CMLS, Cell. Mol. Life Sci. 55 (1999) 812–818
1420-682X/99/050812-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Two new aminopeptidases from *Ochrobactrum anthropi* active on D-alanyl-p-nitroanilide

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Received 7 December 1998; received after revision 15 March 1999; accepted 22 March 1999

Abstract. Two new enzymes which hydrolyse D-alanyl-p-nitroanilide have been detected in *Ochrobactrum anthropi* LMG7991 extracts. The first enzyme, DmpB, was purified to homogeneity and found to be homologous to the Dap protein produced by *O. anthropi* SCRC C1-38 (ATCC49237). The second enzyme, DmpA, exhibits a similar substrate profile when tested on p-nitroanilide derivatives of glycine and L/D-alanine, but the amounts produced by the *Ochrobactrum* strain were not sufficient to allow complete purification. Interestingly, the DmpA preparation also exhibited an L-aminopeptidase activity on the tripeptide L-Ala-Gly-Gly but it was not possible to be certain that the same protein was responsible for both p-nitroanilide and peptide hydrolysing activities. The gene encoding the DmpA protein was cloned and sequenced. The deduced protein sequence exhibits varying degrees of similarity with those corresponding to several open reading frames found in the genomes of other prokaryotic organisms, including Mycobacteria. None of these gene products has been isolated or characterised, but a tentative relationship can be proposed with the NylC amidase from *Flavobacterium* sp. K172.

Key words. Peptidase; stereospecificity; amidohydrolase.

Aminopeptidases release the amino-terminal residue from peptide substrates. Most are L-aminopeptidases and exhibit a wide variety of molecular masses, quaternary structures, catalytic residues and specificity profiles [1–10]. So far, the only aminopeptidase active on peptides containing N-terminal D-residues is that from *Ochrobactrum anthropi* SCRC C1-38. It has been characterized and named D-aminopeptidase (Dap) (E.C 3.4.11.19) [10, 11]. Its sequence exhibits 25% identity with that of the *Streptomyces* R61 DD-carboxypeptidase and the residues most important for the catalytic mechanism of β-lactamases and DD-carboxypeptidases [11–15] appeared to be conserved. Here we report the isolation and purification of a similar D-aminopeptidase from *O. anthropi* LMG7991. Moreover, the same strain produces a second enzyme which hydrolyses D-alanyl-p-nitroanilide but not its N-acetylated derivative, and thus represents a new potential member of the D-aminopeptidase family. The study of D-aminopeptidases will contribute general information on this poorly characterized enzyme group and will help to elucidate the possible evolutionary relationship between D-aminopep-

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tidases and DD-carboxypeptidases. The discovery of D-stereospecific enzymes would also offer new tools for enzymatic stereoselective synthesis in organic chemistry [16, 17].

Materials and methods

Enzymes, chemicals and antibodies. Molecular biology and sequencing kits, oligonucleotides, plasmids, purification gels and columns and Ampholine PAG plates were supplied by Pharmacia Biotech (Uppsala, Sweden). The restriction enzymes were purchased from Life Technologies (Merelbeke, Belgium) and from Eurogentec (Liège, Belgium), and T4 DNA ligase from Boehringer Mannheim (Germany). Substrates and peptides were from Bachem (Bubendorf, Switzerland) and Sigma (Bornem, Belgium). Rabbit anti-DAP antibodies were obtained from Gamma (Liège, Belgium).

Selection of D-aminopeptidase-producing strains. Four strains isolated from soil were obtained from the LMG Culture Collection (Gent, Belgium). Two were *O. anthropi* (LMG3306 and LMG7991) [18] and the others were uncertainly classified as related *Achromobacter* species (LMG1293 which corresponds to ATCC25297 and LMG3499). Cells were grown at 28 °C in nutrient agar medium (1 g beef extract, 2 g yeast extract, 5 g peptone, 5 g NaCl, optionally 15 g agar, 1 l H_2O, adjusted to pH 7.4). Cells were stored at − 70 °C in 20% glycerol (v/v).

Crude cell extracts obtained by sonication were added to 10 mM D-alanyl-p-nitroanilide (D-Ala-p-Na). Samples heated at 100 °C for 5 min were also tested to verify the absence of non-enzymatic cleavage of the substrate. Extracts from *O. anthropi* strains LMG7991 and 3306 exhibited D-aminopeptidase activity. *Achromobacter* extracts and heated *O. anthropi* samples did not significantly hydrolyse D-Ala-p-Na.

Production and partial purification of the aminopeptidase activities produced in *O. anthropi* LMG7991 cells. The cells stored at − 70 °C were plated on nutrient medium and grown at 28 °C. Three colonies were resuspended directly in 250 ml of BPY medium and grown for 16 h at 28 °C. This preculture was used to inoculate 15 l of BPY medium in a 20-l fermentor and the culture was grown for 20 h at 28 °C. Cells were collected, resuspended in 750 ml of Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA buffer and disrupted with a Constant System Basic Disintegrator (Cell°D, France). The D-Ala-p-Na-hydrolysing activity was found in the supernatant. The total extract was incubated for 16 h with benzonase in the presence of 1 mM $MgCl_2$ on ice and the insoluble material eliminated by centrifugation and filtration. The D-aminopeptidase activity was subsequently recovered in the fraction precipitating between 30 and 95% $(NH_4)_2SO_4$ saturation. After centrifugation, the pellet was redissolved in Tris-EDTA buffer and dialysed against the same buffer. The solution was then diluted fivefold in 10 mM Tris pH 8.0 and the proteins adsorbed on DEAE cellulose. The ion exchanger was filtered, washed with 10 mM Tris-HCl pH 8.0 containing 0.1 M KCl and eluted batchwise with 10 mM Tris-HCl pH 8.0 containing 0.3 M KCl. After filtration, concentration and dialysis against 10 mM potassium phosphate pH 6.0, proteins were loaded on a QSFF column equilibrated with 50 mM potassium phosphate pH 6.0. The exchanger was washed with 0.1 M NaCl in the same buffer and eluted with a linear 0.1–0.3 M NaCl gradient. Two separate peaks exhibiting activity towards D-Ala-p-Na were eluted at NaCl concentrations of 0.15 M and 0.25 M (fig. 1).

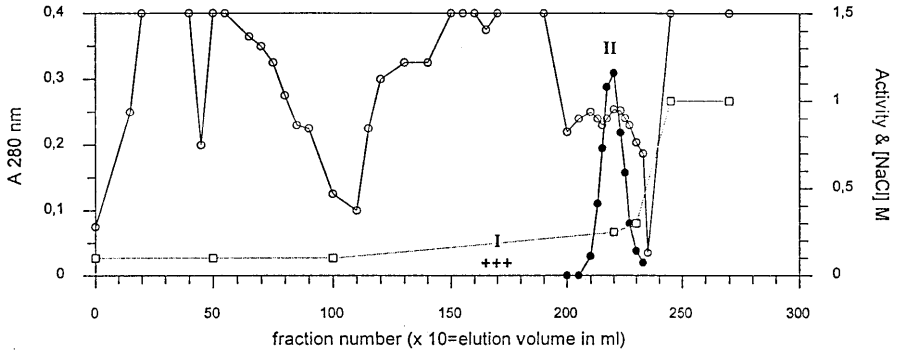

Figure 1. Elution of the DmpA and DmpB proteins from the QSFF column at pH 6.0. Activity is the v_0 value (ΔA/s) measured for 25-µl aliquots added to 500 µl of 80 µM D-Ala-p-Na in 100 mM Tris-HCl pH 8.0, at 30 °C [○, A 280; ●, measured activity ($\Delta A \times 10^3$ s^{-1}); + + +, detected activity (this activity was too low to be accurately quantified); □, NaCl concentration]. I, 0.15 M NaCl peak containing DmpA; II, 0.25 M NaCl peak containing DmpB.

(1) The active fractions eluted at 0.15 M NaCl were pooled, dialysed against 10 mM Tris-HCl pH 8.0 containing 0.2 M KCl and concentrated. This sample was filtered through a Superdex 75 molecular sieve column equilibrated in the same buffer, and the active fractions were analysed by electrophoresis on denaturing and non-denaturing polyacrylamide gels, followed by Coomassie blue staining. Zymograms were obtained with the gels run under non-denaturing conditions, by contact with a piece of filter paper previously soaked in a 10 mM solution of D-Ala-p-Na. This enzyme was named DmpA.

(2) The enzyme eluted at 0.25 M NaCl was purified to 90% by successive chromatographies on QSFF, Phenyl Sepharose Fast-Flow and Sephadex G75 with the help of an Åkta Explorer apparatus (Pharmacia Biotech, Sweden). Active fractions were loaded on a non-denaturing 8% polyacrylamide gel and the activity detected by a D-Ala-p-Na zymogram. The protein was eluted from the piece of gel containing the activity, concentrated and analysed by SDS-PAGE. This enzyme was named DmpB.

Preparation of genomic DNA. *O. anthropi* cells were grown in the nutrient medium to a final absorbance at 600 nm (A_{600}) of 1.5. Genomic DNA was prepared as described for the preparation of *Streptomyces* chromosomal DNA [19, 20], and was dissolved in water by gentle overnight stirring at 20 °C.

***O. anthropi* LMG7991 DNA library construction in pUC18.** An incomplete digestion of *O. anthropi* LMG7991 DNA by *Eco*RI was performed by incubating the genomic DNA for 30 min at 37 °C with *Eco*RI (0.5 U *Eco*RI and 0.1 μg DNA/μl) yielding fragments ranging from 1 to 10 kb. This digested genomic DNA (2 μg) was purified by phenol-chloroform extractions, and precipitated. A total of 500 ng of *Eco*RI-precut and dephosphorylated pUC18 plasmid, T4 DNA ligase and ligation buffer were directly added to the pellet and mixed. After overnight incubation at 16 °C, the ligation mixture was heated at 65 °C for 10 min and used to transform *Escherichia coli* DH5α competent cells [21]. Transformed cells were spread on LB + agar plates containing 50 μg/ml ampicillin, 20 μg/ml X-gal and 100 μM isopropyl thiogalactoside. Untransformed DH5α cells did not hydrolyse D-Ala-p-Na. After replication onto nitrocellulose filters (Hybond C), the D-aminopeptidase activity was detected as described by Asano et al. [11]. Among the 4000 analyzed colonies, one yellow clone (resulting from the hydrolysis of D-Ala-p-Na into p-nitroaniline) was found. After transformation with the plasmid isolated from this clone, the recombinant DH5α cells exhibited high hydrolytic activity on D-Ala-p-Na.

Plasmid analysis, subcloning and sequencing. The plasmid (pDML1100) extracted from the positive colony contained a 4.0-kb insert. The insert size was successively reduced to 2.1 kb (pDML1101) and 1.6 kb (pDML1102). Further details concerning these constructs are available from the authors. The colonies harbouring pDML1102 produced the desired activity and it was concluded that the corresponding gene was located in this fragment. Inserts of reduced sizes were obtained with the help of the Double-Stranded Nested Deletion Kit and were directly sequenced with Reverse (RP) and universal (UP) primers. Sequencing reactions were carried out directly on the two strands with the AutoRead Sequencing Kit and the Thermo Sequenase Labelled Primer Cycle Sequencing Kit for PCR sequencing. Sequences were read on an Automated Laser Fluorescent DNA Sequencer (Pharmacia). Some DNA compression areas were sequenced with ^{35}S-dATP using the T7-Sequencing Kit and Deaza G/A Sequencing Mixes. Analysis of the sequences was performed with the help of the GCG GelAssemble program.

Chemical procedures. Protein N-terminal sequences were determined as described elsewhere [22] on an Applied Biosystems (Perkin Elmer, Foster City, CA) pulsed liquid sequenator. M_r values were estimated by SDS-PAGE (10% polyacrylamide). Isoelectric points were measured by isoelectrofocusing (IEF) on Ampholine PAG plates pH 3.5–9.5, detection of the active bands with the D-Ala-p-Na substrate and measurement of the pH at the position of the active protein. D-aminopeptidase activity could be similarly detected after electrophoresis on non-denaturing 8% polyacrylamide gels by the same zymogram technique [23]. Protein concentrations were estimated on the basis of the absorbance at 280 nm or with the help of the BCA Protein Assay kit (Pierce, Rockford, IL).

D-Aminopeptidase assays and kinetic measurements. The enzyme activity on D-Ala-, L-Ala- and Gly-p-nitroanilides was measured in 100 mM Tris-HCl pH 8.0, at 30 °C by monitoring the variation of absorbance at 405 nm ($\Delta\varepsilon = 11{,}500\ M^{-1}s^{-1}$) [24]. Estimated errors on v_0 values were $\pm 5\%$. The degradation of non-chromogenic peptides was followed by thin layer chromatography analysis (TLC) on silica gel and the amine group of the products visualised by reaction with ninhydrin.

Substrate acetylation. D-Ala-p-Na was dissolved in 50 mM potassium phosphate pH 8.0 buffer. The pH was then adjusted to 9.5 with 1 M KOH, and the solution was chilled on ice. One equivalent of acetic anhydride was added dropwise to the substrate solution under continuous stirring. The reaction mixture was then incubated on ice under vigorous stirring until no ninhydrin-positive compound could be detected. The pH of the solution was adjusted to 8.0 with 1 M KOH. The

Table 1. Activity of the DmpA protein on p-nitroanilide substrates. The *O. anthropi* partially purified enzyme (4–40 μg) or crude extracts (corresponding to 50–500 μl of culture) of *E. coli* XL1-blue cells harbouring the pUC18 or pDML1102 plasmids were incubated with 1 mM substrate in a total volume of 400 μl of 50 mM potassium phosphate buffer pH 8.0 containing 10% dimethylsulphoxide. v_0 values are in nmol/min and SD values ± 5%

Substrate	Partially purified enzyme		Crude extracts			
	v_0	Relative v_0	pUC18 v_0	pDML1102 v_0	DmpA* v_0	Relative v_0
Gly-p-Na	3.2	100	0.011	0.70	0.69	100
D-Ala-p-Na	0.95	30	Not detectable	0.18	0.18	26
L-Ala-p-Na	0.29	9	0.14	0.20	0.06	9

* The DmpA activity was obtained by subtracting the activity of cells harbouring pUC18 from that of cells harbouring pDML1102.

acetylated substrate solution thus contained 1 equivalent of potassium acetate. It was verified that this salt did not influence enzyme activity.

Results

Presence of two distinct D-Ala-p-Na-hydrolysing activities in *O. anthropi* LMG7991. Elution of the QSFF column at pH 6.0 yielded two distinct active fractions, centred at 0.15 and 0.25 M NaCl respectively, (fig. 1). The active protein in the latter (DmpB) was purified to 90% homogeneity and its N-terminal sequence, MKFDLSALETFVRTIPQHYKTPXRAVAV(L)KD-X(K), exhibited nearly 60% identical residues with the Dap protein described by Asano et al. [11]. Moreover, its size (55 kDa as determined by SDS-PAGE) and specific activity against D-Ala-p-Na were also similar to those of Dap, although its isoelectric pH value was slightly higher (4.9 vs 4.6 measured for the two enzymes under the same conditions). DmpB cross-reacted with rabbit anti-Dap antibodies.

The DmpA protein. After the Superdex 75 molecular sieve chromatography, the DmpA protein was still quite impure as shown by both non-denaturing and SDS-gel electrophoreses. In fact, the activity revealed by the zymograms obtained after non-denaturing electrophoresis did not even correspond to one of the major bands revealed by Coomassie blue staining but this activity migrated to a position clearly distinct from those of DmpB and Dap (not shown). Since the amount of DmpA in the preparation was not sufficient, no further attempt was made to purify the protein. A zymogram obtained after gel electrofocusing indicated a pI value of about 5.0.

Preliminary characterisation of the catalytic properties of the enzyme was, however, performed and showed the following relative activities with 1 mM substrates: Gly-p-Na > D-Ala-p-Na > L-Ala-p-Na (table 1). N-Acetyl-D-Ala-p-Na was not significantly hydrolysed, thus indicating a clear aminopeptidase specificity. This was confirmed by the fact that hydrolysis of the tripeptide Ala-Gly-Gly yielded alanine and Gly-Gly as shown by TLC analysis. However, significant hydrolysis was only observed with L-Ala-Gly-Gly and not with D-Ala-Gly-Gly, suggesting that the stereospecificity was modified compared to that prevailing with the p-nitroanilide derivatives.

Cloning and sequencing of the *dmpA* gene. To obtain larger amounts of the DmpA protein, the corresponding gene was cloned. After growth at 37 °C, the *E. coli* cells harbouring the pDML1102 plasmid contained inclusion bodies but also a high amount of soluble D-Ala-p-Na-hydrolysing activity. Crude extracts exhibited an activity pattern similar to that observed with the original DmpA protein produced by *O. anthropi* (table 1). When the *E. coli* cells harboured pUC18, the crude extracts only hydrolysed L-Ala-p-Na significantly. With pDML1102, the hydrolysis of Gly-p-Na and D-Ala-p-Na could easily be measured and the relative reaction rates corresponded well with those observed with the partially purified enzyme. Neither of the crude extracts hydrolysed D-Ala-Gly-Gly. The 2.1-kb *Eco*RI fragment of pDML1101 was completely sequenced on both strands (fig. 2). The sequence has been deposited in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under the accession number X97669. Since production of the desired activity occurred in only one orientation of the insert in pDML1102, it was concluded that the open reading frame (ORF) encoding the enzyme was under the control of the *lacZ* promoter of the plasmid and was thus on the DNA strand downstream of this promoter. This strand was translated in the three reading frames and the codons in each frame were compared with a codon frequency table deduced from the sole presently sequenced gene of *O. anthropi* (the dap D-aminopeptidase gene of *O. anthropi* SCRC C1-38). This analysis revealed one ORF encoding a 375-residue polypeptide in the 1.6-kb fragment (fig. 2), downstream of the *lacZ* promotor, strongly suggesting that this ORF corresponded to the *dmpA* structural gene and that the 280 bp preceding the initiation codon did not contain the native promoter. The 375-residue

polypeptide did not exhibit any similarity with the sequence of the Dap protein determined by Asano et al. [11].

Search for Dap (DmpB) proteins in various *O. anthropi* strains. Western blot analysis of cell extracts of *O. anthropi* LMG3306, LMG7991 and SCRC C1-38 strains with anti-Dap antibodies showed that all strains contain a Dap-like protein exhibiting an M_r value similar to that of Dap (DmpB in the LMG7991 strain). No such protein

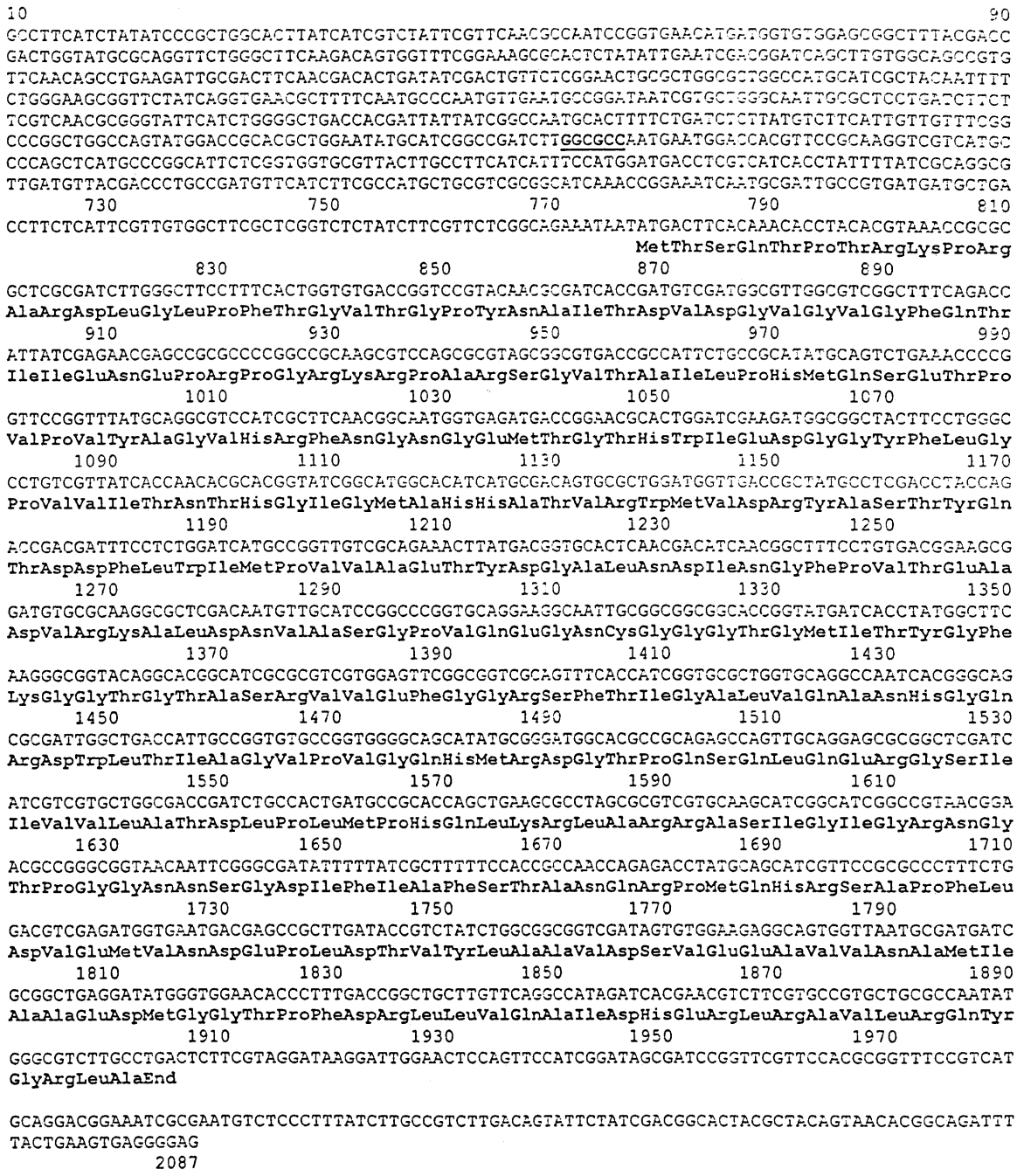

Figure 2. Sequenced *O. anthropi* LMG7991 genomic DNA fragment containing the *dmpA* gene, and the derived DmpA protein sequence. Base numbering starts at the first base of the complete sequence. Start codon ATG: 778–780; stop codon TGA: 1903–1905; *dmpA* open reading frame: 778–1903; putative Shine-Dalgarno sequence: 769–773. GGCGCC (bold type and underlined) at positions 501–506: *Nar*I site used for the construction of pDML1102. When inserted in pUC18, the 1.6-kb fragment starting at the *Nar*I site is sufficient to result in overproduction of the activity in *E. coli* cells.

was found in the *Achromobacter* extracts, in agreement with the absence of D-Ala-p-Na-hydrolysing activity in these strains.

Discussion

A new enzyme which hydrolyses Gly-p-Na, D-Ala-p-Na and L-Ala-p-Na has been purified from *O. anthropi* LMG7991 extracts. Its N-terminal sequence, M_r value and catalytic properties indicate that it is similar to the Dap protein, previously described by Asano et al. [11]. Crystallization attempts are now underway. The crystal structures of these proteins should shed further light on their relationship to the DD-peptidase of *Streptomyces* R61.

However, the LMG7991 strain produces a second protein which also hydrolyses the same glycine and alanine p-nitroanilide derivatives. In the latter case, preliminary experiments indicated a clear specificity for the D-isomer and acetylation of the free amino group of D-Ala-p-Na decreased the activity to non-detectable levels. Surprisingly, when assayed on simple tripeptides, the preparation only liberated the N-terminal L-Ala residue of L-Ala-Gly-Gly. It is possible that the two activities are due to different proteins, since the partially purified sample was far from homogeneous but the fact that the cellular extracts of *E. coli* harbouring the pDML1102 plasmid exhibited a similar specificity pattern strongly suggests that the same protein was responsible for the hydrolysis of the p-nitroanilides and the peptide. If this is confirmed, an interesting reversal of the stereospecificity occurs upon modification of the substrate structure. However, the small amounts available precluded purification of the p-nitroanilide-hydrolysing enzyme to protein homogeneity.

Cloning experiments yielded a 1.6-kb fragment whose insertion in pUC18 yielded high production of D-Ala-p-nitroanilide-hydrolysing activity only when the insert was oriented so that an ORF encoding a novel 375-residue polypeptide was under the control of the *lacZ* promoter of pUC18, indicating that this ORF corresponded to the *dmpA* gene. A search of the data banks revealed that the genomes of *Pseudomonas fluorescens*, *Pyrococcus horikoshii*, *Bordetella pertussis* and *Pseudomonas aeruginosa* contained ORFs encoding putative proteins exhibiting a very high degree of similarity with DmpA. Pairwise comparisons of these putative proteins with DmpA performed with the Bestfit program showed 40–47% identity with scores ranging from 37 to 45 standard deviation units (sdu). Since scores higher than 15 sdu are considered as highly indicative of significant isology, these proteins probably possess enzymatic activities similar to that of DmpA which would then not be specific to *O. anthropi*. Thus, other presently uncharacterized DmpA-like proteins may well be present in several other bacteria some of which are well-known pathogens.

Moreover, the genomes of *M. tuberculosis* and *M. leprae* contain ORFs which also encode unknown proteins similar to DmpA, although with somewhat lower scores (10 and 13 sdu, and 20 and 30% identity, respectively). Surprisingly, these two putative mycobacterial proteins exhibit a reliable similarity (about 58% identity with a score of 15 sdu) with the well-characterized NylC amidase from *Flavobacterium* sp. K172 [25]. This latter enzyme hydrolyses nylon oligomers by a progressive removal of 6-aminohexanoic acid units from the amino terminus. Although this amidase does not hydrolyse other linear amides, dipeptides, tripeptides or casein, it is considered as a linear amidase. It can therefore be hypothesised that DmpA, NylC and the other proteins mentioned above are members of a new amidase superfamily.

Acknowledgments. This work was supported, in part, by the Belgian Program of Interuniversity Poles of Attraction (PAI no. P4/03), by a grant of the FNRS (Brussels) which allowed the purchase of the Åkta-Explorer apparatus, an Action Concertée with the Ministère de l'Education, de la Recherche et de la Formation (ARC 93/98-170) and a Geconcerteerde Onderzoeksactie of the Flemish Government (120.522.93). L.F., B.J. and C.G. are respectively Aspirant and Chercheurs qualifiés, respectively, of the Fonds National de la Recherche Scientifique (FNRS, Brussels, Belgium). We thank C. Duez for her help and suggestions in molecular genetic methods.

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