

# Immediate Entrance to the Export Pathway after Synthesis as a Requirement for Export of the *sak* Gene Product in *Escherichia coli*

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Export through the cytoplasmic membrane and processing of the *sak* product in *Escherichia coli* cells were investigated with *E. coli* strains carrying pTS301, which produce large amounts of staphylokinase at 42°C. High-level synthesis of the *sak* product caused transient accumulation not only of the staphylokinase precursor (pSAK) but also of the maltose-binding protein and outer membrane protein A precursors. Thus it was concluded that the *sak* product shares the export pathway with *E. coli* secreted proteins at least at a certain step. During high-level synthesis of the *sak* product, a significant amount of the newly synthesized pSAK remained unprocessed after a chase period, possibly causing the observed accumulation of pSAK. Accumulating pSAK did not mature for a long period, whereas the newly synthesized *sak* product was exclusively detected in the mature form. These results suggest that it is necessary for the *sak* product to enter the export pathway during or immediately after synthesis to be exported and processed normally.

Many secreted and membrane proteins of both procaryotic and eucaryotic cells are synthesized as larger precursors which have amino-terminal extensions termed signal peptides or leader peptides. It has been suggested that signal peptides serve to transfer polypeptide chains to the membrane and translocate them across the membrane (2, 6, 9, 30, 31). The signal peptides of both procaryotic and eucaryotic secreted proteins have quite similar structural features, some of which indeed promote protein translocation across the membrane of heterologous host cells. In one case, recent works have shown that the product of the *penP* gene from *Bacillus licheniformis* is exported and modified at the cysteine residue of the amino terminus, as are lipoproteins of *Escherichia coli*, in both *B. licheniformis* and *E. coli* (4, 8) and that the product is localized in the outer membrane of *E. coli*, as are lipoproteins (26), showing that at least these two bacteria have quite similar export and processing machinery for diglyceride-modified lipoproteins. There have been reported, at the same time, several examples in which heterospecific secreted proteins are exported into the periplasmic space in *E. coli* (15, 28, 29, 32). However, the molecular nature of these phenomena remains to be solved.

Recently, we have cloned the structural gene for staphylokinase (SAK), which is an extracellular protein produced by certain strains of *Staphylococcus aureus*, onto plasmid vector pBR322 and have shown that functional SAK is produced and exported into the periplasmic space of *E. coli* cells (24). From the amino acid sequence of the gene product deduced from the nucleotide sequence (25) and SAK purified from *E. coli* cells (23), we have identified the signal peptide of 27 amino acids. The signal peptide is different from the lipoprotein signal peptide and functions in both *S. aureus* and *E. coli*. However, when the *sak* product was synthesized at high level, large amounts of the precursor form of SAK (pSAK) accumulated in *E. coli* cells (23). Similar phenomena of precursor accumulation have been reported for the *phoS* (17) and *traT* (16) products. The reason for precursor accumulation has not been determined in either case. To show whether or not there are any differences in export processes between heterospecific secreted proteins and the secreted proteins of *E. coli* and to clarify the reasons why various precursors accumulate during high-

level synthesis, it is worthwhile analyzing the export mechanism of the *sak* product under conditions of high-level synthesis in *E. coli*. In the present study I investigated the mode of export and processing of the *sak* product in *E. coli* cells and found no evidence that export and processing of the *sak* product have peculiar properties not observed in the secreted proteins of *E. coli*. I also found evidence that the pSAK remaining unprocessed during high-level synthesis is localized in the cytoplasm with weak association to the membrane and is no longer capable of export and processing.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains used were derivatives of *E. coli* K-12. WA802 was described previously (24). MC4100 [ $F^-$  *araD139 thi*  $\Delta$ (*argF-lac*)*U169 rpsL150 relA1 fbbB5301 ptsF25 deoC1*] (3) was obtained from K. Ito, Institute for Virus Research, Kyoto University, Kyoto, Japan. Plasmid pTS301 (23) carries the *sak* gene under the control of lambda P<sub>R</sub> promoter. Transformation by plasmid DNA was done by the previously described method (24).

**Media, enzymes, and chemicals.** M9 medium supplemented with 10  $\mu$ g of thiamine per ml and appropriate sugars was used as the basal medium throughout the experiments. Sugars were used at concentrations of 0.4% (glucose and maltose) or 0.5% (glycerol), and amino acids were used at concentrations of 80  $\mu$ g/ml. LB broth was described previously (23). For strains carrying pTS301 plasmid, ampicillin was added to the medium at 100  $\mu$ g/ml. Lysozyme and trypsin were purchased from Sigma Chemical Co., St. Louis, Mo. Protein A-Bacterial Adsorbent (Formalin-fixed *S. aureus* Cowan I cells), En<sup>3</sup>Hance, and Kodak XAR-5 films were purchased from Miles Laboratories, Inc., Elkhart, Ind., New England Nuclear Corp., Boston, Mass., and Eastman Kodak Co., Rochester, N.Y., respectively. L-[4,5-<sup>3</sup>H]lysine was purchased from Amersham, Buckinghamshire, England.

**Pulse-labeling.** Cells were grown to about  $2 \times 10^8$  cells per ml at 30°C in M9-glucose medium supplemented with all amino acids but lysine, concentrated to  $5 \times 10^8$  cells per ml in the same medium, and cultivated at 30°C for an additional

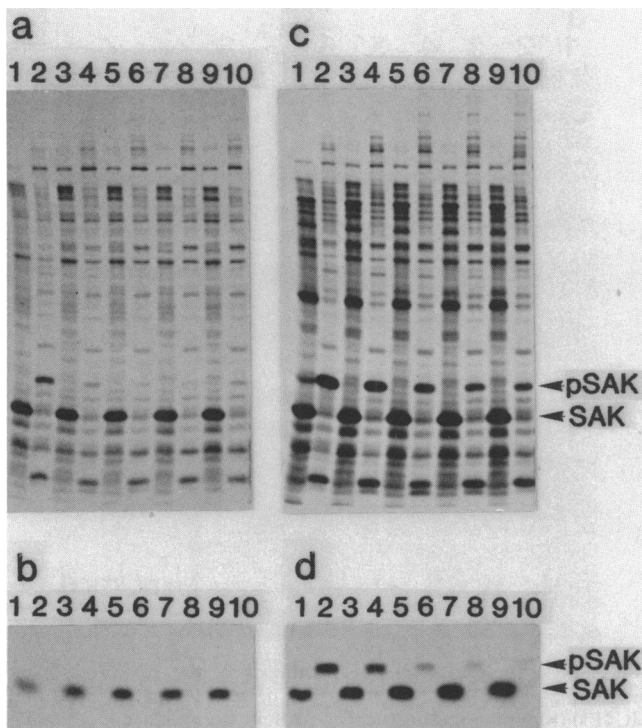


FIG. 1. Kinetics of conversion of pSAK to SAK. At 5 (a and b) and 45 (c and d) min after transfer to 42°C, WA802(pTS301) cells were pulse-labeled and chased. After 10 (lanes 1 and 2), 30 (lanes 3 and 4), 90 (lanes 5 and 6), 270 (lanes 7 and 8), or 810 (lanes 9 and 10)-s chase periods, aliquots were mixed with equal volumes of 0.2 M NaOH and fractionated into soluble (odd-numbered lanes) and insoluble (even-numbered lanes) fractions by centrifugation. Immune precipitation, SDS-PAGE, and fluorography were done as described in Materials and Methods. a and c, Whole cell proteins; b and d; immune precipitates.

30 min. To induce expression of the *mal* regulon, M9-glycerol medium was used instead of M9-glucose medium, and maltose was added when the cells were transferred to fresh medium. The culture was shifted to 42°C, and aliquots were pulse-labeled for 20 s with 100  $\mu$ Ci of L-[4,5-<sup>3</sup>H]lysine (specific activity, 86 Ci/mmol) per ml, unless otherwise mentioned, and chased by adding nonradioactive lysine at 500  $\mu$ g/ml. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid (TCA), 0.2 M NaOH, or ice.

**Subcellular fractionation.** Two methods were used. First, cells were fractionated into soluble and insoluble fractions by adding an equal volume of 0.2 M NaOH followed by centrifugation (23). This method was originally described by Russel and Model (22) and can probably fractionate the periplasmic and cytoplasmic proteins into the soluble fraction and the membrane proteins into the insoluble fraction. Second, the method of Osborn et al. (19) was used. Briefly, cells were suspended in a solution of 25% sucrose in 10 mM Tris hydrochloride (pH 8.0). Lysozyme was added at a concentration of 100  $\mu$ g/ml followed by gradual addition of 40 volumes of cold 1.5 mM EDTA (pH 7.5) with gentle mixing. The mixture was incubated for an additional 10 min on ice and sonicated. After removal of unbroken cells by low-speed centrifugation, the mixture was centrifuged at 100,000  $\times g$  for 2 h to obtain the soluble and membrane fractions. To fractionate the membrane further, isopycnic

sucrose density gradient centrifugation was done as previously described (19).

**Trypsin accessibility test.** Cells were pulse-labeled and chased, and the reaction was stopped by adding solid ice. The harvested cells were treated with 100  $\mu$ g of lysozyme per ml in a buffer of 20% sucrose–50 mM Tris hydrochloride (pH 8.0)–5 mM EDTA in an ice water bath for 20 min. Trypsin was then added to the resulting spheroplasts at various concentrations. After incubation in an ice water bath for 30 min, the reaction was stopped by adding diisopropyl-fluorophosphate to a concentration of 8 mM followed by addition of TCA to 5%. Diisopropylfluorophosphate was added to all buffers used in immune precipitation of these samples.

**Immune precipitation.** Protein samples were dissolved in 20 to 50  $\mu$ l of 3% sodium dodecyl sulfate (SDS) and boiled for 3 min. Aliquots (10  $\mu$ l) were diluted with 300  $\mu$ l of Triton buffer (0.1 M potassium phosphate [pH 8.0], 150 mM NaCl, 2% Triton X-100, 1 mM EDTA) and used for immune precipitation as described previously (25). Antisera to the maltose-binding protein (MBP) and outer membrane protein A (OmpA) were gifts of K. Ito and W. Wickner, University of California, Los Angeles, respectively.

**SDS-PAGE.** The samples were dissolved in sample buffer (62.5 mM Tris hydrochloride [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), boiled for 3 min, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 13.5% SDS-polyacrylamide gels as described by Laemmli (14). Fluorography was done as described previously (25).

## RESULTS

**Mode of translocation and processing of the *sak* product.** As reported previously (23), induction of synthesis of the *sak* product in an *E. coli* strain resulted in accumulation of large amounts of pSAK. This seemed to be attributable to a delay in export of the *sak* product across the cytoplasmic membrane of *E. coli* cells during high-level synthesis. To determine why pSAK accumulates during high-level synthesis, I measured the kinetics of pSAK export and processing in *E. coli* cells.

A log-phase culture of WA802 harboring pTS301 was shifted from 30 to 42°C to induce synthesis of the *sak* product, and the cells were pulse-labeled for various periods beginning 10 min after the temperature shift. Anti-SAK monoclonal antibody was added, and the immune precipitates were analyzed by SDS-PAGE and fluorography. I found that during a 10-s pulse most of the *sak* product existed in the pSAK form and that the ratio of SAK to pSAK increased as the labeling period increased (data not shown). This showed that the signal peptide of the *sak* product can be cleaved off posttranslationally. I used a 20-s pulse-labeling period in the following analyses.

The precise rate of processing, as well as the subcellular localization of the newly synthesized *sak* product, was determined by pulse-labeling and chase followed by fractionation of the cells. In this experiment, an NaOH solution was added instead of TCA to stop protein synthesis, and the cells were fractionated by centrifugation into soluble and insoluble fractions as described previously (23). Figure 1 shows the fluorograms of the gels on which the whole-cell proteins (Fig. 1a and c) or immune precipitates (Fig. 1b and d) were electrophoresed. Most of the *sak* product was detected as SAK soon after the temperature shift, and the remaining pSAK matured rapidly. By densitometric scanning of the fluorogram in Fig. 1a, the half-life of pSAK was calculated to

be 7 s, a value comparable to that of *E. coli* exported proteins (11, 12). However, at 45 min after the temperature shift, approximately half of the product remained as pSAK just after pulse-labeling, and this pSAK matured at the calculated initial half-life of about 30 s. This delay in processing pSAK 45 min after induction may be caused by high-level SAK synthesis (see Fig. 4).

Figure 1 also shows that the location of the *sak* product is independent of the processing rate; that is, pSAK and SAK were exclusively localized in the insoluble and soluble fractions, respectively, by 0.1 M NaOH treatment. Note that a portion of pSAK was recovered in the soluble fraction at the earliest chase point during high-level synthesis (Fig. 1c and d, lanes 1). This indicates that the association of pSAK with the membrane is not an artificial event in the experiment but that the pSAK species is really transferred to the membrane after synthesis is completed. On the other hand, when the cells were fractionated by the procedure of Osborn et al. (19), more pSAK was recovered in the soluble (periplasmic and cytoplasmic) fraction than in the membrane fraction (data not shown). These results appear to be quite contrary to each other. However, as suggested by Ito et al. (10), this may mean that pSAK is not covalently integrated into the membrane but instead is only weakly associated with the membrane. If so, transfer of pSAK to the membrane may occur during or immediately after synthesis even during high-level SAK synthesis. Whether or not the accumulating pSAK had been translocated from the inner to the outer side of the cytoplasmic membrane was determined by treating intact spheroplasts with trypsin. Trypsin digestion of either pSAK or SAK produced a molecule having slightly higher mobility than SAK on an SDS-polyacrylamide gel (23). pSAK was more resistant than SAK to trypsin added to intact spheroplasts, especially at lower trypsin concentrations (Fig. 2). Since pSAK is more sensitive than SAK to trypsin added to osmotically lysed spheroplasts (data not

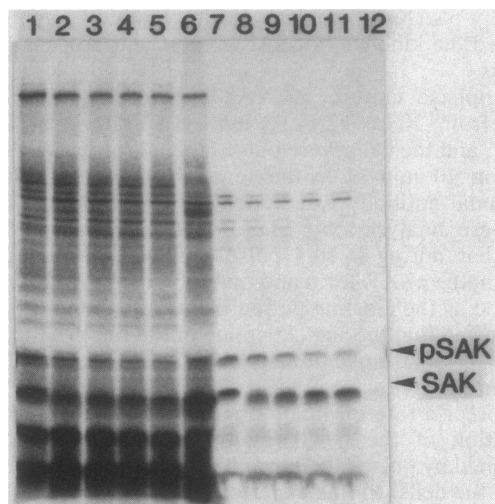


FIG. 2. Accessibility of the *sak* product to trypsin. Pulse-labeled WA802(pTS301) cells were spheroplasted, and trypsin was added at concentrations of 0 (lanes 1 and 7), 5 (lanes 2 and 8), 15 (lanes 3 and 9), 50 (lanes 4 and 10), or 150 (lanes 5 and 11)  $\mu\text{g/ml}$  in an ice water bath. After 30 min the reaction was stopped by adding 8 mM diisopropylfluorophosphate and an equal volume of 10% TCA. As a control, trypsin (15  $\mu\text{g/ml}$ ) was added in the presence of 1% Triton X-100 (lanes 6 and 12). Lanes: 1 to 6, whole-cell proteins; 7 to 12, immune precipitates.

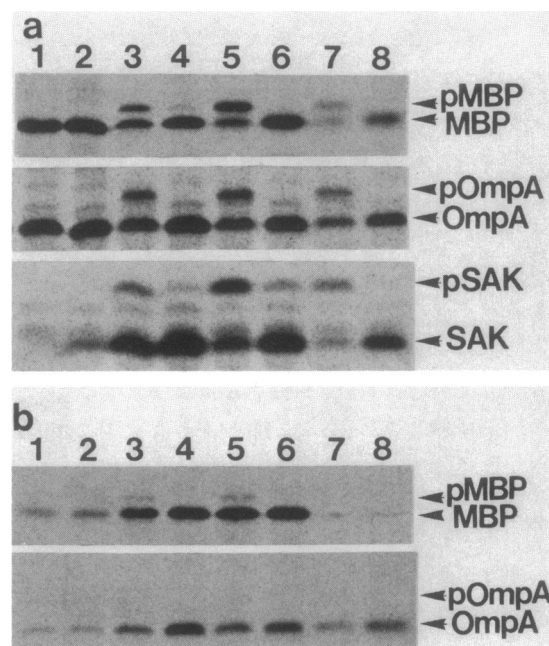


FIG. 3. Effects of high-level synthesis of the *sak* product on processing of MBP and OmpA. Cells of MC4100(pTS301) (a) and MC4100 (b) grown in M9-glycerol medium were induced by adding 0.4% maltose and then transferred to 42°C. At 2 (lanes 3 and 4), 45 (lanes 5 and 6), or 90 (lanes 7 and 8) min, cells were pulse-labeled for 20 s and chased for 10 (lanes 3, 5, and 7) or 600 (lanes 4, 6, and 8) s. The reaction was stopped by adding an equal volume of 10% TCA. Immune precipitation, SDS-PAGE, and fluorography were done as described in Materials and Methods. Lanes 1 and 2 are the samples at 30°C for 10- and 600-s chase periods, respectively. The positions of pSAK, SAK, pMBP, MBP, pOmpA, and OmpA are indicated.

shown), newly synthesized pSAK, which accumulates transiently, must be localized in the cytoplasm or on the cytoplasmic face of the membrane.

**Effect of high-level SAK synthesis on export of other secreted proteins.** If the delay in export of the *sak* product during high-level SAK synthesis is due to an effect of high levels of SAK on the normal export machinery, then it is probable that the export of other secreted proteins is also affected. To determine whether this is the case, I analyzed the processing of two secreted proteins, MBP and OmpA (Fig. 3). Although precursor forms of MBP and OmpA (pMBP and pOmpA, respectively) were hardly detected by pulse-labeling when expression of *sak* was at a low level, they became visible by pulse-labeling when SAK synthesis was induced. Thus the *sak* product seems to share the protein export pathway with *E. coli* secreted proteins at least at a certain step, and this step appears to be rate limiting. At the 10-min chase point, however, pMBP and pOmpA were completely converted to the mature forms, while a significant amount of pSAK remained unprocessed. The reason for this will be discussed later.

**Accumulation of pSAK.** From the results described above, it seemed possible that the accumulated pSAK might consist of pSAK in an unprocessable form rather than as a backlog of pSAK awaiting processing. To determine whether this was the case, I analyzed whether or not newly synthesized pSAK was being processed while accumulating pSAK remained unprocessed. Accumulating pSAK could be detected on the gel stained by Coomassie blue 30 min after induction,

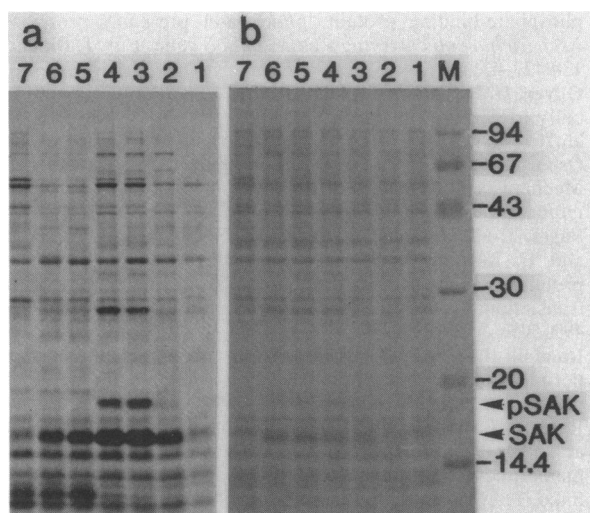


FIG. 4. Accumulation of pSAK. WA802(pTS301) cells were transferred to 42°C. At 0 (lanes 2), 30 (lanes 3), 60 (lanes 4), 120 (lanes 5), or 180 (lanes 6) min, aliquots of the cells were labeled for 5 min. TCA-insoluble materials were electrophoresed on an SDS-polyacrylamide gel, and the gel was stained with Coomassie brilliant blue R (b) followed by treatment for fluorography (a). Lanes 1 and 7 are the samples labeled at 30°C at 0 and 180 min, respectively. Numbers on the right are molecular masses (in kilodaltons) of standard proteins (lane M).

increased until 120 min, and then remained constant until at least 180 min (Fig. 4b). On the other hand, newly synthesized pSAK could be detected by pulse-labeling at 0, 30, and 60 min but not at 120 and 180 min after induction (Fig. 4a), indicating that by this time all newly synthesized pSAK was being immediately processed. Therefore the pSAK seen by Coomassie blue staining at 120 and 180 min which had accumulated during the high synthesis rate did not mature even after a slowdown of the synthesis rate allowed all newly synthesized pSAK to mature rapidly. Induction for 3 h in LB broth followed by fractionation as described by Osborn et al. and detection of SAK activity on a fibrin plate (23) showed that pSAK was localized both in the cytoplasm and on the membrane in similar amounts. The pSAK on the membrane comigrated with the inner membrane in isopycnic sucrose density gradient centrifugation (data not shown). When the cells were fractionated by 0.1 M NaOH, pSAK was exclusively localized in the insoluble fraction (see reference 23). Thus, accumulating pSAK is retained within the cytoplasm, attached weakly to the membrane, and cannot enter the normal export and processing pathway.

#### DISCUSSION

The *sak* product is exported and processed in *E. coli* essentially as fast as *E. coli* secreted proteins (11–13). Cleavage of the signal peptide can proceed posttranslationally, in agreement with the observations reported by Josefsson and Randall (11) and Randall (21), in which they concluded that translocation of a secreted protein, even if it is translocated cotranslationally, occurs after the polypeptide chain has reached about 300 amino acid residues. However, during high-level synthesis of the *sak* product the rate of synthesis exceeded that of export and processing, resulting in transient accumulation of pSAK just after synthesis. Conversion of accumulated pSAK proceeded slower than normal. These are very similar to results reported by Morita

et al. (17) for the *phoS* product and by Minkley (16) for the *traT* product. Moreover, transient accumulation of pSAK caused accumulation of pMBP and pOmpA as well, indicating that pSAK is exported through a pathway common to the secreted proteins of *E. coli*. That pSAK is exported by a common pathway is supported by an observation that the processing of the *sak* product, like that of *E. coli* secreted proteins, depends on at least the *secY* (*priA*) and *secA* functions (unpublished data). Although several cases of interference in processing of normal secreted proteins have been reported (1, 5, 10, 18, 27), the transient accumulation of precursor forms of normal secreted proteins due to high-level synthesis of a secreted protein is a new type of interference and suggests that there exists a relatively slow step in the export pathway. The fact that the transiently accumulating pSAK was localized in the cytoplasm associated weakly with the membrane strongly suggests that the restrictive step is located at the entry to or at a very early point in the export pathway.

After transient accumulation of pSAK as well as pMBP and pOmpA during high-level synthesis of the *sak* product, the latter two converted to the mature forms within 10 min, whereas a significant amount of pSAK localized in the cytoplasm remained unprocessed after a 10-min chase. This difference in processing rate between pSAK and pMBP or pOmpA may reflect differences in the efficiencies with which individual secreted proteins are recognized by a certain factor in the export machinery of *E. coli*. Such differences in the strengths of interaction between a cellular factor and a secreted protein have been demonstrated with various mutant strains (6, 18). Because a significant amount of pSAK remained unprocessed, large amounts of pSAK accumulated in the cytoplasm or on the cytoplasmic membrane after prolonged cultivation. At the same time, newly synthesized pSAK was being processed rapidly as indicated by a failure to detect pSAK by pulse-labeling. Thus, accumulated pSAK is clearly distinct from newly synthesized pSAK. These results suggest that pSAK must enter the *E. coli* export pathway during or immediately after synthesis to be normally processed. Recently, Pages et al. (20) also showed that the precursor of the *phoS* product, which accumulated in the cytoplasm during its own high-level synthesis, was not processed normally. Two interpretations, which must be experimentally verified, can be presented to explain these results. First, there may exist a mechanism that couples translation of an exported protein to translocation such that translocation is linked to translation but complete translation does not require translocation. Alternatively, it is possible that conformational change or folding of the precursor protein in the cytoplasm prevents its translocation across the membrane by preventing its being recognized by a certain export factor.

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