In Vivo Conversion of L-Serine to d-Alanine in a Ribosomally Synthesized Polypeptide*

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The in vivo conversion of the bacteriocin lactocin S and its encoding gene, discovered three alani-ne-for-serine substitutions which, apparently, is a violation of the genetic code. Subsequent chiral analysis of lactocin S hydrolysates revealed a correlation between d-alanine content and the three substitutions, implying a conversion of L-serine to d-alanine in lactocin S maturation. In order to explain this observation, we suggest a conversion of L-serine to d-alanine in lactocin S maturation. In order to explain this observation, we suggest a conversion of L-serine to d-alanine in lactocin S maturation. In order to explain this observation, we suggest a conversion of L-serine to d-alanine in lactocin S maturation.

In the course of characterizing the bacteriocin lactocin S and its encoding gene, we discovered three ala-nine-for-serine substitutions which, apparently, is a violation of the genetic code. Subsequent chiral analysis of lactocin S hydrolysates revealed a correlation between d-alanine content and the three substitutions, implying a conversion of L-serine to d-alanine in lactocin S maturation. In order to explain this observation, we suggest a sequence of events initiated by the dehy dration of serine, which is common in the biosynthesis of the lanthi-onine-containing polycyclic lantibiotics (Schnell, N., Entian, K.-D., Schneider, U., Götz, E., Zähner, H., Hellner, R. & Jung, G. (1988) Nature 333, 276-278; Jung, G. (1991) Angew. Chem. Int. Ed. Engl. 30, 1051-1068; Bierbaum, G. & Sahl, H.-G. (1993) Zentralbl. Bakteriol. 278, 1-22) and completed by the stereospecific reduction of dehydroala nine residues. The occurrence of non-lanthionine α-carbon stereoinversion in lactocin S maturation substantiates the hypothetical α-epimerization scheme originally put forward by Bycroft (Bycroft, B. W. (1969) Nature 224, 595-597), and we propose a revision of this model to accommodate the lactocin S-type stereoinversion. Lactocin S is the first prokaryotic exception to the rule that only L-amino acids are included in ribosomally synthesized peptides.

The lantibiotics (1) are polypeptide bacterial antagonists characterized by the presence of (25,6R)-meso-lanthionine and/or (25,3S,6R)-3-methylthanolalanine residues, which give the molecules a polycyclic structure through intrachain sulfide bridges. Also common to the lantibiotics are the presence of the α,β-didehydroamino acids, α,β-didehydroalanine and/or α,β-didehydrobutyric acid. The biosynthesis of any lantibiotic proceeds via normal ribosomal assembly of a prepeptide, which is post-translationally modified and processed prior to release of the active lantibiotic from the producer cell (1-3).

Lactocin S, produced by Lactobacillus sake strain L45, is a polypeptide exhibiting bacteriocidal activity toward closely related bacteria (5). At present, only small amounts of lactocin S can be isolated (50 nmol/liter culture), thus limiting the number of practical approaches to structure elucidation. Previous partial characterization (5, 6) revealed an N-terminal blocking group preventing direct Edman degradation of lactocin S. The partial amino acid sequence obtained after CNBr cleavage (6), however, allowed the synthesis of an oligonucleotide, which was used to identify a restriction fragment containing the lactocin S encoding gene, lasA. This fragment was subsequently cloned in Escherichia coli and sequenced.1

EXPERIMENTAL PROCEDURES

Nucleic Acid Manipulation, Amplification, and Sequencing—The lasA-containing restriction fragment was isolated and cloned in E. coli DH5α (7) with pUC18 as a cloning vector using standard cloning techniques (8). The nucleotide sequence of lasA was determined by sequencing both cloned (9) and amplified DNA with Sequenase3 (U. S. Biochemical Corp.) according to the manufacturer’s instructions. Single-stranded templates for sequencing were isolated from amplification reactions using the Dynabeads M-280 system according to the manufacturer’s instructions (Dynal AS). A contiguous sequence from both strands was obtained for both cloned and amplified DNA.

Amino Acid Sequence Determination—Protein sequence analysis of lactocin S was performed as follows. Prior to analysis in a 476A protein sequencer (Applied Biosystems), purified (6) lactocin S was modified following a three-step procedure (10) including thiol addition (H2O2; EtOH:5 N NaOH:propane-thiol, 3:4:1:1, v/v), peroxidation with per-trifluoroacetic acid, and a second thiol addition. In order to detect the modified PTH-amino acids PTH-S-propylcysteine (from dehydroalanine) and PTH-S-propyl-β-methylcysteine (from dehydrobutyric acid), a prolonged high pressure liquid chromatography gradient was used during PTH analysis. Amino Acid Chirality Analysis—Lactocin S was purified from the supernatant of an overnight culture as described previously (6). Peptide fragments were generated by cleaving approximately 20 nmol of purified lactocin S with CNBr (11) or endoproteinase Glu-C (Boehringer Mannheim, cleavage performed according to the manufacturer’s instructions). The cleavage products were separated by SMART (Pharmacia Biotech Inc.) reverse phase chromatography (0.1% trifluoroethanol, linear gradient of 0-100% 2-propanol), and the identities of the individual peaks were established by amino acid composition and electrospray mass spectrometry analysis, which were carried out as described previously (12). After hydrolysis (6 N HCl, 110 °C, 24 h under nitrogen), the samples were dried and derivatized to yield N-trifluoroacetyl-alanine-n-propyl esters, which were separated by gas chromatography on glass capillaries coated with the chiral phase Chiraal-Val (13) (temperature, 75-190 °C). For detection and unequivocal characterization of the peaks we used on-line mass spectrometry with selected ion monitoring, e.g. m/z = 140 for N-trifluoroacetyl-alanine-n-propyl ester.

The chiral analysis of hydrolysates of nisin, which is a lantibiotic related to lactocin S, revealed that the extent of racemization due to hydrolysis and derivatization is below 2% (14). However, in order to address the possible problem of racemization we included nisin Z as a control (see Table I) in the chirality analysis of lactocin S. An addi-

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2 The abbreviation used is: PTH, phenylthiohydantoin.
In Vivo Conversion of L-Serine to D-Alanine

RESULTS AND DISCUSSION

lasA (Fig. 1) was identified on the sequenced fragment by aligning the translated DNA sequence with the partial sequence of lactocin S, which indicated the positions of the residues involved in lanthionine formation as well. Surprisingly, the alignment also revealed a discrepancy involving the residue in position 19 (Fig. 1), where alanine is apparently substituted for the encoded serine (codon UCU). The nucleotide sequence was verified by sequencing DNA amplified from three different producer strains. The amino acid sequence was verified by subjecting uncleaved lactocin S to a modified sequencing protocol through which two additional alanine-for-serine substitutions (in positions 7 and 11, codons UCA and UCC, respectively, Fig. 1) were identified.

Although the possibility that alanine is directly incorporated into these positions of the lactocin S precursor protein cannot be excluded a priori, a more reasonable explanation for the phenomenon is suggested by the model for meso-lanthionine formation. In this process (1), the α,β-unsaturated amino acid didehydroalanine is formed through sequence-specific dehydration of the diol group of a neighboring cysteine residue, which is added to the double bond, thereby forming the meso-lanthionine residue, which may be described as two alanine halves connected by a thioether bridge in addition to the peptide chain. Experimental proof that 2,3-dideoxy-amino acids do serve as intermediates in meso-lanthionine and 3-methylanthionine formation has been provided through the isolation of dehydrated Pep5 and epidermin precursor peptides (12, 15).

The addition reaction takes place with full stereospecificity, as the moity derived from serine appears in the β configuration only (16–19). The apparent similarity of the alanine-for-serine substitutions to the process of lanthionine formation raised the question of whether α-carbon stereoconversion might take place in the former case also. In order to test this possibility, derivatized total hydrolysates of native lactocin S and of isolated cleavage fragments of the peptide were separated by gas chromatography using a chiral stationary phase and online detection with mass spectrometry. The results of these experiments (Table I) show that the 3, 4, and 11, 12 alanine content in lactocin S hydrolysates is indeed high, and from the correlation between measured d-alanine content in the different regions of the molecule and the positions of Ala-for-Ser substitutions identified by mRNA and protein sequencing, we conclude that the positions 7, 11, and 19 are all occupied by d-alanine. The proposed structure of mature lactocin S is presented in Fig. 2.
In Vivo Conversion of L-Serine to D-Alanine

not yet know the precise nature of the lactocin S bacteriocidal activity, and the evaluation of the structural and functional significance of the lactocin S d-alanines is therefore premature at present.

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Fig. 3. Model for stereoinversion of peptide-bound amino acids. Hypothetical scheme for the post-translational introduction of D-alanine into polypeptides. The top line shows the Bycroft α-epimerization sequence (4), which applies to peptides where the corresponding L-enantiomer (alanine here) is incorporated initially, whereas the bottom line outlines the stereoinversion involved in lanthionine formation. The reactions included are: α, dehydrogenation; β, hydrogenation; γ, tautomerization; δ, dehydration; and ϵ, Cys addition/thiol ether bridge formation. The Roman numerals refer to the imine intermediates hypothesized by Bycroft. As indicated, the two schemes in the figure can be connected by the common intermediate dehydroalanine (II), suggesting a mechanism by which the D-alanines of lactocin S may be introduced.