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

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In the course of characterizing the bacteriocin lactocin S and its encoding gene, we discovered three alanine-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 dehydration of serine, which is common in the biosynthesis of the lanthionine-containing polycyclic lantibiotics (Schnell, N., Entian, K.-D., Schneider, U., Götz, F., Zähner, H., Kellner, 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 dehydroalanine 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 (2*S*,6*R*)-*meso*-lanthionine and/or (2*S*,3*S*,6*R*)-3-methyllanthionine 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, *las*A. This fragment was subsequently cloned in *Escherichia coli* and sequenced.[1]

EXPERIMENTAL PROCEDURES

Nucleic Acid Manipulation, Amplification, and Sequencing—The *las*A-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 *las*A was determined by sequencing both cloned (9) and amplified DNA with Sequenase™ (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 (H_2O: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[2]-amino acids PTH-*S*-propylcysteine (from dehydroalanine) and PTH-*S*-propyl-β-methylcysteine (from dehydrobutyrine), 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-amino acid-*n*-propyl esters, which were separated by gas chromatography on glass capillaries coated with the chiral phase Chirasil-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. As an addi-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X79889.

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[2] The abbreviation used is: PTH, phenylthiohydantoin.

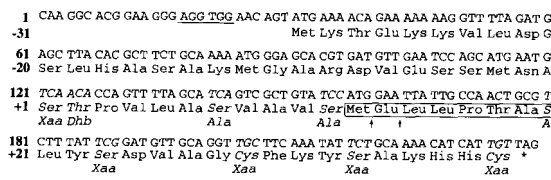

FIG. 1. **Nucleotide sequence and translation of *lasA*.** Below the translation are indicated the positions where the sequence of mature lactocin S (as determined by the modified Edman sequencing, see "Experimental Procedures") deviates from the amino acid sequence predicted by the gene. The numbering of nucleotides is arbitrary, while the numbering of amino acids refers to the deduced point of leader peptidase cleavage. The probable Shine-Dalgarno sequence is *underlined*, and the amino acid sequence from which the oligonucleotide probe was derived is *boxed*. The cleavage points for CNBr and endoproteinase Glu-C are indicated by *vertical arrows*.

tional control, lactocin S was hydrolyzed under deuterium conditions (substituting DCl/D_2O for HCl/H_2O) prior to chromatography and mass spectrometry analysis, which confirmed that the extent of racemization is in the order of 1–2%.

RESULTS AND DISCUSSION

*las*A (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 in 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 serine residues. In a subsequent addition reaction, the thiol group of a neighboring cysteine 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-methyllanthionine 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 moiety derived from serine appears in the D 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 stereoinversion 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 on-line detection with mass spectrometry. The results of these experiments (Table I) show that the D-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 DNA 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.

TABLE I
D-Alanine content of lactocin S

D-Alanine content and molecular masses (measurements carried out in triplicate) of native lactocin S and of lactocin S fragments are shown. Peptides 2 and 3 are the N and C-terminal fragments, respectively, generated by cleavage of lactocin S with cyanogen bromide, whereas peptides 4 and 5 are the corresponding fragments after cleavage with endoproteinase Glu-C. Hydrolyzed nisin Z (27, 28) was included as control in the Ala chirality analysis. D-Alanine content is presented as the percentage of total alanine content. All analyses except for peptides 4 and 5, which were analyzed twice and once, respectively, were carried out in triplicate with excellent base-line separations, and the results were compared with control analyses. MS, mass spectrometry; ND, not determined.

Peptide	D-Ala content	Molecular mass	
		Electrospray MS	Estimated
	%	Da	
1. Lactocin S	35.2 ± 0.9	3764.3 ± 0.7	3764
2. Lactocin S, CNBr-N	48.6 ± 0.3	1046.8	1046
3. Lactocin S, CNBr-C	24.2 ± 0.2	2687.5 ± 0.4	2688
4. Lactocin S, GluC-N	47.9 ± 1.8	1222.8	1223
5. Lactocin S, GluC-C	22.5	2558.4	2559
6. Nisin Z	2.2 ± 0.5	ND	ND

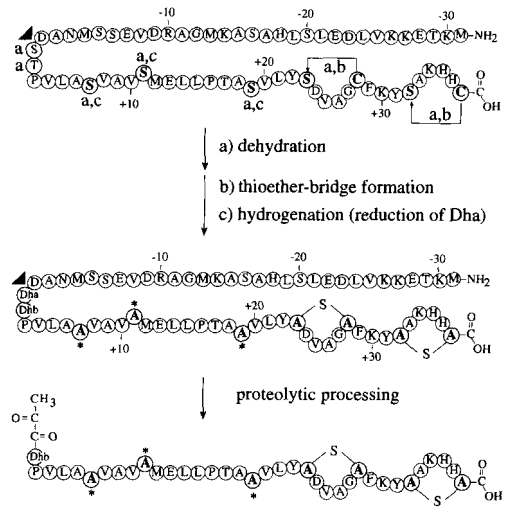

FIG. 2. **Post-translational modification and processing of lactocin S.** Hypothetical maturation of lactocin S and the proposed structure of the mature peptide, which is the longest lantibiotic known and the only known lantibiotic containing D-configurated protein amino acids. The suggested order of events is in agreement with the current model (1) for the maturation of lantibiotics, as are the indicated positions and orientations of the lanthionine bridges. The numbering of amino acids refers to the processing site, which is marked with a *black triangle*. Modified residues are indicated in *boldface*, and the non-lanthionine D-alanine residues are indicated with *asterisks*. The proposed structure of mature lactocin S is based on the experimental results presented above (Fig. 1 and Table I); the presence of the 2-oxopropionyl group in the "N terminus" is deduced from the known instability of N-terminally situated 2,3-didehydro-amino acids.

The results presented above suggest that the lactocin S D-alanines are introduced through a two-step α-carbon stereoinversion reminiscent of lanthionine formation, where the initial dehydration of serine is the key reaction in both cases. However, because the subsequent step(s) in the conversion cannot be explained in terms of known lantibiotic modification reactions, we propose that the process is completed through a stereospecific hydrogenating activity.

As indicated in Fig. 3, this allows the integration of the lactocin S-specific modification with an α-epimerization scheme suggested as a mechanism for introduction of D-amino acids into peptide antibiotics (4, 20). This model, which has been advanced to explain the occurrence of D-amino acids in eukaryotic gene-encoded peptides also (21), postulates a 2,3-

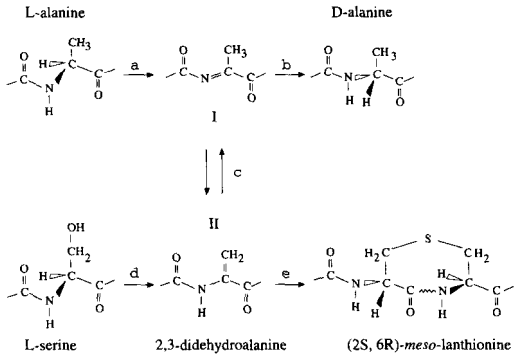

FIG. 3. **Model for stereoinversion of peptide-bound amino acids.** Hypothetical scheme for the post-translational introduction of D-alanine into polypeptides. The *top line* shows the Bycroft α-epimerization sequence (4), which applies to peptides where the corresponding L-enantiomer (alanine here) is incorporated initially, whereas the *bottom line* outlines the stereoinversion involved in lanthionine formation. The reactions included are: *a*, dehydrogenation; *b*, hydrogenation; *c*, tautomerization; *d*, dehydratization; and *e*, Cys addition/thioether bridge formation. The *Roman numerals* refer to the imine (*I*) and enamine (*II*) 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.

didehydro-amino acid as an intermediate in the α-epimerization sequence. Whereas the existence of such an intermediate in D-amino acid formation still remains to be demonstrated, it is highly probable that the D-alanines of lactocin S are derived from α,β-didehydroalanine, and our findings thereby substantiate the principles of the model.

The only known natural peptides where D-alanine is introduced via (an unknown) post-translational modification are the opioid dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) (22) and the related [D-Ala^2]deltorphins I and II (23) isolated from frog skin. In these cases, however, the precursor residues are L-Ala *and not* L-Ser as *in* pre-lactocin S.

Whereas substituting L-Ala for the D-enantiomer in dermorphin completely abolishes biological activity (24), the antimicrobial activity of other D-amino acid-containing peptides secreted from amphibian skin is independent of the chiral status of the affected residues (25). Although lactocin S is structurally similar to the pore-forming (26) type A lantibiotics (2, 3), we do 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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