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Effects of Site-Directed Mutations on Processing and Activities of Penicillin G Acylase from *Escherichia coli* ATCC 11105

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Penicillin G acylase from *Escherichia coli* ATCC 11105 is synthesized from its precursor polypeptide into a catalytically active heterodimer via a complex posttranslational processing pathway. Substitutions in the pair of aminoacyl residues at the cleavage site for processing the small and large subunits were made. Their processing phenotypes and penicillin G acylase activities were analyzed. By the introduction of a prolyl residue at either position, the processing of the small subunit was blocked without a change in enzymatic activity. Four other substitutions had no effect. At the site for processing the large subunit, four substitutions out of the seven examined blocked processing. In general, penicillin G acylase activity seemed to be proportional to the efficiency of the large-subunit-processing step. Ser-290 is an amino acid critical for processing and also for the enzymatic activity of penicillin G acylase. In the mutant pAATC, in which Ser-290 is mutated to Cys, the precursor is processed, but there is no detectable enzymatic activity. This suggests that there is a difference in the structural requirements for the processing pathway and for enzymatic activity. Recombination analysis of several mutants demonstrated that the small subunit can be processed only when the large subunit is processed first. Some site-directed mutants from which signal peptides were removed showed partial processing phenotypes and reduced enzymatic activities. Their expression showed that the prerequisite for penicillin G acylase activity is the efficient processing of the large subunit and that the maturation of the small subunit does not affect the enzymatic activity.

Penicillin G acylase (PGA) is an enzyme that catalyzes the hydrolysis of benzylpenicillin to give 6-aminopenicillanic acid, an intermediate in the production of semisynthetic penicillin (28). PGA from *Escherichia coli* ATCC 11105 is a periplasmic enzyme that is composed of the small subunit and the large subunit. For PGA to reach its catalytically active dimer form in the periplasm, multistep proteolytic processing from a single polypeptide must be accomplished by removing a signal peptide (26 residues) and a spacer peptide (54 residues) (24, 25). The proteolytic cleavage sites are located between the Ala-235–Ala-236 and Thr-289–Ser-290 pairs (18). This maturation mechanism is peculiar to PGA as a bacterial enzyme, though many viral proteins and eucaryotic polypeptide hormones require activation by proteolytic cleavage processes (6, 7). But the enzymes involved in each processing step and their locations within the cell have not yet been identified.

In this report, site-specific mutations at the proteolytic cleavage sites of PGA were constructed in order to obtain the necessary information for cleavage activation, especially (i) the amino acids critical to PGA processing and PGA activity, (ii) the amino acid specificity of each cleavage step, and (iii) the order of the cleavage steps. Through the expression of various recombinant *pga* genes, the characteristics of each processing step were predicted and the relationship between the catalytically active form and the processing steps was able to be discussed.

MATERIALS AND METHODS

Materials. Restriction endonucleases and T4 ligase, DNA polymerase I (Klenow fragment), and T4 polymerase were purchased from KOSCO Biotech or Promega Corp. Most chemicals were purchased from Sigma Chemical or Promega Corp. The isotope [α-^{35}S]dATP was purchased from Amersham Corp. Oligonucleotides were synthesized on a Beckman System 1 Plus DNA synthesizer.

Bacterial strains, plasmids, and growth conditions. The bacterial strains in this study were *E. coli* HB101 (*supE44 hsdS20*(r^- m^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*), JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)/F′ *traD36* $ProA^+B^+$ $lacI^qZ\Delta M15$), and RZ1032 (*dut ung thi-1 relA spoT1 supE44*/F *lysA*).

For the expression of wild-type PGA, pKS63 (4), in which the *pga* gene had been cloned on the pACYC184 vector, was used. M13mp18 derivatives (30) were used for template DNA preparations in mutagenesis experiments. For the construction of a plasmid containing the *pga* structural gene lacking the signal peptide-coding region, pKK233-2 (13) was used. For the replacement of the antibiotic marker with kanamycin, pUC-4K (29) was used.

All strains were grown in Luria broth (15), and the cultivation temperature was kept at 28°C except during DNA preparation. When required, the following antibiotics were added (at the following concentrations): ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), and kanamycin (50 μg/ml).

Construction of the site-directed mutations at the cleavage sites. For mutagenesis, a replicative form of M13 HT was constructed by insertion of a 1.1-kb *Hin*dIII-*Taq*I fragment which contained the small-subunit- and the spacer peptide-coding regions of the *pga* structural gene into *Hin*dIII-*Acc*I-restricted M13mp18. Uracil-containing single-stranded DNA of M13 HT was prepared from the RZ1032 strain, and the mutagenesis protocol was based on that described by Kunkel et al. (10). The sequences of the mutagenic nucleotides are listed in Table 1. Incorporation of the correct

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TABLE 1. Mutants generated by site-directed mutagenesis

Mutant plasmid[a]	Sequence of mutagenic oligonucleotides[b]	Amino acid substitution
pPATS	TCGCAAACTCCAGCTCTGT	Ala-235 → Pro
pTATS	TCGCAAACTACAGCTCTGT	Ala-235 → Thr
pSATS	TCGCAAACTTCAGCTCTGT	Ala-235 → Ser
pAPTS	CAAACTGCACCTCTGTTGC	Ala-236 → Pro
pATTS	CAAACTGCAACTCTGTTGC	Ala-236 → Thr
pASTS	CAAACTGCATCTCTGTTGC	Ala-236 → Ser
pAASS	ATCCAACGAGCAGCAATAT	Thr-289 → Ser
pAAGS	TATCCAACGGGCAGCAATA	Thr-289 → Gly
pAACS	TATCCAACGTGCAGCAATA	Thr-289 → Cys
pAATT	CAACGACCACCAATATGTG	Ser-290 → Thr
pAATR	CCAACGACCCGCAATATGT	Ser-290 → Arg
pAATG	CCAACGACCGGCAATATGT	Ser-290 → Gly
pAATC	CCAACGACCTGCAATATGT	Ser-290 → Cys

[a] See the text for details.
[b] Sequences of mutagenic primers are written 5' to 3'. Underlined nucleotides denote differences from the wild-type sequence.

mutant sequence in M13 HT derivatives was verified by dideoxy sequencing (21) using the primer 5' GCACAATT TGCACAGGGT 3' for mutations at the small-subunit cleavage site and the primer 5' GCCGTACAAGAGAGTAACT 3' for mutations at the large-subunit cleavage site. Each mutant plasmid was constructed by removing the 1.1-kb *Hin*dIII-*Acc*I fragment of pKS63 (4) and replacing it with a mutagenized DNA fragment. In order to confirm the construction of the desired mutants, a modified dideoxy terminator method for double-stranded DNA (3) was used for sequencing. Throughout this study, each constructed plasmid was named on the basis of the position of the mutated amino acid as follows. Two amino acids at the small-subunit cleavage site were listed, and then two amino acids at the large-subunit cleavage site followed as one-letter codes. Each mutant plasmid was transformed into *E. coli* JM109 or HB101 to screen for PGA activity and processing phenotype.

Construction of double mutants at the cleavage sites. To construct the double mutant at the small- and large-subunit cleavage sites, the 0.9-kb *Hin*dIII-*Bst*XI DNA fragment containing the mutation at the small-subunit cleavage site was ligated with the 4.1-kb *Hin*dIII-*Bst*XI fragment containing the mutation at the large-subunit cleavage site. The construction of the desired mutant was confirmed by the modified dideoxy terminator method for double-stranded DNA sequencing (3).

Construction of the mutants lacking signal peptides. From the expression vector pKK233-2 (13), the unnecessary 1.7-kb *Eco*RI-*Pvu*II DNA fragment was deleted. To ligate the ATG initiation site-coding region of the pKK233-2 derivative with the N-terminal region of the small subunit lacking a signal peptide in the *pga* structural gene, 663 bp of *Bsp*MI-digested fragment containing the small-subunit-coding region was treated with Klenow fragment and inserted into the *Nco*I-digested pKK233-2 derivative whose cohesive ends were filled in with Klenow fragment. The plasmid with the correctly oriented insert was confirmed by DNA sequencing around the fusion point. The C-terminal portion of the *pga* structural gene containing the spacer peptide- and the large-subunit-coding regions was inserted into that plasmid at the *Eco*RV restriction site. The constructed plasmid was named pJA.

To replace the antibiotic marker with kanamycin from pJA, a 1.2-kb *Pst*I fragment from pUC-4K (29) containing the kanamycin resistance gene was inserted into the *Pst*I site of the beta-lactamase-coding region in pJA. Finally, pJAK was obtained from the kanamycin-resistant transformants. The essential steps in the construction procedure are summarized in the text.

To remove the signal peptides from the mutants at the cleavage sites, after digestion of pJAK with *Sph*I and *Bgl*II, a 1.5-kb DNA fragment containing the wild-type proteolytic cleavage sites was replaced with that of the site-directed mutants.

Determination of PGA activity. The PGA activity of each mutant was determined by the colorimetric assay described by Bomstein and Evans (2). This method depends on measuring the intensity of the color generated by reacting 6-aminopenicillanic acid with *p*-dimethylaminobenzaldehyde to form Schiff's base. The activity is calculated as A_{415} (A_{600} × reaction time) × 100 and then compared with the activity of the wild type (100%). Each sum is the average of at least three independent determinations.

Analysis of processing phenotypes. Active PGA was purified by the method described by Kutzbach and Rauenbusch, with modifications (11). Each subunit was isolated by preparative sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Antisera directed against the small and the large subunits were raised in rabbits. The immunization protocol was as described by Herbert and Kristensen (9). Western blotting (immunoblotting) was carried out by the procedure described by Towbin et al. (27). SDS cell lysates were prepared by resuspending cells in the sample buffer (12) and incubating them at 95°C for 5 min. Equal amounts of cells chosen for their A_{600}s were applied to each lane of a 10% polyacrylamide slab gel throughout.

RESULTS

Effects of the mutations at the small-subunit cleavage site. The two Ala codons at the cleavage site between the small subunit and the spacer peptide were replaced by Pro, Thr, and Ser (Fig. 1A). The cells harboring mutant *pga* genes were analyzed for their PGA activities and processing phenotypes by immunoblotting with the mixture of antisera directed against the small subunit and the large subunit. Figure 1A shows that there is no striking difference in the PGA activity of the wild type and those of the mutants. In processing patterns, the mutants at positions 235 and 236, except the Pro-substituted mutants, appeared to undergo normal proteolytic processing (Fig. 1B). For pPATS and pAPTS, a protein product in addition to the small subunit, which is presumed to be the processing intermediate of the small subunit, was accumulated. The ratio of the intermediate form to the mature form appeared different for two mutants. The amino acids at the small-subunit cleavage site appear to be somewhat tolerant to substantial diversity in the amino acid side chain present at that position. But a severe structural change, such as breakdown of the alpha-helix structure around the cleavage site, may cause a delay in the processing pathway of the small subunit. But such alterations at the small-subunit cleavage site did not affect PGA activity and processing at the large-subunit cleavage site.

Effects of the mutations at the large-subunit cleavage site. Thr-289 at the C terminus of the spacer peptide was replaced by Ser, Gly, and Cys. Ser-290 at the N terminus of the large subunit was replaced by Thr, Arg, Gly, and Cys. Although the mutations at Thr-289 showed different levels of PGA activity, all mutants at Ser-290 lacked PGA activity (Fig. 2A). The processing phenotypes of the altered precursor polypeptides were analyzed (Fig. 2B). While the processing

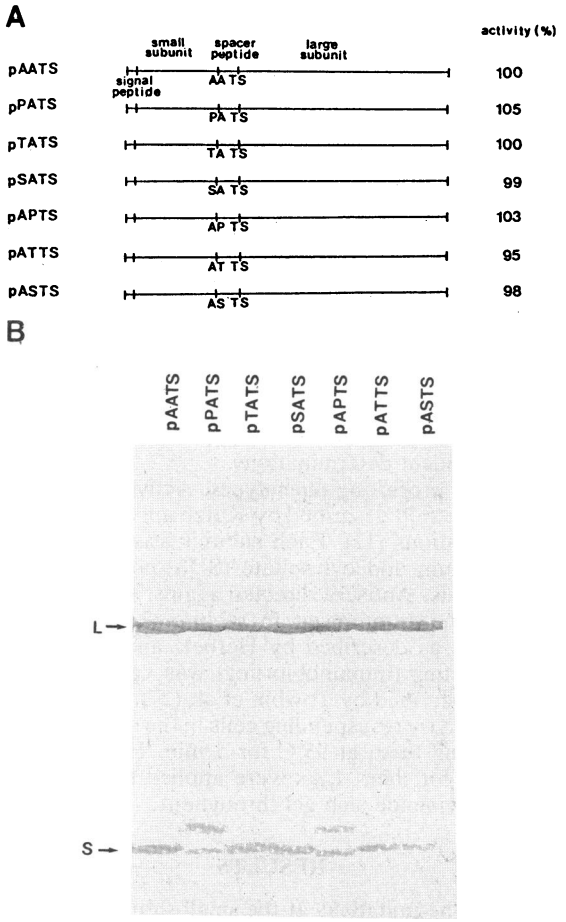

FIG. 1. Structure and PGA activity (A) and immunoblot analysis (B) of Ala-235 and Ala-236 mutants. (A) The PGA activity of each mutant was assayed as described in Materials and Methods. Amino acids at positions 235, 236, 289, and 290 are denoted by one-letter codes. (B) Immunoblot analyses of total cell lysates of *E. coli* JM109 containing the mutant plasmids. L and S mark the positions of the small and the large subunits of PGA, respectively.

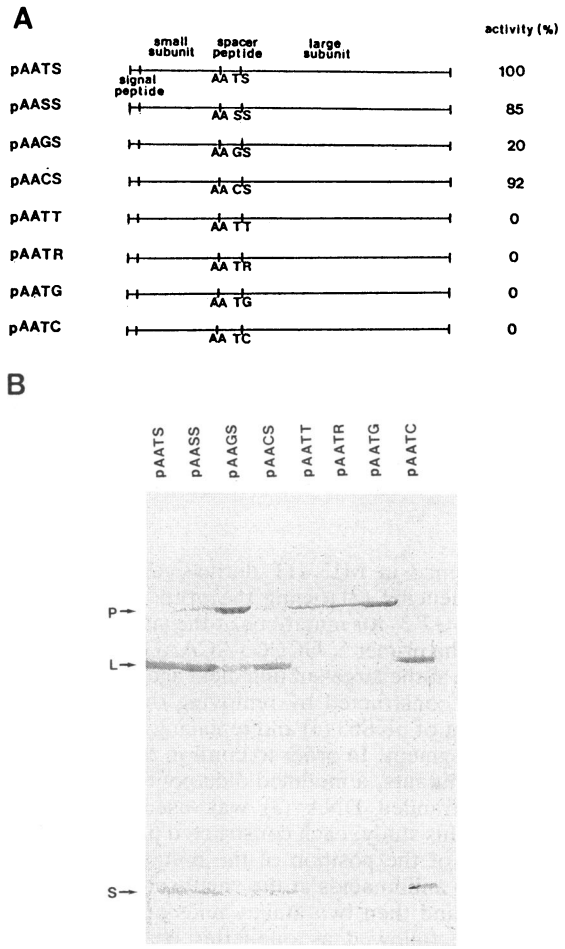

FIG. 2. Structure and PGA activity (A) and immunoblot analysis (B) of Thr-289 and Ser-290 mutants. P, L, and S mark the precursor, the large subunit, and the small subunit, respectively. See the legend to Fig. 1 for further details.

efficiency decreased a little in the cells harboring pAASS and pAACS, in the case of pAAGS, a large amount of precursor was accumulated compared with that in the mature form, and its PGA activity was reduced to 20% of that of the wild type. For the mutations at Ser-290, most substitutions except pAATC completely prevented cleavage of the large subunit from the precursor and resulted in the loss of PGA activity. In the cells harboring pAATT and pAATR, only small amounts of precursor were detected. The possibility that the structural change due to the amino acid substitution could cause the precursor to be more susceptible to nonspecific proteases cannot be excluded. In general, the PGA activity seemed to be proportional to the processing efficiency of the cleavage between the spacer peptide and the large subunit.

Effects of double mutations at two cleavage sites. It was noteworthy that the cells harboring pAATC whose *pga* gene products were fully processed had no PGA activity. This suggests that there is a difference in the structural requirements for the processing pathway and PGA activity. To confirm that the absence of PGA activity in pAATC was caused by the single-amino-acid substitution of Ser-290 with Cys, the double mutants pPATC and pAPTC were constructed as described in Materials and Methods by recombining pAATC with pPATS and pAPTS. The cells harboring pPATS and pAPTS had normal PGA activities, despite the altered processing of the small subunit. But, like those harboring pAATC, the cells harboring pPATC or pAPTC had no PGA activity. The processing phenotype of pPATC was the same as that of pPATS, and that of pAPTC was the same as that of pAPTS (Fig. 3). This result demonstrated that the substitution of Ser-290 with Cys was involved in the loss of PGA activity, in spite of the normal processing of the large subunit. Altered processing of the small subunit caused by a Pro substitution at the small-subunit cleavage site did not influence the processing of the large subunit or PGA activities in these double mutants.

In order to investigate the dependency between the proteolytic cleavage steps, the mutants at the small-subunit cleavage site (pPATS, pTATS, pSATS, pAPTS, pATTS, and pASTS) and the mutants at the large-subunit cleavage site (pAASS and pAATT) were recombined with one another. The PGA activities of the recombinants were affected only by the mutations at the large-subunit cleavage site (Fig. 4A). In the recombinants with pAASS, the large subunits

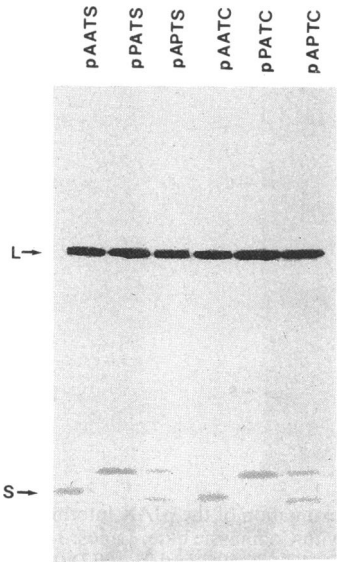

FIG. 3. Immunoblot analysis of the recombinants with pAATC. L and S mark the positions of the small and the large subunits of PGA, respectively.

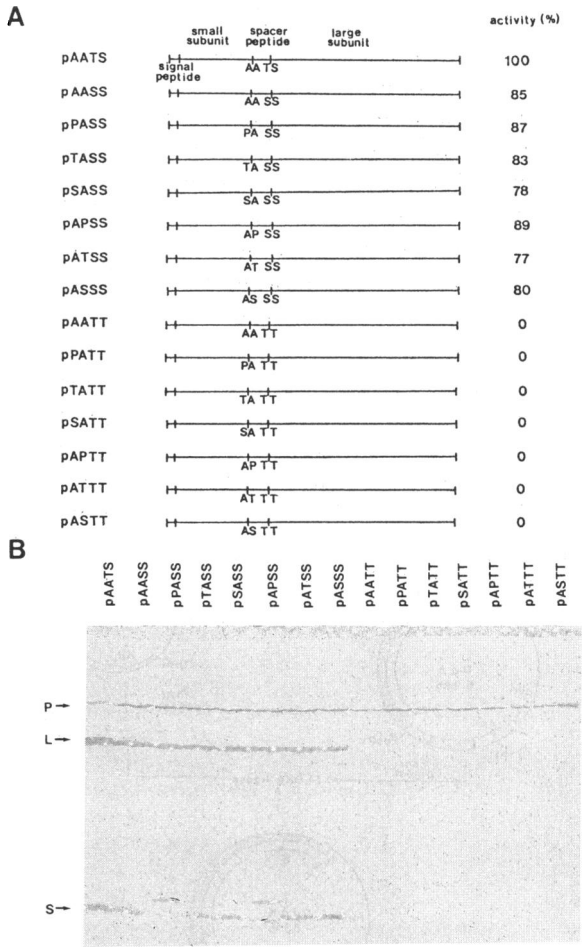

FIG. 4. Structure and PGA activity (A) and immunoblot analysis (B) of the recombinants. P, L, and S mark the precursor, the large subunit, and the small subunit, respectively. See the legend to Fig. 1 for further details.

were partially processed from precursors, and the processing phenotypes of the small subunit caused by amino acid substitutions at the small-subunit cleavage site were reflected. About 80% of the activity of the control JM109/pAATS (wild type) was detected in these mutants, as was the case with pAASS. But in the recombinants with pAATT whose processing was completely blocked, none of the mature products was detected and no PGA activity was found. The effect of amino acid substitutions at the small-subunit cleavage site was not detected when the large subunit could not become mature. Therefore, the above data prove that the small subunit can become mature only after processing of the large subunit.

Effect of the removal of signal peptides from site-directed mutants. The precursor without a signal peptide might be accumulated in the cytoplasm, and a processing study using the overproduced precursors as substrates was expected to be possible.

The constructed plasmid (pJA) was confirmed by sequencing to be destined to code for the precursor without a signal peptide and with a single amino acid (Ala) incorporated before the small subunit. The essential steps in the construction procedure are summarized in Fig. 5. The assay for PGA activity is based on measuring the amount of 6-aminopenicillanic acid produced from penicillin G (2). Ampicillin, which is the antibiotic marker of pJA, and penicillin G are structurally similar beta-lactam antibiotics. The antibiotic marker of pJA was replaced with kanamycin in order to avoid artifacts caused by endogenous beta-lactamase. *E. coli* JM109 cells harboring pJAK were grown to the exponential phase and induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside), and the SDS lysates were prepared for immunoblotting. The size of the gene product of the pJAK intermediate was calculated in comparison with those of the marker proteins and the purified PGA (Fig. 6). Unexpectedly, not only the precursor without a signal peptide but also two protein bands which were presumed to be intermediates of processing were observed (Fig. 6B). One band of processing intermediates is the size of that for the large subunit, and the size of another band was approximately 29 kDa. That intermediate was supposed to contain the small subunit (24 kDa) and the full-sized spacer peptide (54 amino acids). This result indicates that without the complete translocation of the precursor, cleavage between the spacer peptide and the large subunit can be generated at low efficiency.

In order to investigate the relationship between the translocation of *pga* gene products and the processing steps in more detail, signal peptides were removed from the site-directed mutants with different processing phenotypes. Two processing phenotypes were observed (Fig. 7). One was the partial processing pattern at the C terminus of the spacer peptide, as observed for pJAK, and another was the unprocessed form. Without the signal peptide, the small subunit was unable to mature no matter what the mutations were, although maturation of the large subunit could occur partially in the signal peptide-less mutants of Ala-235, Ala-236, and Thr-289. For the Thr mutant at the Ser-290 position, processing from its precursor was completely blocked. These results showed that the absence of a signal peptide in the PGA precursor completely blocked the processing of the small subunit and decreased the processing efficiency of the

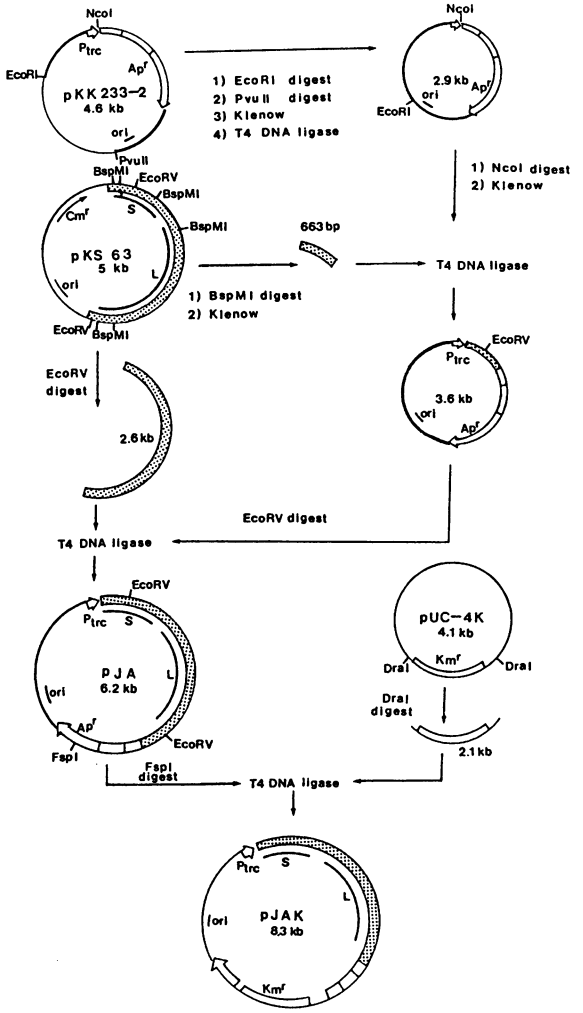

FIG. 5. Construction of the plasmid (pJAK) coding for a PGA precursor which is lacking the signal peptide. For details, see the text.

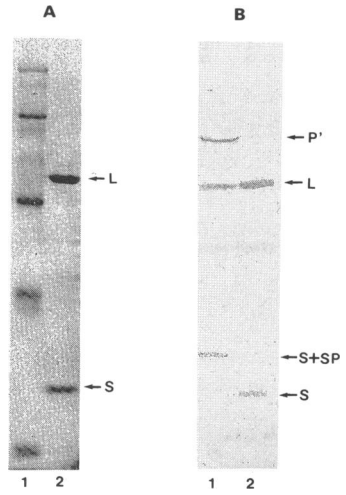

FIG. 6. Size estimation of the pJAK intermediate. (A) NC paper directly stained with Coomassie blue. Lanes: 1, size marker proteins (17,000, 97,400, 55,400, 36,500, and 21,100 Da); 2, purified PGA. (B) Immunoblot analysis. Lanes: 1, cell lysates from JM109 harboring plasmid pJAK; 2, cell lysates from JM109 harboring pKS63 (wild-type plasmid). The approximate protein size of the small subunit intermediate was determined by plotting the relative mobilities of marker proteins and purified PGA against their molecular weights. P', L, S, and SP denote the precursor without a signal peptide, the large subunit, the small subunit, and the spacer peptide, respectively.

large subunit, although the cause of that phenomenon was not clear. Except for the cells harboring pAATT-JAK, the cell extracts of the pJAK series contained about 40 to 50% of the activity of the control JM109/pKS63 (wild-type plasmid). The PGA activities of the pJAK series seemed to be proportional to the processing efficiency of the large subunit.

The *pga* gene product in pJAK may be confined within the cytoplasm or cytoplasmic membrane because of the absence of a signal peptide. In order to investigate the possibility of processing into mature products occurring further when cellular compartmentalization was disrupted, processing kinetics was analyzed in vitro (Fig. 8). The decrease in the amount of the small-subunit intermediate was accompanied by a proportional increase in the amount of the mature small subunit. The small subunit seemed to mature in three steps. However, considering that the amount of precursor did not decrease and the amount of the large subunit did not increase with time, it was expected that processing from the precursor to the large subunit could no longer occur under those conditions. PGA activity did not increase detectably during the assay, despite the maturation of the small subunit.

DISCUSSION

In the present study, we assessed the significance of the primary sequence of amino acids composing the proteolytic cleavage sites in PGA. For the viral polyprotein with the proteolytic processing pathway, a conserved sequence mo-

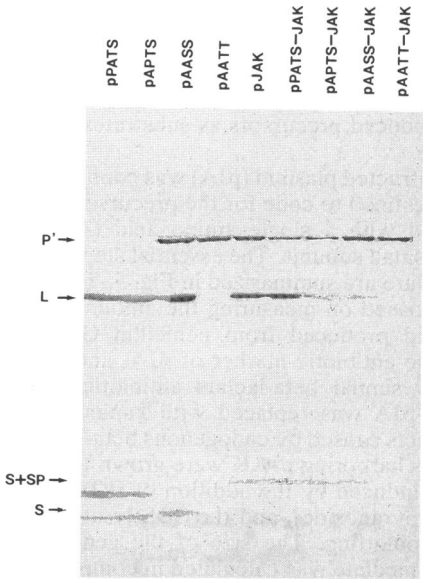

FIG. 7. Immunoblot analysis of site-directed mutants without signal peptide. Cells of the pJAK series were deleted of signal peptides by recombination with the site-directed mutants. P', L, S, and SP denote the precursor without a signal peptide, the large subunit, the small subunit, and the spacer peptide, respectively.

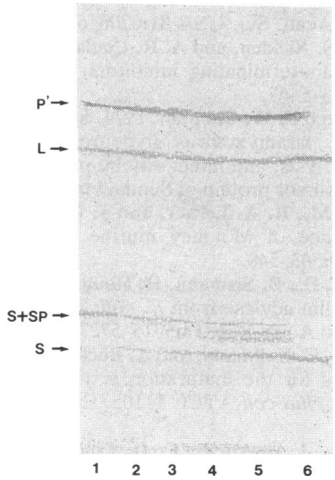

FIG. 8. Immunoblot analysis of in vitro processing products. After culture of the cells harboring pJAK, cell extracts were prepared by mild sonication. Unbroken cells were removed by centrifugation, and broken-cell extracts were incubated at 37°C. Samples were taken at the times indicated below for analysis. Lanes: 1, fractions incubated for 0 h; 2, those incubated for 0.5 h; 3, those incubated for 1 h; 4, those incubated for 2 h; 5, those incubated for 3 h; 6, cell extract of wild-type plasmid.

tif, such as basic amino acids near the cleavage sites, is known to contribute to the structural requirements for cleavage (8, 20, 22, 23). The real conserved sequence motif for PGA processing may not be confined within the dipeptides containing the scissile bonds, because not every Ala-Ala or Thr-Ser pair in PGA is subject to cleavage. The fact that two consensus amino acids (Ser-Asn at positions 290 and 291 in the PGA from *E. coli*) are found in PGA from *Kluyvera citrophila* (1) and *Arthrobacter viscosus* (19), as well as in two cephalosporin acylases from *Pseudomonas* species (16, 17), suggests that the amino-terminal region of the large subunit is important in cleavage site recognition. Although only a few Ser-290 mutants were tested, our data are consistent with the sequence-specific constraints on residues at this position. The processing system might accommodate the change to Cys at Ser-290 because Cys is more similar to Ser than any other amino acid in its size, shape, and chemical properties. In other mutants, cleavage of the precursor might be inefficient because of improper presentation of the site, possibly because the substitution of amino acids at the cleavage site made the site less accessible.

A prerequisite for PGA activity is the efficient processing of the large subunit from its precursor. By comparing the PGA activities and the processing phenotypes of many recombinants, it was shown in this study that the precursor with or without a signal peptide has no PGA activity. But after cleavage of the large subunit, the complex of the large subunit with the small-subunit intermediate should have retained PGA activity at a level similar to that of the final mature dimer, regardless of the length of the spacer peptide appended to the small subunit. This fact was supported by evidence such as the expression of pPATS, pAPTS, and the pJAK series. The fact that reduced cleavage of the large subunit and the small subunit with the spacer peptide is possible in the pJAK series (Fig. 7) suggests that the cleavage reaction of the large subunit occurs in the cytoplasm or the cytoplasmic membrane. On the other hand, the small subunit would be processed in the periplasm. This fact was supported by evidence that the small subunit could mature only after cellular compartmentalization was disrupted, as shown in Fig. 8.

Steps in the processing pathway of PGA are expected to occur as follows. After the translation of the precursor, the signal peptide guides translocation of the N terminus of the precursor to outside the inner membrane, where it is cleaved by signal peptidase. At the next step, the large subunit is processed specifically at the cytoplasmic membrane after the majority of the precursor has traversed the membrane. At this step, the *pga* gene product would take a catalytically active form by conformational change. The association of the large subunit with the small-subunit moiety after the cleavage at the C terminus of the spacer peptide may weaken the cytoplasmic membrane binding of *pga* gene products. The spacer peptide extension may be exposed outside the folded dimer and cleaved by the multiple steps up to the dangling amino acid, which is not involved in the tight association of the dimer complex.

Discussions about the amino acids at the active site have been controversial. The involvement of Trp at the active sites of enzymes from *Proteus rettgeri* and from *E. coli* has been suggested from inactivation studies (14). The Ser reagent phenylmethanesulfonyl fluoride, which structurally resembles the side chain of benzylpenicillin, inactivated the enzyme from *E. coli* (11) and an enzyme from *P. rettgeri* (5). During the preparation of this article, there was a report that Ser-290 may act as a nucleophile in catalysis (26). Results of our site-directed mutagenesis also show that the Ser-290 residue is an amino acid critical both for PGA activity and for processing. The −OH functional group of Ser-290 is especially essential for PGA activity. This finding, that the N-terminal amino acid of one subunit is involved in the active site, is unprecedented.

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