Effects of signal peptide mutations on processing of *Bacillus stearothermophilus* α -amylase in *Escherichia coli*

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***Bacillus stearothermophilus* α -amylase has a signal peptide typical for proteins exported by Gram-positive bacteria. There is only one signal peptidase processing site when the protein is exported from the original host, but when it is exported by *Escherichia coli*, two alternative sites are utilized. Site-directed mutagenesis was used to study the processing in *E. coli*. Processing sites for 13 *B. stearothermophilus* α -amylases carrying mutations in their signal peptide were determined. Processing of the signal peptide was remarkably tolerant to mutations, because switching between the alternative sites was possible. The length and the sequence of the region between the hydrophobic core and the cleavage site was crucial for determining the choice of the processing site. Some mutations more distal to the cleavage site also affected the site preference.**

Keywords: α -amylase, processing, protein secretion, signal peptidase, signal peptide

INTRODUCTION

Signal peptides (leader peptides) serve as localization labels for proteins to be exported outside the cytoplasmic compartment of a bacterial cell. During or shortly after translocation of the protein across or into the membrane, this transient peptide is cleaved off (processed) by an integral membrane protease, the signal peptidase (Wolfe & Wickner, 1984). The primary amino acid sequences of signal peptides are not conserved (von Heijne & Abrahmsen, 1989; Watson, 1984), but they share certain common features. Three distinct regions have been defined by von Heijne (1985): (i) the positively charged N-terminus, the N region; (ii) the hydrophobic core, the H region, usually 12–20 amino acid residues in length; and (iii) the part preceding the signal peptidase processing site, the C region. In endogenous *Escherichia coli* proteins the C region is almost invariably five or six residues long and the cleavage specificity has been proposed to be dictated by residues at positions –1 (Ala, Gly, Ser or Thr)

and –3 (Ala, Leu, Gly, Ser, Thr or Val) (Nilsson & von Heijne, 1991; Shen *et al.*, 1991; von Heijne, 1985, 1986; von Heijne & Abrahmsen, 1989).

Signal peptides of proteins exported by Gram-positive bacteria do not usually fulfil these criteria exactly (see e.g. von Heijne & Abrahmsen, 1989; Watson, 1984); the charge of the N region is often higher than the average (+2 for *E. coli*), the hydrophobic core is longer than in *E. coli* (von Heijne & Abrahmsen, 1989), and the C region can be as long as 9–11 residues. Whether these variations between bacterial species reflect the specificity of processing by signal peptidase has not been systematically studied. To elucidate this problem, van Dijk *et al.* (1991) attempted to express *E. coli* signal peptidase in *Bacillus subtilis*. The *E. coli* signal peptidase appeared to be non-functional in *B. subtilis*. Several other studies have focused on the kinetics of processing during export, but the exact processing sites for the numerous signal-peptide mutations have usually not been determined.

Bacillus stearothermophilus α -amylase has a signal peptide typical for proteins exported by Gram-positive bacteria; it is 34 residues long, with a 14-residue H region and a 9-residue C region (Suominen *et al.*, 1987a). We have previously observed that the signal peptide is processed differently in *E. coli* compared to *B. subtilis* or *B. stearothermophilus* (Suominen *et al.*, 1987a; Vihinen &

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Mäntsälä, 1990). In *E. coli*, a secondary site (-Gly-Gln-Pro↓), three residues upstream from the original processing site (-Ala-Lys-Ala↓), is used in approximately 30% of the exported α -amylase molecules. Although the secondary site is otherwise atypical for exported *E. coli* proteins, it shortens the unusually long C region down to the consensus length of six residues for exported *E. coli* proteins. To study the factors affecting the choice between these two processing sites, we made 13 different site-directed mutations in the α -amylase signal peptide and studied their processing in *E. coli*. The results show that the presence of two alternative processing sites renders the signal peptide exceptionally tolerant to mutations. The C region was shown to play a major role in determining the processing site. A shorter C region increased the amount of α -amylase produced in *E. coli*.

METHODS

Bacteria and culture conditions. *E. coli* strains JM103 (Vieira & Messing, 1982), HB101 (Boyer & Roulland-Dussoix, 1969) and MC4100 (Ito *et al.*, 1981) were used as hosts for cloning and expressing the *B. stearothermophilus* α -amylase gene. *E. coli* RZ1032 (Kunkel, 1985) was used for uridinylation of the template DNA for *in vitro* mutagenesis. For mutagenesis, the DNA fragments were cloned in the M13mp18 phage vector (Yanisch-Perron *et al.*, 1985). Plasmid pCSS4 (Suominen *et al.*, 1987b) carries a 3 kb *PvuII*-*HindIII* chromosomal DNA fragment from *B. stearothermophilus* ATCC 12980, including the gene for α -amylase with complete 5'- and 3'-flanking regions. Bacteria were grown in 2 × YT medium, in LB medium or in M9 minimal medium (Sambrook *et al.*, 1989), supplemented where appropriate with 100 µg ampicillin ml⁻¹. For uridinylation of the template to be mutagenized, 2 × TY medium was supplemented with 0.25 µg ml⁻¹ uridine. Insoluble corn starch was included (to 1%, w/v) in the plates when α -amylase activity was to be detected directly by staining with iodine vapour.

DNA manipulations. Standard cloning techniques (Sambrook *et al.*, 1989) were employed, using enzymes and reagents from various commercial sources. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) either for fragments cloned in M13 vectors or for double-stranded plasmids (Zagursky *et al.*, 1985). All the sequence data were manipulated and secondary structure predictions were made using the GCG software package (Devereux *et al.*, 1984). *In vitro* mutagenesis was done using uridinylated M13 templates (Kunkel, 1985) with the single priming technique described by Suominen *et al.* (1987b). Mutagenic oligonucleotides (17- or 18-mers) were used to introduce point mutations and 31-mers were used for deletions. Construction of the pCSS20ss plasmids with signal-sequence mutations in the α -amylase gene was similar to the method described for pCSS20ss1 by Suominen *et al.* (1987b). Briefly, the 740 bp *DraI*-*KpnI* fragment coding for the N-terminal portion of α -amylase was cloned from pCSS4 into M13mp18. After mutagenesis, the contiguous α -amylase gene was reconstructed, resulting in the pCSS20ss series of plasmids. The mutations were double-checked by sequencing after reconstituting the pCSS20ss plasmids.

Fractionation of bacterial cultures. *E. coli* cultures were fractionated by two methods. For purification of the enzymes, cells were separated from the culture medium by centrifugation, and washed and treated by the osmotic-shock procedure of Cornelis *et al.* (1982) to obtain the periplasmic fraction. To

determine total α -amylase production levels, the washed cells were directly sonicated and centrifuged (12000 r.p.m., 15 min in a microcentrifuge) to obtain the total soluble cell extract.

Enzyme activity measurements. Activities for α -amylase were assayed at 70 °C using soluble starch (Merck) as substrate as previously described by Suominen *et al.* (1987b).

Purification of α -amylase and N-terminal sequence analysis.

N-terminal amino acid sequences were determined for α -amylases purified from cultures of *E. coli* JM103 or MC4100 carrying each of the pCSS20ss plasmids with a signal-sequence mutation. Cultures of *E. coli* carrying the plasmids were grown separately overnight in 2 × YT medium. The periplasmic fraction (obtained as described above) was then heated at 70 °C for 1 h in the presence of 5 mM Ca²⁺, resulting in denaturation and precipitation of most of the host-derived proteins, while α -amylase remained active and soluble (Vihinen *et al.*, 1990). The α -amylase was then adsorbed to insoluble corn starch (Sigma; 5 g per 400 ml shock fluid) in the presence of 10% (NH₄)₂SO₄ by gentle agitation for approximately 6 h at 4 °C. The starch slurry was collected on a membrane filter and washed thoroughly with 10 mM Tris/HCl, pH 7.5, 10% (NH₄)₂SO₄, 1 mM CaCl₂. The enzyme was eluted from starch with 4 M urea in 10 mM Tris/HCl, pH 7.5, 1 mM CaCl₂. The eluate was collected in a flask containing an equal volume of the same buffer without urea, so that the urea concentration was immediately reduced to 2 M. The eluate was dialysed extensively against 10 mM Tris/HCl, pH 7.5, 1 mM CaCl₂. As α -amylase is a very stable enzyme (Vihinen & Mäntsälä, 1990), the short exposure to 4 M urea did not denature it or decrease its activity. The yield of α -amylase with this procedure was approximately 80%, resulting in more than 90% pure enzyme preparations as judged from reverse-phase HPLC profiles. From 400 ml cultures, milligram amounts of pure protein were obtained for the subsequent N-terminal amino acid sequence analysis. For sequencing, the enzyme preparations were further purified by reverse-phase HPLC (Bakerbond C₁₈ column, J. T. Baker, Phillipsburg, NJ, USA). A linear gradient of acetonitrile (3–100% in 60 min) in 0.1% trifluoroacetic acid was used for elution. N-terminal amino acid sequence analysis was performed essentially as described by Suominen *et al.* (1988). The frequency of utilization of either of the two processing sites was determined from the relative amounts of the amino acid derivatives released in each cycle of amino acid sequencing. When two parallel sequences were obtained, the relative amounts between the two sequences remained constant throughout all ten cycles of Edman degradation, indicating that the percentage utilization of each cleavage site could be reliably determined.

RESULTS AND DISCUSSION

Construction of α -amylase signal peptide mutations

Site-directed mutagenesis (Kunkel, 1985) was used to make a series of 13 mutations (single and double amino acid replacements or deletions) in the signal peptide region of the *amyS* gene (Suominen *et al.*, 1987a) coding for *B. stearothermophilus* α -amylase. The mutations were verified by DNA sequencing (Sanger *et al.*, 1977; Zagursky *et al.*, 1985). The resulting series of plasmids, designated pCSS20ss, each plasmid having a different mutation in the α -amylase signal peptide, is depicted in Fig. 1. The point mutations were assigned consecutive numbers (ss1, ss2, etc), and the deletions were denoted by Δ and a letter referring to the signal peptide region followed by a code number (ss Δ N1-7, ss Δ H1, etc). The

Fig. 1. Map of pCSS20ss series of plasmids carrying α -amylase signal peptide mutations. All pCSS20ss plasmids are identical except for the mutations in the *amyS* signal peptide coding region. pCSS20ss is derived from pCSS4 (Suominen *et al.*, 1987b). *B. stearo-thermophilus* DNA, contained in the 2526 bp (DraI)–HindIII fragment (the DraI site was lost due to the cloning strategy), was joined to the 2325 bp PvuII–HindIII fragment of pBR322 containing the ColE1 replicon. ss denotes the signal-peptide coding region of *amyS*; *bla* is the β -lactamase coding region of pBR322.

amino acid sequences of the wild-type and mutant signal peptides are presented in Fig. 2. The main features of the mutations are as follows. ss2 and ss8 alter the charge, and ssAN1·7 alters the charge and the length of the N region. ss1 was designed to make two replacements in the extreme N-terminus without altering the charge. ssAN1·6, not merely a deletion, but a fortuitous rearrangement mutation (duplication of the N-terminal Val-Leu-Thr-Phe, followed by the same deletion as that in ssAN1·7) found while screening for ssAN1·7, elongates the signal peptide by one residue, extends the H region by six residues and reduces the net positive charge of the N region. ss3 and ss4 disrupt the hydrophobic H region with a charged (Glu) and a polar (Ser) residue, respectively. In ssAH1 and ssAH2, the first and the second half of the H region are deleted, respectively. In ss6 and ss7 the C region is changed. ss5 is actually in the mature part of the enzyme replacing the Pro₊₃ with Ala. ssAC1 shortens the C region from nine to five residues. In Fig. 2, secondary structure and hydropathy predictions for the wild-type signal peptide are also shown. The predictions show a well-ordered, mainly β , structure, along the entire length of the signal peptide. Although there also seems to be a strong tendency for α -helix formation in the hydrophobic core region, the Chou & Fasman (1978) N- and C-terminal prediction suggests that β -structure is dominant (not

shown). The cleavage site is preceded by a β -turn followed by a short α -helical region. Similar predictions were also made for the mutant signal peptides (not shown). Most mutations had only a minor effect on the predicted structures, with the following exceptions. ssAN1·7 was predicted to be highly random from the N-terminus until the β -turn preceding the cleavage site; ss3 and ss4 had lower probabilities for β -structure around the mutations; ssAC1 almost completely abolished the predicted β -turn preceding the processing site.

When high expression levels of *amyS* are promoted in *E. coli* utilizing, for example, the λ p_L promoter (Suominen *et al.*, 1987b), α -amylase leaks from the periplasm into the culture medium. However, the lower constitutive level of *amyS* expression in *E. coli* carrying any of the pCSS20ss plasmids did not result in leakage of α -amylase activity into the growth medium. Consequently, the production of the wild-type and signal peptide mutant α -amylases was determined by measuring enzyme activities from total-cell lysates after sonication. Bacteria were grown in 2 $\times$ YT medium at 37 °C to early stationary phase. None of the mutations interfered with growth and all cultures reached similar cell densities. The α -amylase activity produced by the wild-type culture amounted to 40 U ml⁻¹. The activity levels reached by the signal-peptide mutants are presented (data from one representative set of experiments) in Table 1. The effects of the point mutations on the level of α -amylase production were in most cases only moderate. The largest effects were observed for deletions that neutralized the N-terminal positive charge (ssAN1·7, 78 % lower), deleted the second half of the H region (ssAH2, 75 % lower), or shortened the C region so that its length was closer to the consensus length for typical *E. coli* exported proteins (ssAC1, 41 % higher). We did not study directly whether the unprocessed precursor of *B. stearo-thermophilus* α -amylase is active or not, so there is a possibility that intracellular accumulation of the precursor is reflected in the activity levels in some of the mutants. However, we found that there were no major deviations in the activity levels measured from the total-cell lysates, compared to the relative yields of the enzymes purified from the periplasm (see Methods).

Processing of the signal peptide in *E. coli*

To assess the consequences of the α -amylase signal peptide mutations for the choice of their processing site, the exported α -amylases from each of the mutant strains were purified from stationary-phase cultures and their N-terminal amino acid sequences were determined. The results are shown in Table 1. The net positive charge of the N-terminus functions in the correct positioning of the signal peptide relative to the membrane for initiating the export (Puziss *et al.*, 1992). The fact that the point mutations in the N region, even those decreasing the net positive charge (ss2 or ss8), did not alter the site preference significantly, is in agreement with the findings of Puziss *et al.* (1992). However, the rearrangement that decreased (ssAN1·6), or the deletion that abolished (ssAN1·7), the N-terminal charge, shifted the cleavage preference towards

Fig. 2. The *B. stearothermophilus* α -amylase signal peptide. The amino acid sequence of the wild-type signal peptide is shown in one-letter code and the original signal peptidase cleavage site (solid arrow) and the secondary cleavage site (dashed arrow) are indicated. Amino acid replacements for each single and double mutation are shown above. Deletions are indicated by two-headed arrows. Mutation ss Δ N1-6 has a duplication of the first eight residues from the wild-type sequence followed by the same deletion as that in ss Δ N1-7; after the indicated residues, the sequence is identical to the wild-type signal peptide. Below the wild-type sequence, the structure predictions in the GCG program PepPlot (Devereux *et al.*, 1984) are shown. Top, α - (dashed line) and β -structure (solid line) prediction; middle, β -turn prediction according to Chou & Fasman (1978); bottom, the Kyte & Doolittle (1982) hydrophobicity index. Although the hydrophobic core region (the H region) also has the tendency to form an α -helix structure, it is predicted to be β -structure, since it is flanked by typical N- and C-termini of β -structure (data not shown; Devereux *et al.*, 1984).

the secondary site and also caused a decrease in the production level of the enzyme (40 and 78 %, respectively). We also conclude that the N-terminal portion of the signal peptide is able to contribute to the cleavage specificity via a mechanism that is not presently understood. The overall length of the signal peptide seems to be irrelevant, since the lengths of ss Δ N1-6 and ss Δ N1-7 differ by eight amino acid residues and yet the secondary processing site is preferred for both of them.

The most striking results were obtained for the C region mutations ss6, ss7 and ss Δ C1, for ss5 residing in the mature part in the vicinity of the original processing site and, surprisingly, for the H region mutation, ss4. ss5 and ss6 mutations apparently increased the specificity of the *E. coli* signal peptidase for the original processing site. Thus, even though a variety of different residues have been found at position -2 in signal peptides and at position +3 in the mature part in other cleavage regions, residues in these positions in α -amylase also appear to contribute to the recognition site for the signal peptidase. ss7, in which the original processing site was altered by a double point mutation, was processed exclusively at the secondary site. In this case, neither one of the sites has the sequence of a typical cleavage site, but the secondary site is closer to the C/H boundary. It has been shown that proline must be excluded from position +1 (Barkocy-Gallagher & Bassford, 1992; Barthelemy *et al.*, 1993). Since glycine is frequently found at position -1, it must be the threonine

at +1 in ss7 that apparently makes the original cleavage site unfavourable. Preference for a shorter C region by *E. coli* signal peptidase is especially evident from the results obtained with the ss Δ C1 mutation. This signal peptide has a five-residue C region and it was processed only at the original site, although both sites were still intact.

An unexpected effect was observed with the ss4 mutation, especially when compared to the ss3 mutation. Both of these mutations cause a small local disturbance in the predicted secondary structure (Chou & Fasman, 1978; Devereux *et al.*, 1984) and both have almost identical overall hydrophobicity over the H region (Devereux *et al.*, 1984; Kyte & Doolittle, 1982). Nevertheless, with ss4 the original processing site is exclusively used, while with ss3 the cleavage preference remains much like that in the wild-type. The result obtained with ss4 is the more puzzling, since truncation of the H region by either ss Δ H1 or ss Δ H2 had almost no effect on the site preference. How a mutation in the middle of a long H region is able to alter the cleavage site preference is not clear. It has been shown by Dev *et al.* (1990) that features other than the minimal cleavage region are important for efficient processing by signal peptidase. Obviously, there are factors affecting the choice of the cleavage site that are yet to be characterized.

The C region of *B. stearothermophilus* α -amylase signal peptide is predicted (Fig. 2) to be composed of a β -turn structure followed by an α -helix which includes the

Table 1. Effects of signal peptide mutations of *B. stearothermophilus* α -amylase on processing of the enzyme in *E. coli*

Mutant	Signal sequence					Relative α -amylase activity (%)*	Processing at site (%)	
	Total	Length			N-terminal net charge		Original†	Secondary‡
		N	H	C				
Wild-type	34	11	14	9	+4	100	70	30
N region mutations:								
ss1	34	11	14	4	+4	53	60	40
ss2	34	11	14	9	+2	59	50	50
ss8	34	11	14	9	+1	92	50	50
ss Δ N1-6	35	6	20	9	+2	60	10	90
ss Δ N1-7	27	4	14	9	0	22	30	70
H region mutations:								
ss3	34	17	8	9	+3	77	50	50
ss4	34	11	14	9	+4	112	100	0
ss Δ H1	27	11	7	9	+4	110	80	20
ss Δ H2	27	11	7	9	+4	25	80	20
C region mutations:								
ss5	34	11	14	9	+4	100	100	0
ss6	34	11	14	9	+4	61	100	0
ss7	31	11	14	6	+4	82	0	100
ss Δ C1	29	11	13	5	+4	141	100	0

* Relative to wild-type, 100% = 40 U ml⁻¹.

† Original wild-type processing site, -Ala-Lys-Ala₃₄↓Ala (see Fig. 2).

‡ Secondary wild-type processing site, -Gly-Gln-Pro₃₁↓Ala (see Fig. 2).

cleavage site. It has been suggested that a β -turn structure in the cleavage site is crucial for protein export (see e.g. Saunders *et al.*, 1991). However, it is possibly the flexibility around the processing site that is important, rather than the actual presence of a β -turn structure. Not much is known about the effects of varying the signal peptide C region of exported proteins (Nilsson & von Heijne, 1991; Sakakibara *et al.*, 1993; Shen *et al.*, 1991), but the dramatic effects of mutations in the C region of *E. coli* β -lactamase (Kadonaga *et al.*, 1985) and the absence of mutations in this region among those that have been screened genetically (see e.g. Benson *et al.*, 1987), strongly suggest that this is the most stringent region in signal peptides. In the signal peptides of *Bacillus* exoproteins this region is generally longer than the average (von Heijne & Abrahmsen, 1989). Recently, Sakakibara *et al.* (1993) reported processing in *B. subtilis* and in *E. coli* of *B. subtilis* α -amylase signal peptide mutants fused to *amyS*. Their results are highly consistent with the major conclusion derived from our study that *E. coli* favours shorter C regions than those usually found in proteins exported by Gram-positive bacteria. Although the authors suggest that the overall length of the signal peptide was the main determinant in cleavage-site recognition, their results showed that when two processing sites were available, *E. coli* always used the cleavage site for which the C region was five residues long. When the bacterium was forced to cleave at a site having a seven- to nine-residue C region, the processing rate declined dramatically.

Concluding remarks

The results presented here demonstrate that in *E. coli*, the presence of two alternative processing sites in the α -amylase signal peptide makes it possible for signal peptidase to cleave at the less favoured site, if the preferred site is made unfavourable by mutation. This provides flexibility in processing of the signal peptide. The C region was shown to constitute the most critical part determining the choice between two alternative processing sites. An optimal length, and also the sequence of the C region, were shown to be particularly crucial in *E. coli*. Surprisingly, some mutations in the H region and even in the N region affected the cleavage-site preference. Based on these results, it may be worthwhile to optimize the length and the sequence of the C region, when planning heterologous protein export in *E. coli*.

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