Proenkephalin-A has been described to generate enkelynins, opioid peptides, and several derived peptides, which display various biological effects, including antinociception and immunological enhancement. Recent-ly, we have isolated from bovine chromaffin granules a new antibacterial peptide, named enkelynin, which corresponds to the bisphosphorylated form of PEAP_{209–237} (Goumon, Y., Strub, J. M., Moniatte, M., Nullans, G., Poteur, L., Hubert, P., Van Dorssemla, A., Aunis, D., and Metz-Boutigue, M. H. (1996) Eur. J. Biochem. 235, 516–525). In this paper, the three-dimensional solution structure of synthetic PEAP_{209–237} was investigated by NMR. These studies indicate that this peptide, which is unstructured in water, folds into an \( \alpha \)-helical structure in trifluoroethanol/water (1/1). NMR data revealed two possible three-dimensional models of PEAP_{209–237}. In both models, the proline residue Pro-227 induces a 90° hinge between two \( \alpha \)-helical segments (Ser-221 and Glu-228 to Arg-232) leading to an overall L-shaped structure for the molecule. The negative charge of PEAP_{209–237} and the low amphipathicity of the two \( \alpha \)-helical segments imply new mechanisms to explain the antibacterial activity of enkelynin.

In order to characterize the molecular mechanism by which enkelynin inhibits bacteria growth, it is important to determine the three-dimensional structure of this antibacterial peptide. In this paper we report the three-dimensional structure of the synthetic nonphosphorylated PEAP_{209–237} in aqueous trifluoroethanol solution, which is the first requirement for the structural analysis of enkelynin. We focused on the conformational changes induced in the active molecule by the phosphorylation of the two serine residues Ser-221 and Ser-223.

In a recent study we have related the antibacterial activity of enkelynin with structural features (16). Using various synthetic peptides, we were able to conclude that the antibacterial activity of enkelynin results from three structural parameters: (i) the length of the peptidic chain, (ii) the endogenous conformations of the three proline residues Pro-212, Pro-214, and Pro-227, and (iii) the phosphorylation of the two serine residues Ser-221 and Ser-223.

In order to characterize the molecular mechanism by which enkelynin inhibits bacteria growth, it is important to determine the three-dimensional structure of this antibacterial peptide. In this paper we report the three-dimensional structure of the synthetic nonphosphorylated PEAP_{209–237} in aqueous trifluoroethanol solution, which is the first requirement for the structural analysis of enkelynin. We focused on the conformational changes induced in the active molecule by the phosphorylation of the two serine residues, Ser-221 and Ser-223. This study leads to the characterization of the COOH-terminal domain of PEA whose biological significance in neuroimmuno-
modulation is revealed by its intact presence in organisms 500 million years divergent in evolution (14). 

EXPERIMENTAL PROCEDURES

Peptide Synthesis—PEAP<sub>209–237</sub> was synthesized in our laboratory on an Applied Biosystems 432A peptide synthesizer, SYNERGY, using the stepwise solid-phase synthetic approach (17) with 9-fluorenylmethoxycarbonyl (Fmoc chemistry). Peptide was further purified by reverse-phase high performance liquid chromatography on a preparative Macherey-Nagel column, Nucleosil RP 300–7C18 (10 million years divergent in evolution (14). 2

### RESULTS

NMR Spectroscopy—The complete proton assignment is presented in Table I and Ha chemical shifts, short and medium range NOE patterns, and <sup>3</sup>J<sub>NH-Ha</sub> coupling constants are summarized in Fig. 1. Only one set of resonances was found for all spin systems of the peptide except for the backbone protons of Leu-213. The structural picture that emerges from the NMR

| Amino acid | <sup>3</sup>J<sub>NH-Ha</sub> (Hz) | Chemical shifts (ppm) |
|------------|-----------------|---------------------|
|            | NH   | Ha   | Hb<sub>1</sub> | Hb<sub>2</sub> | Hb<sub>3</sub> | Hb<sub>4</sub> | Other |
| Phe-209    | 6.2  | 8.48 | 4.24         | 3.28         | 3.16         |           | Hb   | 7.32 |
| Ala-210    | 5.2  | 8.31 | 4.56         | 2.06         | 1.94         | 2.40      | He   | 7.39 |
| Glu-211    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Pro-212    | 7.8  | 7.99 | 4.67         | 1.59         | 1.54         | 1.70      | 0.92  |
| Leu-213 cis| 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Leu-213 trans| 7.8 | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Pro-214    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Ser-215    | 5.2  | 8.31 | 4.24         | 2.06         | 2.04         | 2.44      | 2.38  |
| Glu-216    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Glu-217    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Gly-218    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Gly-219    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Phe-236    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Glu-235    | 7.8  | 8.01 | 4.65         | 1.59         | 1.54         | 1.70      | 0.92  |
| Ser-221    | 4.0  | 8.12 | 4.28         | 3.91         |               |           | Hb   | 7.12 |
| Tyr-222    | 6.0  | 8.14 | 4.46         | 3.07         | 3.11         |           | He   | 6.80 |
| Ser-223    | 6.0  | 8.14 | 4.46         | 3.07         | 3.11         |           | Hb   | 7.12 |
| Lys-224    | 5.2  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Glu-225    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Val-226    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Pro-227    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Glu-228    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Met-229    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Glu-230    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Lys-231    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Arg-232    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Tyr-233    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Gly-234    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Gly-235    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Phe-236    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |
| Met-237    | 7.8  | 8.01 | 4.10         | 2.14         | 0.98         | 0.95      |       |

<sup>a</sup> ND, not determined.
data presented in Fig. 1 corresponds to a conformation with two α-helical segments hinged around Pro-227. The first helical segment extends from residue Ser-215 to Ser-223 and the second one from Glu-228 to Arg-232.

Prolines Cis-Trans Isomerism—Two NOE patterns characteristic of the cis- and trans-isomers of the Leu-213-Pro-214 peptide bond are detected. This observation, together with the presence of two sets of resonances obtained for Leu-213, clearly indicates the presence of a slow conformational exchange between the cis- and trans-conformations for Pro-214. In contrast, this exchange is not observed for the first proline residue Pro-212, for which the trans-isomer could be unambiguously characterized. The analysis of the NOE pattern involving the Pro-227 residue is in favor of Val-226-Pro-227 peptide bond being in the cis-conformation. However, due to the peak overlap that affects the Val-226 Hα-Pro-227 Hα cross-peak, one cannot exclude completely a possible trans-conformation for the Val-226-Pro-227 peptide bond. Therefore two three-dimensional models corresponding to both cis- and trans-isomers of Val-226-Pro-227 peptide bond are presented.

Three-dimensional Model—169 distance constraints were derived from the analysis of the 500-ms NOESY map. Among them, 91 were (i, i + 1) distances, 41 (i, i + 2), 26 (i, i + 3), and 10 were detected between residues separated by more than 3 amino acids. Additional dihedral angle constraints were used for amino acids where NOE pattern, Hα chemical shifts, and $^3J_{NH-H\alpha}$ coupling constants indicated the presence of an helical conformation. Simulated annealing calculations were carried out with both cis- and trans-topology for Pro-227, resulting in two possible conformations for PEP$_{209-237}$. For cis- and trans-isomers of Pro-227, the 25 conformers with the lowest overall energy were selected. None of these structures had distance violations greater than 0.5 Å and showed no dihedral angle deviations greater than 10° from the target value. Average energies for both sets of selected structures are

| Residues | cis | trans |
|----------|-----|-------|
| Residues 1–29 | 2.81 | 2.12 |
| Residues 7–23 | 1.58 | 1.03 |
| NOE (Å) | 0.065 (0.0065) | 0.07 (0.0044) |
| Dihedral restraints (°) | 3.31 (0.42) | 3.04 (0.29) |
| Bond angles (°) | 0.94 (0.025) | 0.85 (0.027) |
| Improper dihedral angles (°) | 0.64 (0.061) | 0.54 (0.066) |
| Noe energies (kcal · mol$^{-1}$) | 19.7 (5.0) | 16.5 (3.3) |
| Dihedrals$^a$ | 14.8 (2.8) | 10.5 (2.6) |
| Lennard-Jones van der Waals$^a$ | -22.0 | -7.0 |
| Ramachandran plot$^b$ | Residues in most favored regions | 95.2% | 85.7% |
| Residues in additional allowed regions | 4.8% | 14.3% |

$^a$ NOE and dihedral energies were calculated using the XPLOR 3.1 force field. The final values of the target function force constants were $k_{NOE} = 50$ kcal · mol$^{-1}$ and $k_{dih} = 200$ kcal · mol$^{-1}$ · rad$^{-1}$.

$^b$ The Lennard-Jones van der Waals energies were calculated on energy minimized average structures using CHARMM version 22 (47). The Ramachandran plot parameters were taken from the PROCHECK-NMR software (15).
given in Table II. These results indicate that NMR experimental data define a single set of structures for each isomer and that both sets of structures are compatible with experimental distance constraints. The superimposition of the 25 lowest energy structures for each Pro-227 conformation is shown in Fig. 2. In both conformations, the two helical segments (Ser-215-
FIG. 3. Ribbon pictures of PEAP<sub>209–237</sub> structures corresponding to the two isomers of the Val-226-Pro-227 peptide bond. A, the cis-isomer; B, the trans-isomer. The side chains corresponding to Ser-221 and Ser-223, which, after phosphorylation, may lead to electrostatic interactions with neighboring glutamate side chains, are indicated. The side chains of proline, tyrosine, and phenylalanine, residues that may play a role in antibacterial activity, are also indicated.
Ser-223, Glu-228-Arg-232) of the peptide are bent with an angle of 90°. This orientation is defined by 8 NOE between residues from both sides of Pro-227. However, the orientation of the second helix (Glu-228-Arg-232) in regard to the first one differs, for the trans- and cis-isomers of Pro-227, by an angle of 120°.

A ribbon diagram of the average structure of the PEAP 209–237 is presented in Fig. 3 for both isomers of Pro-227. Three and a half-canonical helical turns are found in both models, which is in agreement with CD data predicting that a third of the peptide is folded in helical conformation (data not shown). It is worth noting that all aromatic side chains are located on the same side of the helix. The NH₂-terminal part has no regular secondary structure due to the lack of medium range NOE observed in this region. The COOH-terminal part of the peptide, which contains the Met-enkephalin motif, is likely to experience some motional averaging that affects $3J_{\text{NH-H}_a}$ coupling constants and NOE values in a different way. Due to the $1/r^6$ dependence of the dipolar interaction, the helical conformation indicated by the observation of $\text{nn}(i + 3)$ and $\text{nn}(i + 3)$ NOE may be overestimated in the model (Fig. 3) and might correspond to only a fraction of all possible conformations.

**DISCUSSION**

The structural analysis of PEAP<sub>209–237</sub> demonstrates that this peptide adopts an $\alpha$-helical fold in trifluoroethanol/water solution. The folding of peptides in the presence of trifluoroethanol is a behavior that is often found for peptides interacting with membranes and has been widely used to study the three-dimensional structure of these molecules. In relation to antibacterial peptides, trifluoroethanol has been used to study the structure of various antibacterial peptides, including cecropin A (29), pardaxin P-2 (30), magainin 2 (31), and ranalexin (32). Furthermore, comparative study of NMR structures of magainin 2 and ranalexin in the presence of phospholipid micelles confirmed the helical structure previously found in trifluoroethanol/water solution (32, 33).

NMR data indicate that PEAP<sub>209–237</sub> is composed of two helical segments (Ser-215-Ser-223 and Glu-228-Arg-232) separated by the loop Lys-224-Pro-227, the proline residue Pro-227 inducing a 90° bend in the structure. The analysis of PEAP<sub>209–237</sub> models indicates that the helical structures are stabilized by various favorable side chain interactions. The motif Glu-220-Lys-224 located at the end of the first helical segment corresponds to an electrostatic (i, i + 4) interaction that has been found to be a major contribution in stabilizing short helices (34). A similar interaction is found in the second segment, where a salt bridge between Glu-228 and Arg-232 could stabilize the helical conformation. In addition, Pro-227 located at the NH₂-terminal end of the second helix appears to be a good helix initiator as based on the NH₂-capping process (35, 36). Furthermore, the fractional helical conformation observed in the enkephalin motif from NOE data is probably stabilized by the side chain interactions of the two COOH-terminal aromatic residues Tyr-233 and Phe-236 (37). The hydrophobic character is expected to mediate the antibacterial activity. In enkelytin, hydrophobic residues Phe-209, Phe-236, and Met-237 are located on the NH₂- and COOH-terminal ends of the molecule and furthermore the aromatic side chain of Phe-209, Tyr-233, Phe-236, and Met-237 are located on the same helical face. Recently, it has been demonstrated that the residue F1 in the ranalexin peptide is essential for the expression of the antimicrobial activity (32), and studies about brevinins, gaegurins, and temporin (38) suggest the importance of this residue to induce peptide insertion.

The position of the two helical segments observed for PEAP<sub>209–237</sub> peptide correlates well with a previous NMR study performed in our laboratory on the synthetic PEAP<sub>224–237</sub> in 70% trifluoroethanol (data not shown). In this shorter peptide, NMR data indicated the presence of a helical conformation for residues Glu-228 to Gly-234. Thus, the comparison...
of the NMR data obtained for the short (PEAP$_{209-237}$) and longer (PEAP$_{209-237}$) COOH-terminal fragments of PEA leads to the conclusion that the folding of the COOH-terminal part of the PEAP$_{244-237}$ is not affected by the addition of the NH$_2$-terminal sequence.

Many studies have focused on the three-dimensional structure of the Met-enkephalin sequence (YGGFM), because of its properties to be able to bind to opioid receptors and induce morphine-like effects (1). A wide range of conformations has been found for enkephalin peptides in crystal (39), in Me$_2$SO solution, or bound to membranes (for review, see Ref. 40), most of them involving $\beta$ turn and $\gamma$ turn conformations, but no helical conformation has yet been described. It has been reported that any sequence addition to the NH$_2$ terminus of enkephalin peptides leads to the inhibition of binding to the opiate receptor (41). This inhibition may either be due to a steric hindrance or result from the induction of an helical conformation by the additional NH$_2$-terminal sequence, a phenomenon also suggested by our present results.

The PEAP$_{209-237}$ sequence contains three proline residues, two of these (Pro-214, Pro-227) being strictly conserved in two of these. This feature is in favor of a different mechanism relative to the first one by an angle of 120°. This conformational change, which preserves a spatial proximity between two potentially phosphorylated serine residues (Ser-221, Ser-223), located in the first helical segment, and two glutamic residues, located in the COOH-terminal helical segment (Glu-228, Glu-230), may be involved in triggering the antibacterial activity of the peptide. The electrostatic interactions induced by the spatial proximity between phosphorylated serine and glutamate residues may also be critical in inducing the active conformation.

The antibacterial bisphosphorylated PEAP$_{209-237}$, named enketylkin, shares properties with some other antibacterial peptides: helix-kink-helix (30, 45), hydrophobic residues located at the ends of the molecule. However, the surface potential calculated for this peptide highlights its overall negative charge (Fig. 4). This feature is in favor of a different mechanism than the one proposed previously for positively charged compounds like ceporins or defensins, which form large pores in the bacterial membrane by direct peptide-membrane electrostatic interactions (46). Moreover, the amphipatic character of the two helical segments of PEAP$_{209-237}$ is very low compared with pore-forming peptides.

Thus, the structural features of this COOH-terminal PEAP$_{209-237}$ indicate that enketylkin may express its antibacterial activity by different potential mechanisms: (i) a pore-forming or carpet-like mechanism by interaction of its three basic residues with the negative phospholipids and/or glycolipids on bacterial membrane; (ii) a linkage with potential bacterial membrane receptors, including enzymes, pumps, or transporters; (iii) the binding of di- or trivalent ions necessary for the bacteria growth. The $^1$H NMR study of the synthetic bisphosphorylated PEAP$_{209-237}$ and of (Ser $\rightarrow$ Glu) mutants are currently in progress in our laboratory to address the role of electrostatic interactions involved in the antibacterial activity of enketylkin.
