JOURNAL OF BACTERIOLOGY, Feb. 1994, p. 973–984
0021-9193/94/$04.00+0

Vol. 176, No. 4

The Thioredoxin System of *Penicillium chrysogenum* and Its Possible Role in Penicillin Biosynthesis

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Received 16 August 1993/Accepted 5 December 1993

***Penicillium chrysogenum* is an important producer of penicillin antibiotics. A key step in their biosynthesis is the oxidative cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) to isopenicillin N by the enzyme isopenicillin N synthase (IPNS). bis-ACV, the oxidized disulfide form of ACV is, however, not a substrate for IPNS. We report here the characterization of a broad-range disulfide reductase from *P. chrysogenum* that efficiently reduces bis-ACV to the thiol monomer. When coupled in vitro with IPNS, it converts bis-ACV to isopenicillin N and may therefore play a role in penicillin biosynthesis. The disulfide reductase consists of two protein components, a 72-kDa NADPH-dependent reductase, containing two identical subunits, and a 12-kDa general disulfide reductant. The latter reduces disulfide bonds in low-molecular-weight compounds and in proteins. The genes coding for the reductase system were cloned and sequenced. Both possess introns. A comparative analysis of their predicted amino acid sequences showed that the 12-kDa protein shares 26 to 60% sequence identity with thioredoxins and that the 36-kDa protein subunit shares 44 to 49% sequence identity with the two known bacterial thioredoxin reductases. In addition, the *P. chrysogenum* NADPH-dependent reductase is able to accept thioredoxin as a substrate. These results establish that the *P. chrysogenum* broad-range disulfide reductase is a member of the thioredoxin family of oxidoreductases. This is the first example of the cloning of a eucaryotic thioredoxin reductase gene.**

Penicillins are sulfur-containing β-lactam compounds that are produced by certain filamentous fungi and industrially by *Penicillium chrysogenum*. The initial steps in the biosynthesis of these antibiotics involve the condensation of α-aminoadipic acid, cysteine, and valine to form a tripeptide by the enzyme δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase and then the cyclization of ACV to make isopenicillin N by the enzyme isopenicillin N synthase (IPNS) (3). 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. Within the cell, it may also make mixed disulfides with other thiol-containing compounds. Significantly, only the reduced form of ACV is converted in vitro to isopenicillin N by IPNS (36). Furthermore, IPNS is less active under nonreducing conditions, and a similar situation may exist for ACV synthetase (4, 36). Therefore, synthetic reducing agents, such as dithiothreitol (DTT), are used to maintain reducing conditions in cell-free systems used for producing penicillins (41). At present, it is not known how each of the above-mentioned enzymes and ACV are kept in their reduced state in *P. chrysogenum* during β-lactam synthesis. Moreover, the high yields of penicillins made by commercial strains suggest that a substantial part of the reducing power of the cell may be diverted to their synthesis. We recently searched for an identified an enzymatic disulfide reductase system in *Streptomyces clavuligerus*, a potent producer of penicillins and cephalosporins, that is able to replace the need for DTT in IPNS reactions (2). This system efficiently reduces bis-ACV to its thiol form and modulates the activity of IPNS (4). When coupled with IPNS, it quantitatively converts bis-ACV to isopenicillin N. Because ACV structurally resembles glutathione, γ-glutamyl-cysteinyl-glycine, the most common intracellular low-molecular-weight (LMW) thiol, we originally supposed that this disulfide reductase might be related to glutathione reductase. However, we have now shown that streptomycetes lack both glutathione and glutathione reductase (2, 35). Also, a biochemical characterization of the *S. clavuligerus* disulfide reductase revealed that, in contrast to glutathione reductase, the *S. clavuligerus* disulfide reductase reduces a wide range of disulfides in LMW compounds and in proteins and is composed of two nonidentical polypeptides. The high-molecular-weight (HMW) component is a 70-kDa flavoprotein containing two identical subunits. In the presence of NADPH, it catalyzes the transfer of electrons to the LMW component. The latter is a heat-stable 12-kDa protein that is a general disulfide reductant. These properties of the *S. clavuligerus* broad-range disulfide reductase led us to propose that it belongs to the thioredoxin-thioredoxin reductase class of flavoprotein disulfide oxidoreductases. This proposal was recently confirmed by cloning the *S. clavuligerus* HMW and LMW protein genes and determining their DNA sequences (6). The purpose of the present paper is to describe the biochemical and genetic properties of a broad-range disulfide reductase system in *P. chrysogenum* that reduces bis-ACV, is distinguishable from glutathione reductase, and may play a role in promoting the biosynthesis of β-lactam compounds. We show that *P. chrysogenum* possesses both glutathione reductase and thioredoxin-based disulfide reductase systems and that the thioredoxin system is able to efficiently reduce bis-ACV and disulfides in proteins. The two genes encoding the *P. chrysogenum* thioredoxin system were cloned and sequenced. To the best of our knowledge, this is the first reported cloning of a eucaryotic thioredoxin reductase gene.

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MATERIALS AND METHODS

Microorganisms, media, and growth conditions. *P. chrysogenum* NRRL 1951 was used for preparing the LMW and HMW proteins of the general disulfide reductase. Cultures were grown at 30°C in tryptic soy broth (Biolife) containing 10 g of soluble starch per liter (TSBS). Fifty-milliliter amounts of TSBS in 250-ml flasks were inoculated with spores (adsorbed on rice seeds) and incubated for 24 to 48 h at 250 rpm. These seed cultures were transferred at 2% (vol/vol) into fresh TSBS (usually 1-liter amounts in 4-liter flasks) and incubated for a further 24 to 48 h at 150 rpm. Cultures were harvested by filtration, washed with 0.9% NaCl, lyophilized, and stored at −70°C.

Materials. Chromatography resins, DEAE-Sephacel, Blue-Sepharose CL-6B, and 2′,5′-ADP-Sepharose were supplied by Pharmacia. Ultrogel AcA54 was obtained from LKB. DTT, NADPH, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and bovine insulin were purchased from Sigma. Chemically synthesized ACV was obtained from Bachem Feinchemicalien AG, Bubendorf, Switzerland, in the oxidized form (bis-ACV). Thioredoxins from *Spirulina platensis* and *Escherichia coli* were obtained from Sigma and Calbiochem, respectively. Thioredoxin reductase from *S. clavuligerus* was purified as previously described (2).

Enzyme assays. (i) Disulfide reductase activity. The *P. chrysogenum* general disulfide reductase contains two protein components, denoted LMW and HMW. Its activity was determined by use of the DTNB assay described by Holmgren (13). Activity was monitored by measuring the increase in the A_{412} at 25°C during the initial 2 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 (disolved in 95% ethanol). Depending on which component activity was to be assayed, various amounts of the HMW or LMW protein were added to the mixture, and the reaction was started by the addition of the complementary component. Control reactions in which either one of the two protein components was omitted or in which NADPH was omitted showed no significant increase in absorbance. The assay could be adapted easily to a microtiter plate scale by reducing the reaction mixture volume to 0.2 ml and monitoring the increase in the absorbance by use of an automatic microtiter plate reader. One unit of activity is the amount of protein that oxidizes 1 μmol of NADPH per min, calculated as $\Delta A_{412}/(13.2 \times 2)$ (27), in a standard reaction mixture containing known amounts of the LMW or HMW component.

(ii) Insulin reduction. The reduction of protein disulfides by the LMW component of the *P. chrysogenum* general disulfide reductase was done with insulin as the substrate and DTT as the terminal reductant (14). The reaction was monitored by measuring the turbidity at 650 nm of a solution containing, in a final volume of 1 ml, 0.5 mM bovine insulin, 100 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 1 mM DTT, and the LMW protein. Control reactions in which DTT or the LMW protein was omitted were carried out in parallel. In other experiments, DTT was substituted for by the HMW protein and NADPH, and the reaction was monitored by measuring the change in the A_{340}.

(iii) Glutathione reductase. Reactions for the determination of glutathione reductase activity were performed with a mixture containing, in a final volume of 1 ml, 50 mM Tris-HCl (pH 8.0), 1 mM oxidized glutathione (GSSG), 1 mM EDTA, 0.2 mM NADPH, and enzyme as required. Activity was measured by monitoring the change in the A_{340}.

(iv) IPNS assay. The conversion of ACV to isopenicillin N by IPNS was measured with reaction mixtures containing, in a final volume of 0.08 ml, 0.345 mM 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, and purified preparations of IPNS and the LMW and HMW protein components of the general disulfide reductase. The reaction mixtures were incubated for 30 to 60 min at room temperature, and the reactions were terminated by the addition of 0.08 ml of methanol. The amount of isopenicillin N produced was estimated by the agar diffusion bioassay with *Micrococcus luteus* ATCC 9341 as the indicator strain (17).

Purification of the *P. chrysogenum* general disulfide reductase system. Dry mycelium was ground in a mortar, suspended in 4 volumes of 50 mM Tris-HCl (pH 8.0)–0.1 mM EDTA (TE buffer), and centrifuged in the cold for 15 min at 14,000 × *g*. 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, adjusted to 80% saturation with solid ammonium sulfate, stirred again 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 that 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 a substrate. Whereas disulfide reductase activity was readily detected in the resuspended ammonium sulfate pellet, it was absent in the individual fractions collected from the gel filtration column. Activity could be recovered only by combining certain fractions containing an LMW protein (<15,000) with certain fractions containing an HMW protein (>60,000). The latter also contained glutathione reductase activity. The LMW and HMW proteins were further purified as described below, and their activities were determined by assaying for each in the presence of the complementary component.

(i) Purification of the HMW protein. Gel filtration fractions containing the HMW protein were combined and applied to a DEAE-TSK (Merck) column that had a 20-ml bed volume and 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 and separately for GSSG reduction (glutathione reductase activity). The fractions containing activity in the DTNB assay were pooled, diluted with water to adjust their conductivity to that of the buffer in the next step, and applied to a Blue-Sepharose CL-6B column (1 by 10 cm) that had been preequilibrated with 20 mM Tris-HCl (pH 7.25)–1 mM EDTA. The HMW protein was eluted with the same buffer containing 5 mM NADPH. Active fractions were pooled and kept frozen. The yield of activity at this stage was about 50%, and a purification of about 500-fold was achieved.

(ii) Purification of the LMW protein. Gel filtration fractions containing the LMW protein were combined and incubated with 2 mM DTT at room temperature before application to a DEAE-Sephacel column that had been preequilibrated with 10 mM Tris-HCl (pH 8.0)–1 mM EDTA. The column was developed with a linear gradient of 0 to 0.3 M NaCl in the same buffer. Fractions were tested for DTNB reduction in the presence of the HMW protein and NADPH. Active fractions were combined and stored frozen at −20°C. A further ion-

exchange step was sometimes done and included CM-Sephacel (10-ml bed volume) as the gel matrix. For this step, the pooled material was dialyzed against 15 mM sodium acetate buffer (pH 4.9)–1 mM EDTA by use of tubing having a molecular weight cutoff of 3,000. A gradient of 15 to 600 mM sodium acetate (pH 4.9) was used for elution. The yield of activity at this step was about 20%, and a purification of about 700-fold was achieved.

The purity of the HMW and LMW preparations at the different steps was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (20). The NH_2-terminal portions of the purified LMW and HMW proteins were determined after electrophoresis in denaturing gels, staining with Coomassie blue R-250, electroblotting to a polyvinylidene difluoride membrane, and sequencing with a gas-phase sequencer at Eurosequence, Groningen, The Netherlands.

Cloning and gene manipulations. A genomic library of *P. chrysogenum* GB8 in the lambda cloning vector EMBL-3 (9) was screened by hybridization to isolate the LMW and HMW protein genes as described elsewhere (39). Synthetic mixed degenerate oligodeoxynucleotides were prepared by use of an Applied Biosystems 380B DNA synthesizer according to the NH_2-terminal sequences of the purified LMW and HMW proteins. Phage and plasmid DNA preparations were made by standard procedures (28). Plasmid pMY-8 is pUC18 containing the entire *S. clavuligerus* HMW reductase gene (*trxB*) (6). Restriction enzymes and DNA-modifying enzymes were purchased from International Biotechnologies, Inc., and Promega and used as recommended by the manufacturers. Subcloning and Southern analyses were done as described previously (28).

DNA analysis. Nucleotide sequencing was performed by the dideoxy chain termination procedure after subcloning fragments into M13 phage vectors (32) or by direct sequencing from double-stranded DNA. Both strands of open reading frames were sequenced with Sequenase version 2.0 (United States Biochemical Corp.) and synthetic oligonucleotide primers.

Sequence analysis. Published protein sequences were accessed from the PIR protein or Swiss-Prot data banks with the Genetic Computer Group, Inc. (GCG) (Madison, Wis.), sequence analysis software package. Sequence analysis was performed with the Clone Manager software package, version 3.0 (Scientific and Educational Software, National Institutes of Health, Bethesda, Md.). Paired sequence alignments were carried out with the FASTA (26) program in the GCG package. Multiple sequence alignments were made with the Pileup program in the same GCG package.

Nucleotide sequence accession number. Sequence data reported in this work have been submitted to EMBL and assigned accession numbers X76119 for the *P. chrysogenum trxB* gene and X76120 for the *P. chrysogenum trxA* gene.

RESULTS

***P. chrysogenum* disulfide reductases.** Gel filtration chromatography of crude extracts of *P. chrysogenum* revealed that they contain two NADPH-dependent disulfide reductases, one able to reduce GSSG and the other able to reduce GSSG and DTNB. The specific activities of the whole-cell extracts for GSSG and DTNB were 0.095 and 0.0015 to 0.0045 U/mg of protein, respectively. Glutathione reductase recognizes GSSG but not DTNB as a substrate. It was found to coelute with the main protein profile in AcA54 chromatography of the ammonium sulfate fraction (Fig. 1). A second reductase able to accept both GSSG and DTNB with similar efficiencies was initially not detected in any of the individual fractions from the column but was subsequently identified when certain well-separated fractions were combined. The NADPH-dependent DTNB reductase activity was recovered when a fraction containing an HMW protein (>60,000) that coeluted with glutathione reductase was combined with an LMW protein (<15,000) that eluted later (Fig. 1). The fact that DTNB reductase activity requires two nonidentical components, whereas a single protein species is sufficient for GSSG reductase activity, readily allows discrimination between the two enzyme systems and forms the basis of the assay used for the purification of the LMW and HMW proteins. We henceforth refer to the former system as the *P. chrysogenum* general disulfide reductase system for reasons that will become clear.

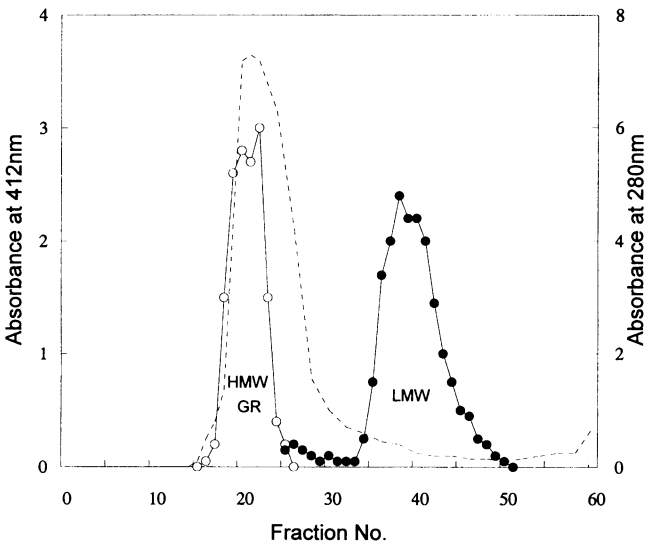

FIG. 1. Separation of the LMW and HMW proteins of the *P. chrysogenum* NADPH-dependent general disulfide reductase by AcA54 chromatography of the ammonium sulfate precipitate of cell extracts. The broken line represents the A_{280}. The activities (×5) of the LMW protein (●) and the HMW protein (○) were determined by the DTNB assay (see Materials and Methods). For detection of the HMW component, samples from the pooled fractions containing the LMW protein (fractions 38 to 42) were included in the assay. For detection of the LMW component, samples from the pooled fractions containing the HMW protein (fractions 18 to 22) were included in the assay. Glutathione reductase (GR) was assayed in individual fractions with GSSG as a substrate (see Materials and Methods). It coeluted with the HMW protein.

Purification of the LMW and HMW proteins of the *P. chrysogenum* general disulfide reductase. Table 1 summarizes the steps in the purification of the LMW and HMW components of the general disulfide reductase. In the initial stages of purification, up to the ammonium sulfate step, reductase activity was assayed with both LMW and HMW components present in the extract with DTNB as a substrate. Because of the high level of nonspecific reductase activity in the ammonium sulfate fractions and the difficulty in determining the relative amounts of the two components, the activities at the AcA54 step were used as a reference for calculating relative yields during purification. At that step, the proteins were separated by gel filtration chromatography, and HMW enzyme activity was monitored by including in the reaction mixture the LMW component. Because the fractions containing HMW enzyme activity from the gel filtration step had high levels of glutathione reductase activity, we preferred not to use them at

TABLE 1. Purification of the HMW and LMW proteins of the *P. chrysogenum* general disulfide reductase

Protein and purification step	Total amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
HMW protein					
Ammonium sulfate	95.1	0.398	0.0042	93	1
Ultrogel AcA54	25.2	0.429	0.0170	100	4
DEAE-TSK	2.7	0.283	0.104	66	24
Blue-Sepharose CL-6B	0.1	0.207	2.070	48	493
LMW protein					
Ammonium sulfate	169.6	0.105	0.00062	27	1
Ultrogel AcA54	6.9	0.390	0.056	100	90
DEAE-Sephacel	1.5	0.244	0.162	62	261
CM-Sephacel	0.23	0.083	0.355	21	572

this stage for assaying LMW enzyme activity. The separation of glutathione reductase from HMW enzyme activity by DEAE-TSK chromatography is shown in Fig. 2. Glutathione reductase elutes first at 0.15 to 0.23 M NaCl and is followed by HMW reductase at 0.24 to 0.32 M NaCl. To further purify HMW reductase from residual levels of highly active glutathione reductase, pooled HMW fractions were applied to a 2',5'-ADP-Sepharose column that had previously been found to be effective at this step for different disulfide reductases. HMW reductase bound poorly to this resin but was retained much more effectively by Blue-Sepharose CL-6B and could be eluted with a buffer containing NADPH. A purification of 500-fold at this stage was achieved, with an overall yield of 50%. Analysis of the HMW preparation by electrophoresis in denaturing gels revealed a single band with an estimated molecular weight of 36,000 ± 2,000. Gel filtration chromatography of the native HMW protein showed that it is a dimer. The purification of the LMW component of the disulfide reductase is summarized in Table 1. A purification of about 570-fold was obtained, with an overall yield of 20%. Analysis of the LMW preparation by electrophoresis in denaturing gels showed it to be greater than 90% pure and to have a molecular weight of about 12,000. The size of the native LMW protein determined by gel filtration chromatography was indistinguishable from that of the denatured protein. The LMW protein is heat stable. Incubation of the LMW protein (DEAE-Sephacel step) for 25 min at 78°C had no significant effect on its DTNB-reducing activity.

The *P. chrysogenum* general disulfide reductase has broad substrate specificity. Several LMW disulfide-containing compounds were tested as substrates with the purified *P. chrysogenum* NADPH-dependent disulfide reductase; they included DTNB, GSSG, bis-ACV, CoASSCoA (oxidized coenzyme A), CoASSG (coenzyme A glutathione disulfide), and CysGlySS CysGly (bis-CysGly). The standard reaction mixture contained 0.2 mM NADPH, 18 μg of HMW protein (from the DEAE purification step), 2.5 μg of LMW protein (from the same step), and various concentrations of disulfide substrates. The

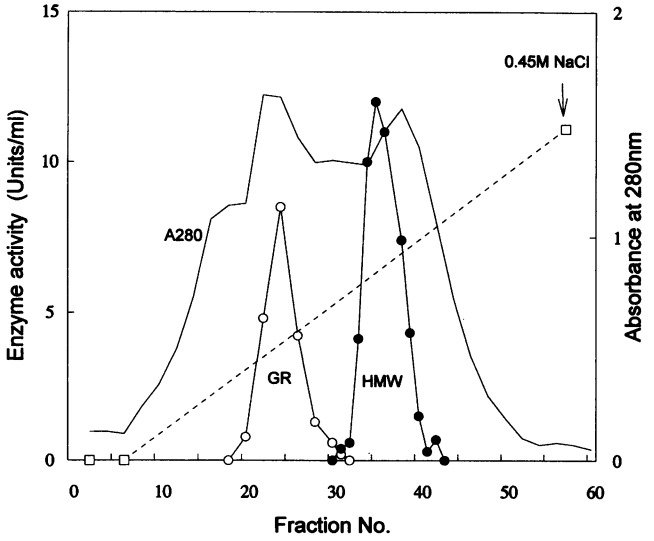

FIG. 2. Separation of the *P. chrysogenum* HMW reductase and glutathione reductase by DEAE-TSK chromatography of pooled AcA54 fractions (see Fig. 1). The activity (×100) of HMW reductase (●) was determined by the DTNB assay in the presence of the LMW protein (see Materials and Methods). Glutathione reductase (GR) (○) was assayed with GSSG as a substrate (see Materials and Methods).

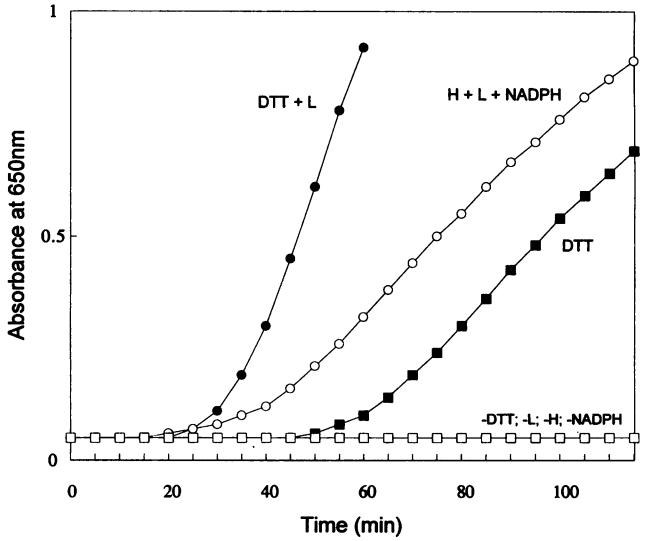

FIG. 3. Reduction of disulfide bonds in insulin by the *P. chrysogenum* LMW protein with NADPH and the HMW protein or DTT as the source of reducing power. Symbols: ■, 1 mM DTT; ○, 22 μg of LMW protein (L) (DEAE step) plus 60 μg of HMW protein (H) (DEAE step) and NADPH; ●, 22 μg of LMW protein (L) (DEAE step) plus 0.5 mM DTT; □, control experiments in which DTT, the LMW protein, the HMW protein, and NADPH were omitted.

levels of activity were highest for DTNB and progressively lower for CoASSCoA, bis-ACV, CoASSG, and CysGlySSCys-Gly, in that order, with apparent K_m values of 1.4, 83, 125, 125, and 800 μM, respectively. The specific activity of disulfide reductase (the DEAE step) was 2.3-fold higher for bis-ACV than for GSSG, 0.129 and 0.056 μmol of NADPH oxidized per mg of protein per min, respectively. In contrast, the specific activity of glutathione reductase was 70-fold higher for GSSG than for bis-ACV, 5.78 and 0.082 μmol of NADPH oxidized per mg of protein per min, respectively.

The LMW component of the purified *P. chrysogenum* disulfide reductase stimulates the reduction of disulfide bonds in bovine insulin by DTT. Figure 3 shows the effect of the LMW protein on the dissociation of the two polypeptide chains of insulin. The increase in turbidity is due to precipitation of the free insulin B chain. By itself, DTT reduces insulin disulfides at a characteristic rate. However, in the presence of the LMW protein, the rate of insulin disulfide reduction is considerably enhanced, much in the same manner as that which occurs with thioredoxins (14) (leftmost curve). Moreover, NADPH and the HMW protein are able to effectively substitute for DTT as the source of reducing power in that reaction, and when any one of the LMW, HMW, and NADPH components is omitted, the rate of insulin disulfide reduction falls to the background level. These experiments show that the LMW component of the *P. chrysogenum* general disulfide reductase is a protein disulfide reductant.

Ability of the *P. chrysogenum* HMW and LMW proteins to accept and to serve as substrates for other thioredoxins and thioredoxin reductases, respectively. The *P. chrysogenum* HMW protein was tested for disulfide reductase activity with *E. coli*, *S. platensis*, and *S. clavuligerus* thioredoxins as substrates, as well as the *P. chrysogenum* LMW protein, and with DTNB as an electron acceptor. In the presence of saturating amounts of NADPH, the fungal HMW protein partially accepted the *E. coli* and *S. platensis* thioredoxins, with apparent K_m values of 27 and 65 μM, respectively, compared with 0.27 μM for its cognate LMW protein, but surprisingly failed to accept the *S. clavuligerus* thioredoxin. In contrast, the *P. chrysogenum* LMW protein was unable to serve as a substrate for either the *E. coli* or the *S. clavuligerus* HMW reductase.

The in vitro biosynthesis of isopenicillin N from bis-ACV is driven by the *P. chrysogenum* general disulfide reductase and IPNS. The conversion of ACV to isopenicillin N by IPNS is strictly dependent on its existing in the free thiol form. bis-ACV is not a substrate for IPNS. To determine whether bis-ACV is cyclized to isopenicillin N by the concerted action of disulfide reductase and IPNS, reactions were carried out in the presence of the complete enzymatic system, the LMW and HMW proteins, and NADPH, and the synthetic DTT reductant was omitted. The composition of the enzymatic reaction mixture was as described in Materials and Methods. To 70 μl of the reaction mixture, 16.8 μg of highly purified recombinant IPNS was added. The latter was obtained by expressing the *Flavobacterium* sp. strain 12.154 IPNS gene in *E. coli* (21). The LMW protein (8 μg of protein) was from the DEAE purification step; the HMW protein (39.6 μg of protein) was from the DEAE purification step. Incubation was done for 30 min at 25°C. Antibiotic production was measured in a bioassay with *M. luteus* as the indicator organism (see Materials and Methods and reference 16). In the presence of the HMW and LMW proteins and NADPH, 72 μg of isopenicillin N per ml was detected; none was detected in the presence of the HMW and LMW proteins, the HMW protein and NADPH, or the LMW protein and NADPH. In a control experiment in which the disulfide reductase system was replaced with 5 mM DTT, the amount of isopenicillin N obtained was 250 μg/ml.

Cloning of the *P. chrysogenum* HMW reductase gene. To isolate the gene coding for the HMW component of the *P. chrysogenum* general disulfide reductase, the purified protein was subjected to Edman degradation and the NH_2-terminal portion was sequenced. The information was used to construct a mixed degenerate oligodeoxynucleotide probe for screening by hybridization with a phage λ EMBL-3 genomic library. The sequences of the HMW peptide and the mixed oligodeoxynucleotide probe are as follows:

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(Val) (His) Ser Lys Val Val Ile Ile Gly Ser Gly Pro Gly Ala His
                                        5'-GGA CCI GGA GCI CAC
                                            C       C       G
                                            T       T

Thr Ala Ala Ile Tyr Leu Ser (Arg) Ala Glu Leu Gln Pro-
ACI GCI GCI ATC GC-3'
        T
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Approximately 9×10^3 recombinant phage plaques were transferred to nitrocellulose filters and hybridized with the 29-mer inosine-containing probe, and eight positively responding phages were recovered. Two further screens were used to purify the recombinant phages, and five were selected for detailed analysis. Southern analysis of the DNA of one phage showed that a 4.7-kb *Sal*I fragment hybridized to both the 29-mer probe and plasmid pMY-8, which has the entire HMW thioredoxin reductase gene (*trxB*) of the *S. clavuligerus* disulfide reductase system. That fragment was cloned into plasmid vector pUC19, and a restriction map was constructed (Fig. 4B). Further subcloning showed that a 0.5-kb *Bam*HI-*Hin*dIII fragment hybridized to the oligodeoxynucleotide probe. The nucleotide sequence of the 0.5-kb *Bam*HI-*Hin*dIII fragment was determined and found to contain an open reading frame with a deduced amino acid sequence that matched the NH_2-terminal portion of the HMW protein, but with the HMW protein initiation methionine removed. The remaining segment of the gene, coding for the carboxy-terminal portion of the HMW protein, resides in an overlapping 1.3-kb *Eco*RV fragment. Figure 4B shows the restriction map of the 1.4-kb *Bam*HI-*Eco*RV fragment containing the entire HMW reductase gene and the strategy used for sequencing. Figure 5 shows the nucleotide sequence of that fragment. It includes 118 nucleotides upstream of the ATG translational initiation codon, 1,150 nucleotides representing the HMW reductase gene, and 153 nucleotides downstream of the chain termination codon. Two introns, denoted I and II and containing 83 and 65 nucleotides, respectively, are located in the amino- and carboxy-terminal coding parts of the gene, respectively. Consensus fungal intron-exon junction sequences and lariat branch sites are found in each case (11). The predicted amino acid sequence of the intronless gene codes for a polypeptide of 334 amino acids (including the initiation methionine). The calculated molecular weight of that protein is 35,643 and is

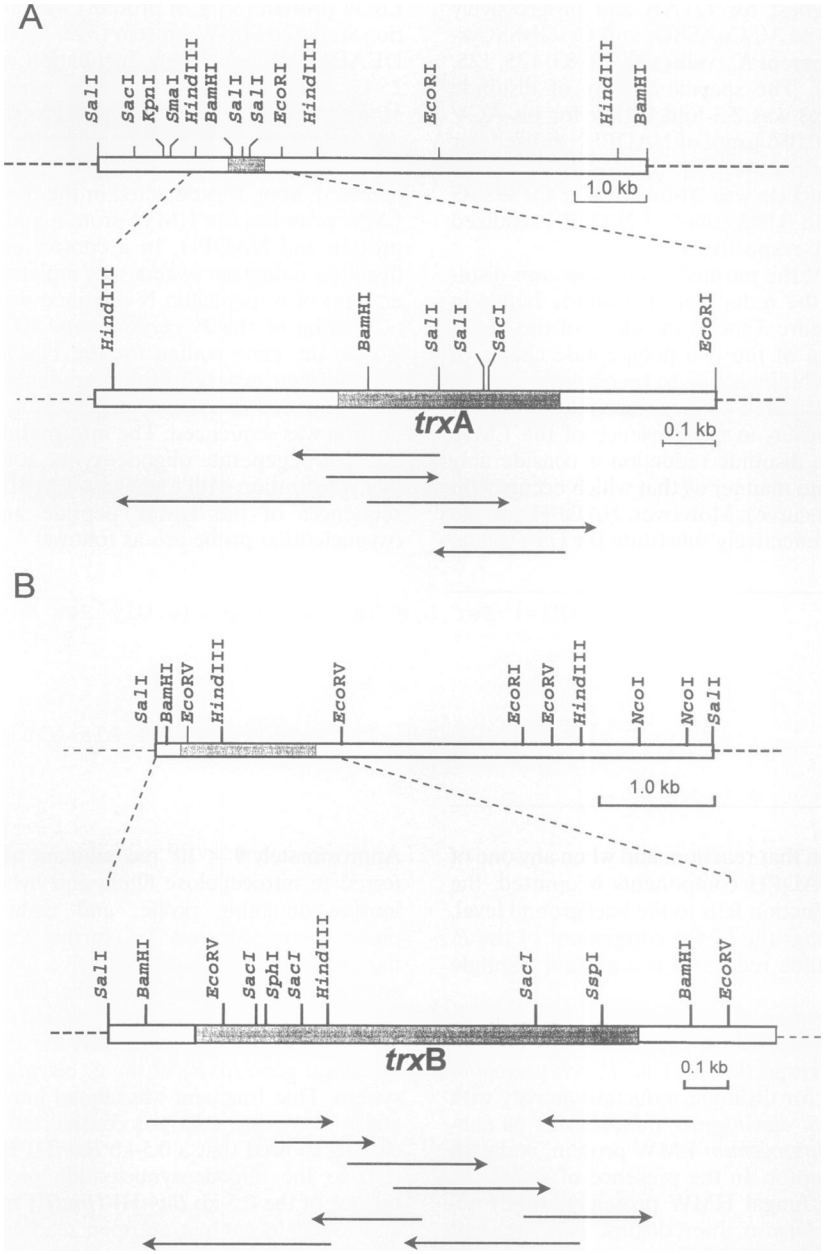

FIG. 4. Restriction maps of and nucleotide sequencing strategy for the LMW (*trxA*) (A) and HMW (*trxB*) (B) genes of *P. chrysogenum*.

in good agreement with that found for the HMW protein by electrophoresis in denaturing gels.

Amino acid sequence comparison of the *P. chrysogenum* HMW protein with thioredoxin disulfide reductases. Figure 6 shows the alignment of the predicted amino acid sequence of the *P. chrysogenum* HMW reductase, from this work, with the sequences of the only other thioredoxin reductases that have been reported, those of *E. coli* (37) and *S. clavuligerus* (6). All three enzymes are closely related. The fungal protein shares 45.5 and 44% sequence identities with the *E. coli* and *S. clavuligerus* enzymes, respectively; the bacterial enzymes share 48.7% sequence identity. Lesser but significant sequence similarity occurs with the 34.2-kDa protein encoded by the rubredoxin operon of *Clostridium pasteurianum*, the NADPH dehydrogenase of a *Bacillus* sp., and the F52a protein of *Salmonella typhimurium* alkyl hydroperoxide reductase (36.6, 30.2, and 30.6% identities, respectively). The *P. chrysogenum* HMW reductase has a redox active site (-CAVC-) similar to that present in the *E. coli* and *S. clavuligerus* thioredoxin reductase (-CATC-) and located in the same position in the polypeptide. Also, the *P. chrysogenum* HMW reductase and the bacterial thioredoxin reductases possess three peptide regions that share extensive sequence similarity, and these are shown shaded in Fig. 6. Related sequences occur in other NADPH-dependent disulfide reductases, including glutathione reductase, mercuric reductase, dihydrolipoamide dehydrogenase, trypanothione reductase, and alkyl hydroperoxide reductase. The function of these peptide regions is discussed below.

Cloning of the *P. chrysogenum* LMW protein gene. The sequences of the NH_2-terminal portion of the LMW protein and

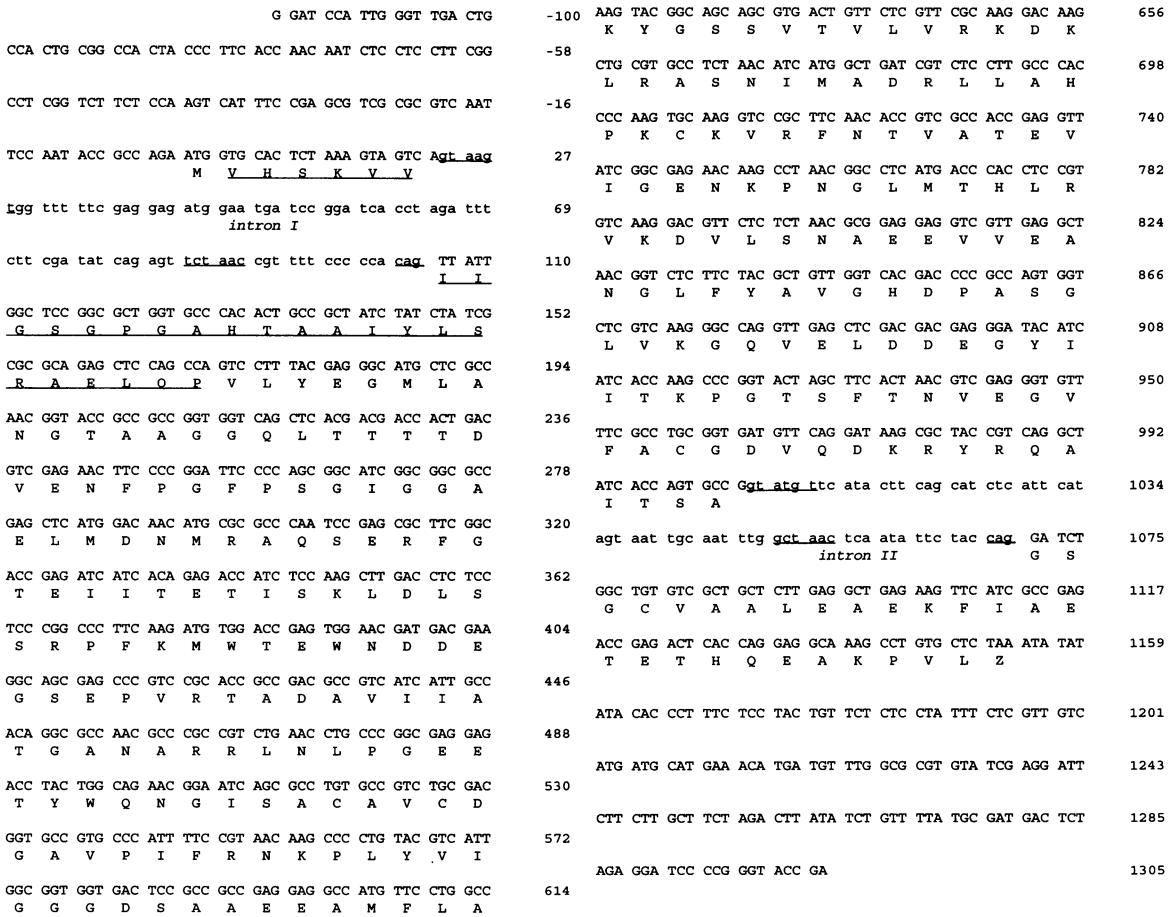

FIG. 5. Nucleotide sequence of the *P. chrysogenum* HMW gene (*trxB*). Nucleotides are numbered from the ATG start codon (A in ATG = 1) and are shown in the right margin. Introns are indicated by lowercase letters, and consensus internal splicing sites and branch sites are underlined. The deduced amino acid sequence is presented below the nucleotide sequence. The NH_2-terminal region of the HMW protein that was sequenced is shown by a line beneath the deduced sequence.

the mixed degenerate oligodeoxynucleotide probe (complementary strand) used for isolating the LMW protein gene are as follows:

```
(Gly) Val Thr Pro Ile Lys Ser Val Ala Glu Tyr Lys Glu Lys
                              3'-CAI CGI CTT ATG TTT CTT TTT
                                       C   A   C   C   C

Val Thr Asp Ala Thr Gly Pro Val Val Val Asp Phe His Ala
CAI TGI CT-5'

Thr Trp Xaa Gly Pro Xaa Lys Ile Ala Pro-
```

Southern analysis of total chromosomal DNA of *P. chrysogenum* with the 29-mer inosine-containing probe identified at a low hybridization stringency a 4.0-kb *Sal*I fragment (data not shown). The same hybridization conditions were used to screen the λ EMBL-3 genomic library, and four positively responding recombinant phage plaques were recovered. After purification, the DNA of one phage was found to contain a 0.65-kb *Sal*I-*Hin*dIII fragment that hybridized to the probe and that was subcloned into vector pUC19. The nucleotide sequence of that fragment was determined and found to contain an open reading frame with a deduced amino acid sequence that matched the NH_2-terminal portion of the LMW protein, but with the LMW protein initiation methionine removed. In parallel, a 5.8-kb *Bam*HI fragment was subcloned from the same phage and shown by Southern analysis to contain a 0.7-kb *Bam*HI-*Eco*RI fragment that hybridized to the 29-mer probe and that overlapped the 0.65-kb *Sal*I-*Hin*dIII fragment. Figure 4A shows the restriction map of the *Hin*dIII-*Eco*RI region containing the entire LMW protein gene and the strategy used for sequencing. Figure 7 shows the nucleotide sequence of that region. It includes 383 nucleotides upstream of the ATG translational initiation codon, 457 nucleotides representing the LMW protein gene, and 74 nucleotides downstream of the

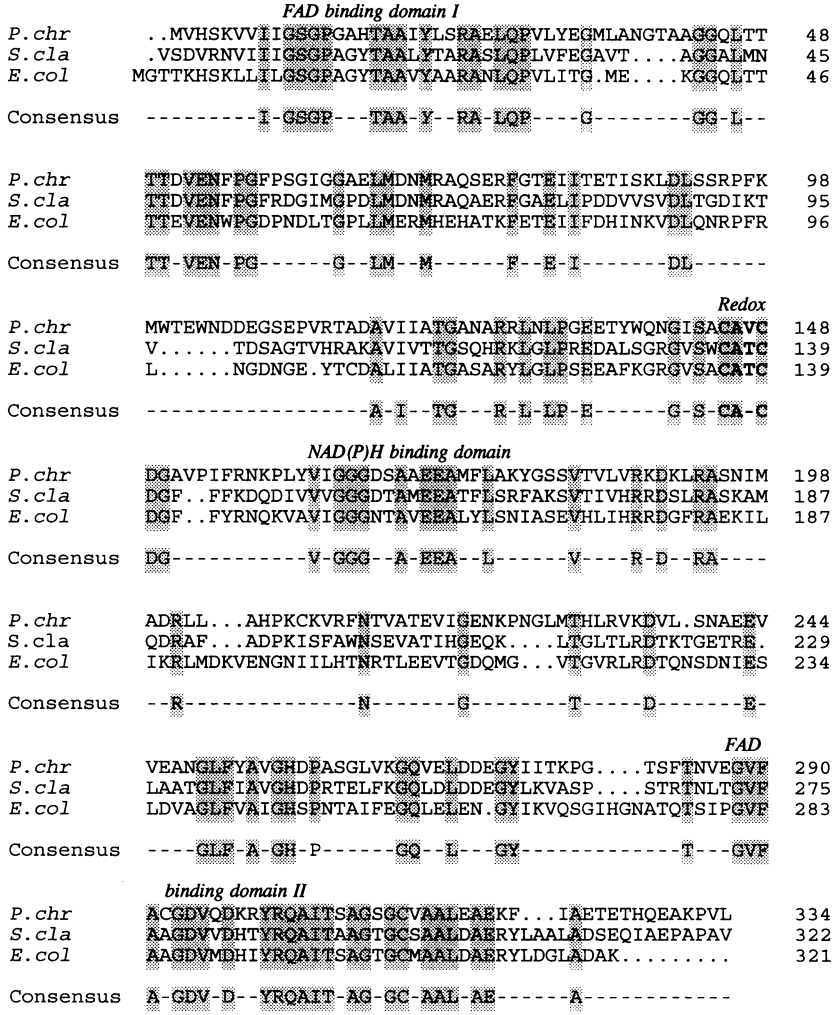

FIG. 6. Amino acid sequence alignment of the *P. chrysogenum* (*P.chr*) HMW reductase and the *E. coli* (*E.col*) (37) and *S. clavuligerus* (*S.cla*) (6) thioredoxin reductases. Identical amino acids occurring at the same position in all three aligned sequences are shown shaded and provide the consensus sequence depicted below. Redox active-site cysteines are shown in boldface type.

chain termination codon. The LMW protein gene contains a single intron of 139 nucleotides located in the NH_2-terminal coding part of the gene. It has the consensus intron-exon junction sequences and lariat branch sites found in fungal genes (11). Other features of this gene are two promoter-like motifs in the 5′-untranslated region, TATAAA at −204 and CCAATT at −350 (11). The predicted amino acid sequence of the intronless gene contains 106 residues (including the initiation methionine) with a calculated molecular weight of 11,333, in good agreement with that found for the LMW protein by electrophoresis in denaturing gels.

Amino acid sequence comparison of *P. chrysogenum* LMW protein with thioredoxins. Figure 8 shows the alignment of the predicted amino acid sequence of the *P. chrysogenum* LMW protein, from this work, with sequences of selected thioredoxins from eucaryotic and procaryotic sources. The latter were obtained from a computer search of protein data banks for sequences related to that of the LMW protein. Thioredoxins contain 106 to 112 amino acids, have a highly conserved redox active region (-D^{30}XXAXWCGPCK/RXXXP^{44}-) (numbering is that of the consensus sequence), and share five other totally conserved residues. The *P. chrysogenum* LMW protein is clearly a thioredoxin. It possesses 106 amino acids, has the identical redox active-site cysteine dithiol of thioredoxins, which is located in the same position in the polypeptide chain, and has each of the five other conserved residues.

DISCUSSION

P. chrysogenum possesses an NADPH-dependent disulfide reductase that has broad substrate specificity and that is effective in reducing disulfides in LMW compounds and proteins. When coupled in vitro with IPNS, it converts bis-ACV to isopenicillin N. We previously proposed that because IPNS reactions occur in vitro only when ACV is in its reduced thiol form, β-lactam-producing organisms may employ a disulfide reductase system to maintain intracellular ACV in its reduced state (2, 6). The broad-range disulfide reductase described in this report may therefore play an important role in promoting penicillin production in *P. chrysogenum*.

The properties of the *P. chrysogenum* general disulfide reductase are incompatible with it being glutathione reductase or any other glutathione-like disulfide reductase. Thus, DTNB, CoASSCoA, and bis-ACV are not substrates or are very poor

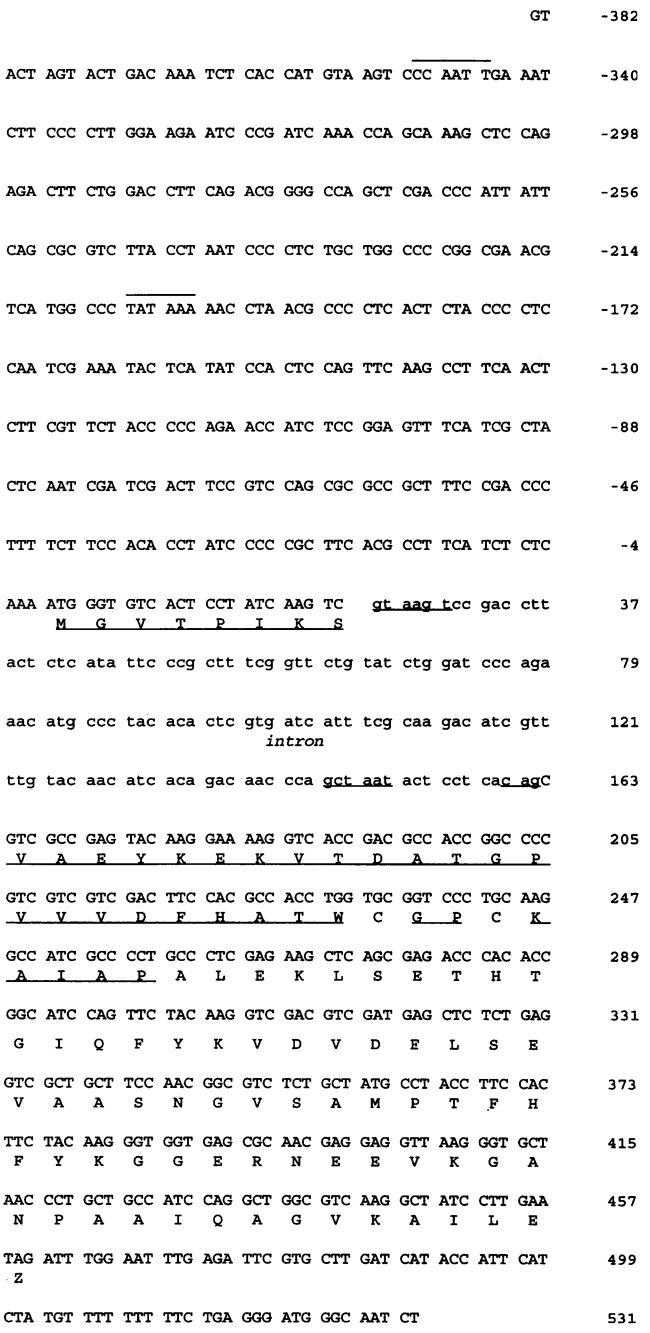

FIG. 7. Nucleotide sequence of the *P. chrysogenum* LMW (*trxA*) gene. Details are as given in the legend to Fig. 5. Two transcription promoter-like motifs in the 5′-untranslated region upstream of the structural gene are overlined.

substrates for glutathione reductase. In addition, glutathione reductase consists of a single protein component (38), whereas the *P. chrysogenum* broad-range disulfide reductase is composed of two nonidentical protein components. The HMW reductase component is a 35-kDa protein that forms dimers. In contrast, the glutathione reductase from *P. chrysogenum* (43) is composed of two 55-kDa subunits. The LMW component is a heat-stable 12-kDa protein that is reduced by the HMW protein and that is a general disulfide reductant. In all these properties, the *P. chrysogenum* disulfide reductase closely resembles the thioredoxin-thioredoxin reductase systems of *E. coli* (15) and *S. clavuligerus* (2). The finding that the *P. chrysogenum* HMW reductase partially accepts the *E. coli* and *S. platensis* thioredoxins supports this view. Surprisingly, the *P. chrysogenum* HMW reductase was unable to accept as a substrate the *S. clavuligerus* thioredoxin, and the *P. chrysogenum* LMW component failed to recognize either of the bacterial thioredoxin reductases. The fact that *P. chrysogenum* contains glutathione reductase led us to consider the possibility that the bis-ACV disulfide reductase activity described in this work is a glutaredoxin-based system. Glutaredoxin is a small disulfide redox active-site protein that is reduced specifically by glutathione (16). However, the biochemical evidence summarized above and the amino acid sequence analysis (see below) rule out this idea. Furthermore, although glutaredoxins (thioltransferases) have been considered to be a class of relatively nonspecific general catalysts of thiol-disulfide exchange reactions, more recent work (10) shows that they possess a restrictive substrate specificity involving a requirement for mixed disulfides containing the glutathione moiety.

To verify that the LMW and HMW components of the *P. chrysogenum* general disulfide reductase are homologs of thioredoxin and thioredoxin reductase, respectively, the genes encoding these proteins were cloned and sequenced. Amino acid sequence analysis showed that the *P. chrysogenum* HMW reductase is closely related to the thioredoxin reductases of *E. coli* and *S. clavuligerus* (Fig. 6). The enzymes possess 334, 321, and 322 amino acids (counting the initiation amino acids), respectively, share 44 to 48.7% amino acid sequence identity, and contain essentially the same catalytic redox active site and FAD and NADPH dinucleotide binding domains. They clearly differ from other NADPH-dependent disulfide reductases, glutathione reductase, dihydrolipoamide dehydrogenase, trypanothione reductase, and mercuric reductase, which are much larger, share limited sequence similarity with each other and with the *P. chrysogenum* HMW reductase and the bacterial thioredoxin reductases, and have rather different redox active regions (6). The consensus dithiol redox active region of the former is -CV/LNVI/GC- (1, 37), and that of the latter is -CAT/VC- (6, 37; this work). We conclude that the HMW component of the *P. chrysogenum* disulfide reductase is a thioredoxin reductase, and the gene coding for it is therefore denoted *trxB*, in accordance with the convention used for the *E. coli* thioredoxin reductase gene (12). This is the first reported example of the cloning of a eucaryotic thioredoxin reductase gene.

Amino acid sequence analysis of the *P. chrysogenum* LMW protein showed that it is closely related to procaryotic and eucaryotic thioredoxins. Although thioredoxins share limited overall sequence similarity, 26 to 58%, they all possess the dithiol amino acid sequence-WCGPC-, containing the catalytic redox active site as well as several other highly conserved residues. The *P. chrysogenum* LMW protein has these features and is most similar to the thioredoxins of *Saccharomyces cerevisiae* and *Aspergillus nidulans*, with which it shares 53.5 and 59.6% sequence identities, respectively (Fig. 8). It shows no significant similarity to *E. coli* and calf thymus glutaredoxins. These observations as well as the biochemical properties of the LMW protein allow us to conclude that it is a thioredoxin. The gene coding for the *P. chrysogenum* LMW protein is therefore denoted *trxA*, in accordance with the convention used for the *E. coli* thioredoxin gene (30).

The nucleotide sequences of the *P. chrysogenum trxA* and *trxB* genes show that both contain introns; the *trxA* gene consists of two exons and one intron, while the *trxB* gene possesses three exons separated by two introns. In each case,

FIG. 8. Amino acid sequence alignment of the *P. chrysogenum* LMW protein and 13 thioredoxins from eucaryotic and procaryotic sources, obtained by the CLUSTAL V and FASTA programs (26). The redox active region is shown in boldface type. Conserved amino acids are shown below in the consensus sequence; similar amino acids are indicated by asterisks. X denotes an unknown amino acid. The percentages of pairwise amino acid sequence identities for the thioredoxins and the LMW protein of *P. chrysogenum* are shown at the ends of the sequences. Data were obtained from the indicated sources: *Chromatium vinosum* (18); *E. coli* (25); *Corynebacterium nephridii* (31); *Anabaena* sp. strain 7199 (24); *Bacillus subtilis* (5); *S. clavuligerus* (6); rat (40); human (42); chicken (19); *S. cerevisiae* (8); *A. nidulans* (22); *Chlamydomonas reinhardtii* CH1 (7); and *P. chrysogenum* (this work).

intron-exon junction sequences and lariat branch sites correspond well with the consensus sequences reported for fungal genes (11). The only previous report of introns in thioredoxin genes is for the human *trxA* gene, which is 13 kb in size and contains five exons encoding a 12-kDa protein (40).

We previously argued that the thioredoxin oxidoreductase system may support the synthesis of penicillins and cephalosporins in β-lactam-producing *Streptomyces* spp. by maintaining the intracellular level of reduced ACV (2). In most eucaryotic organisms, the thiol-disulfide redox balance within the cell is thought to be governed by glutathione, present in millimolar levels, and its associated enzymes and is strongly poised to the reduced state (29). Because streptomycetes lack glutathione and glutathione reductase (35), we suggested that their thioredoxin system may have a similar function. In addition, we noted that thioredoxin could potentially be involved in supporting β-lactam synthesis through its ability to modulate the thiol-disulfide redox state of functionally important cysteine residues in ACV synthetase and IPNS (4). Thus, the activities of ACV synthetase and IPNS, the two early enzymes in the β-lactam biosynthetic pathway, and ACV, their respective product and substrate, may be affected by the thiol-disulfide redox state of the cell.

The potential involvement of disulfide reductase systems in the biosynthesis of penicillins in *P. chrysogenum* may differ in two important ways from that in streptomycetes. Firstly, *P. chrysogenum* contains both glutathione (and presumably glutaredoxin) and thioredoxin redox active disulfide reductase systems, whereas streptomycetes have only the thioredoxin system. At present we know little about the regulatory mechanisms operating on these reductases and less about their involvement in the synthesis of antibiotics. Secondly, in *P. chrysogenum*, the three penicillin biosynthetic enzymes, ACV synthetase, IPNS, and acyltransferase (AT), are physically compartmentalized in the cell (23, 33, 34), whereas in the procaryotic streptomycetes, they appear to be uniformly distributed. The role of disulfide reductase systems in penicillin synthesis in *P. chrysogenum* might therefore be dependent on the redox state which exists at these intracellular sites and which could be a function of the physiological state of the organism. Because antibiotic production typically occurs at a late stage during the growth cycle and in response to nutrient imbalance, the activities of the cellular disulfide reductase systems at this stage and at these different sites are of special interest. These considerations warrant an analysis of the individual roles of the glutathione and thioredoxin systems in penicillin production in *P. chrysogenum* and their regulation. They also point to the possibility of improving β-lactam yields

by increasing expression, for example, of the thioredoxin system. If successful, it would provide an example of the use of pathway engineering of regulatory genes for rational improvement of strains producing β-lactam antibiotics.

ACKNOWLEDGMENTS

We thank Yossi Av-Gay for advice regarding protein sequence analysis.

REFERENCES

1. **Aboagye-Kwarteng, T., K. Smith, and A. H. Fairlamb.** 1992. Molecular characterization of the trypanothione reductase gene from *Crithidia fasciculata* and *Trypanosoma brucei*: comparison with other flavoprotein disulphide oxidoreductases with respect to substrate specificity and catalytic mechanism. Mol. Microbiol. **6:**3089–3099.
2. **Aharonowitz, Y., Y. Av-Gay, R. Schreiber, and G. Cohen.** 1993. Characterization of a broad-range disulfide reductase from *Streptomyces clavuligerus* and its possible role in beta-lactam antibiotic biosynthesis. J. Bacteriol. **175:**623–629.
3. **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.
4. **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. Actinomycetes.
5. **Chen, N. Y., J. J. Zhang, and H. Paulus.** 1989. Chromosomal location of the *Bacillus subtilis* aspartokinase II gene and nucleotide sequence of the adjacent genes homologous to uvrC and trx of *Escherichia coli*. J. Gen. Microbiol. **135:**2931–2940.
6. **Cohen, G., M. Yanko, M. Mislovati, A. Argaman, R. Schreiber, Y. Av-Gay, and Y. Aharonowitz.** 1993. Thioredoxin-thioredoxin reductase system of *Streptomyces clavuligerus*: sequences, expression, and organization of the genes. J. Bacteriol. **175:**5159–5167.
7. **Decottignies, P., J. M. Schmitter, S. Dutka, J. P. Jacquot, and M. Miginiac-Maslow.** 1991. Characterization and primary structure of a second thioredoxin from the green alga, *Chlamydomonas reinhardtii*. Eur. J. Biochem. **198:**505–512.
8. **Gan, Z. R.** 1991. Yeast thioredoxin genes. J. Biol. Chem. **266:**1692–1696.
9. **Gouka, R., W. van Hartingsveldt, R. A. Bovenberg, A. M. Cees, J. J. van den Hondel, and F. M. van Gorcom.** 1991. Cloning of the nitrate-nitrite reductase gene cluster of *Penicillium chrysogenum* and use of the *nia*D gene as a homologous selection marker. J. Biotechnol. **20:**189–200.
10. **Gravina, S. K., and J. J. Mieyal.** 1993. Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. Biochemistry **32:**3368–3376.
11. **Gurr, S., S. Unkles, and J. Kinghorn.** 1987. The structure and organization of nuclear genes of filamentous fungi, p. 93–139. *In* J. Kinghorn (ed.), Gene structure in eukaryotic microbes. IRL Press, Oxford.
12. **Haller, B. L., and J. A. Fuchs.** 1984. Mapping of *trxB*, a mutation responsible for reduced thioredoxin reductase activity. J. Bacteriol. **159:**1060–1062.
13. **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.
14. **Holmgren, A.** 1979. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J. Biol. Chem. **254:**9627–9632.
15. **Holmgren, A.** 1985. Thioredoxin. Annu. Rev. Biochem. **54:**237–271.
16. **Holmgren, A.** 1989. Thioredoxin and glutaredoxin systems. J. Biol. Chem. **264:**13963–13966.
17. **Jensen, S. E., B. K. Leskiw, L. C. Vining, Y. Aharonowitz, D. W. Westlake, and S. Wolfe.** 1986. Purification of isopenicillin N synthetase from *Streptomyces clavuligerus*. Can. J. Microbiol. **32:**953–958.
18. **Johnson, R. S., and K. Biemann.** 1987. The primary structure of thioredoxin from *Chromatium vinosum* determined by high-performance tandem mass spectrometry. Biochemistry **26:**1209–1214.
19. **Jones, S. W., and K. C. Luk.** 1988. Isolation of a chicken thioredoxin cDNA clone. Thioredoxin mRNA is differentially expressed in normal and Rous sarcoma virus-transformed chicken embryo fibroblasts. J. Biol. Chem. **263:**9607–9611.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Landman, O., D. Shiffman, Y. Av-Gay, Y. Aharonowitz, and G. Cohen.** 1991. High level expression in *Escherichia coli* of isopenicillin N synthase genes from *Flavobacterium* and *Streptomyces*, and recovery of active enzyme from inclusion bodies. FEMS Microbiol. Lett. **68:**239–244.
22. **Le Marechal, P., B. M. Hoang, J. M. Schmitter, A. Van Dorsselaer, and P. Decottignies.** 1992. Purification, properties and primary structure of thioredoxin from *Aspergillus nidulans*. Eur. J. Biochem. **210:**421–429.
23. **Lendenfeld, T., D. Ghali, M. Wolschek, E. M. Kubicek-Pranz, and C. P. Kubicek.** 1993. Subcellular compartmentation of penicillin biosynthesis in *Penicillium chrysogenum*. The amino acid precursors are derived from the vacuole. J. Biol. Chem. **268:**665–671.
24. **Lim, C. J., D. Geraghty, and J. A. Fuchs.** 1985. Cloning and nucleotide sequence of the *trxA* gene of *Escherichia coli* K-12. J. Bacteriol. **163:**311–316.
25. **Lim, C. J., F. K. Gleason, and J. A. Fuchs.** 1986. Cloning, expression, and characterization of the *Anabaena* thioredoxin gene in *Escherichia coli*. J. Bacteriol. **168:**1258–1264.
26. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227:**1435–1441.
27. **Luthman, M., and A. Holmgren.** 1982. Rat liver thioredoxin and thioredoxin reductase: purification and characterization. Biochemistry **21:**6628–6633.
28. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Mannervik, B., I. Carlberg, and K. Larson.** 1988. Glutathione: general review of mechanism of action, p. 475–516. *In* D. Dolphin, R. Poulson, and O. Avramovic (ed.), Glutathione, chemical, biochemical and medical aspects, part A. John Wiley & Sons, Inc., New York.
30. **Mark, D. F., J. W. Chase, and C. C. Richardson.** 1977. Genetic mapping of trxA, a gene affecting thioredoxin in *Escherichia coli* K12. Mol. Gen. Genet. **155:**145–152.
31. **Meng, M., and H. P. Hogenkamp.** 1981. Purification, characterization, and amino acid sequence of thioredoxin from *Corynebacterium nephridii*. J. Biol. Chem. **256:**9174–9182.
32. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
33. **Muller, W. H., R. A. Bovenberg, M. H. Groothuis, F. Kattevilder, E. B. Smaal, L. H. Van der Voort, and A. J. Verkleij.** 1992. Involvement of microbodies in penicillin biosynthesis. Biochim. Biophys. Acta **1116:**210–213.
34. **Muller, W. H., T. P. van der Krift, A. J. Krouwer, H. A. Wosten, L. H. Van der Voort, E. B. Smaal, and A. J. Verkleij.** 1991. Localization of the pathway of penicillin biosynthesis in *Penicillium chrysogenum*. EMBO J. **10:**489–495.
35. **Newton, G. L., R. C. Fahey, G. Cohen, and Y. Aharonowitz.** 1993. Low-molecular-weight thiols in streptomycetes and their potential role as antioxidants. J. Bacteriol. **175:**2734–2742.
36. **Perry, D., E. P. Abraham, and J. E. Baldwin.** 1988. Factors affecting the isopenicillin N synthetase reaction. Biochem. J. **255:**345–351.
37. **Russel, M., and P. Model.** 1988. Sequence of thioredoxin reductase from *Escherichia coli*. Relationship to other flavoprotein disulfide oxidoreductases. J. Biol. Chem. **263:**9015–9019.
38. **Schrimer, R., H. Krauth-Siegel, and G. 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.
39. **Shiffman, D., M. Mevarech, S. E. Jensen, G. Cohen, and Y. Aharonowitz.** 1988. Cloning and comparative sequence analysis of

the gene coding for isopenicillin N synthase in *Streptomyces*. Mol. Gen. Genet. **214:**562–569.

40. **Tonissen, K. F., and J. R. Wells.** 1991. Isolation and characterization of human thioredoxin encoding genes. Gene **102:**221–228.
41. **Wolfe, S., A. L. Demain, S. E. Jensen, and D. W. Westlake.** 1984. Enzymatic approach to syntheses of unnatural beta-lactams. Science **226:**1386–1392.
42. **Wollman, E. E., L. d'Auriol, L. Rimsky, A. Shaw, J. P. Jacquot, P. Wingfield, P. Graber, and F. Dessarps.** 1988. Cloning and expression of a cDNA for human thioredoxin. J. Biol. Chem. **263:**15506–15512.
43. **Woodin, T. S., and I. H. Segel.** 1968. Isolation and characterization of glutathione reductase from *Penicillium chrysogenum*. Biochim. Biophys. Acta **167:**64–77.