationship to other flavoprotein disulfide oxidoreductases. J. Biol. Chem. **263:**9015–9019.
18. **Schirmer, R. H., R. L. Krauth-Siegel, and G. E. Schulz.** 1988. Glutathione reductase, p. 553–596. *In* D. Dolphin, R. Poulson, and O. Avramovic (ed.), Glutathione: chemical, biochemical and medical aspects, part A. John Wiley & Sons, Inc., New York.
19. **Shirafuji, H., Y. Fujisawa, M. Kida, T. Kanzaki, and M. Yoshida.** 1979. Accumulation of tripeptide derivatives by mutants of *Cephalosporium acremonium*. Agric. Biol. Chem. **43:**155–160.
20. **Swerdlow, R. D., and P. Setlow.** 1983. Purification and characterization of a *Bacillus megaterium* disulfide reductase specific for disulfides containing pantetheine 4′,4″-diphosphate. J. Bacteriol. **153:**475–484.
21. **Williams, C. H., Jr.** 1976. Flavin-containing dehydrogenases, p. 89–173. *In* P. D. Boyer (ed.), The enzymes, vol. 13. Academic Press, Inc., New York.
22. **Willing, A., H. Follmann, and G. Auling.** 1988. Nucleotide and thioredoxin specificity of manganese ribonucleotide reductase from *Brevibacterium ammoniagenes*. Eur. J. Biochem. **175:**167–173.