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Eukaryotic precursor proteins are processed by *Escherichia coli* outer membrane protein OmpP

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A new specific endopeptidase that cleaves eukaryotic precursor proteins has been found in *Escherichia coli* K but not in *E. coli* B strains. After purification, protein sequencing and Western blotting, the endopeptidase was shown to be identical with *E. coli* outer membrane protein OmpP [Kaufmann, A., Stierhof, Y.-D. & Henning, U. (1994) *J. Bacteriol.* **176**, 359–367]. Further characterization of enzymatic properties of the new peptidase was performed. Comparison of the cleavage specificities of the newly found endopeptidase and that of rat mitochondrial processing peptidase (MPP) showed that patterns of proteolytic cleavage on the investigated precursor proteins by both enzymes are similar. By using three mitochondrial precursor proteins, the specificity assigned to OmpP previously, a cleavage position between two basic amino-acid residues, was extended to a three amino-acid recognition sequence. Positions +1 to +3 of this extended recognition site consist of an amino-acid residue with a small aliphatic side chain such as alanine or serine, a large hydrophobic residue such as leucine or valine followed by an arginine residue. Additionally, structural motifs of the substrate seem to be required for OmpP cleavage.

Keywords: outer membrane protein OmpP; *Escherichia coli*; specific endopeptidase; eukaryotic precursor proteins.

By using radioactively labeled mitochondrial precursor proteins as substrates to detect the function of recombinant rat mitochondrial processing peptidase (MPP) in *Escherichia coli* DH5αF′I^q crude extracts, we became aware of a similar specific proteolytic activity, which was provided by the host bacterium itself. After identifying the newly found specific peptidase as *E. coli* outer membrane protein OmpP by protein purification and protein sequencing, we wished to characterize this enzyme further to learn more about cleavage conditions and specificities.

To date, little is known about the function of *E. coli* outer membrane peptidase OmpP. It is an endopeptidase of 33 kDa that preferentially cleaves between two basic amino-acid residues [1]. OmpP was characterized first as the receptor protein for *E. coli* phage Ox2h12h1.1, a mutant of *E. coli* phage Ox2h1. Digest with proteinase K suggests a 155-amino-acid N-terminal moiety exposed on the cell surface, possibly carrying the center of enzymatic action. Synthesis of OmpP is thermoregulated and under control of the cAMP system.

Of the 25 endopeptidases identified in *E. coli*, only five are known to be associated with the *E. coli* outer membrane [2]. The earliest known *E. coli* outer membrane-associated peptidases were *E. coli* signal peptidases I [3] and *E. coli* signal peptidase II [4] as well as *E. coli* protease V [5]. More recently, outer membrane proteins OmpT [6,7] and OmpP [1], two peptidases sharing 87% identity with each other, have been identified.

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Abbreviations: MPP, mitochondrial processing peptidase; pF^{1}β, F^1-ATPase β-subunit precursor; pFe/S, ubiquinol-cytochrome *c* reductase iron–sulfur subunit precursor; pMDH, malate dehydrogenase precursor; pMe$_2$GlyDH, dimethylglycine dehydrogenase precursor; pMUT, methylmalonyl-CoA mutase precursor; pOTC, ornithine transcarbamylase precursor.

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OmpT is an endopeptidase with a narrow specificity, cleaving predominantly between basic amino-acid residues. Among the protein substrates cleaved are ferric enterobactin receptor protein [8], certain secreted fusion proteins [9] as well as T7 RNA polymerase, where cleavage occurs between adjacent lysine and arginine residues [6]. OmpT activity is sensitive to $CoCl_2$, $ZnCl_2$, $CuCl_2$, $FeSO_4$, and to proteinase inhibitors like diisopropylfluorophosphate, but only slightly to phenylmethanesulfonyl fluoride. In contrast, it is not affected by $MnCl_2$, $CaCl_2$, and $MgSO_4$. In the presence of metal chelators like EDTA or *o*-phenanthroline OmpT activity is enhanced [7]. Like for OmpP, there are only few data about the physiological role of OmpT. It is probably a serine protease; protein purification data hint that it is present within the membrane in an oligomeric form.

OmpP as well as OmpT are not present in all *E. coli* strains. Neither enzyme is required for growth of *E. coli* under normal laboratory conditions.

MATERIALS AND METHODS

Bacterial strains, protein precursors, and chemicals

E. coli DH5α F′I^q [F′/*endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* (ϕ80 *dLac-*Δ(*lacZ*)*M15*)] [10]; *E. coli* CJ236 [F′*cat* (= pCJ105; M13^{s}Cm^r)/*dut ung 1 thi-1 relA1 spoT1 mcrA*] [11]; *E. coli* JM109 [F′*traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/e14$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* ($r_K^-m_K^+$) *relA1 supE44 recA1*] [12]; *E. coli* UH300 [F$^-$ *araD134* Δ*lacU169 rpsL relA thi recA56 proA* or *B* F′ *lacI*^{q} Δ*(lacZ)M15 proA*$^+$*B*$^+$] [13]; *E. coli* UH300 *ompA*, and *E. coli* UH300 *ompC*, provided by Dr U. Henning, Tübingen, Germany; *E. coli* KL442 [F$^-$ *metE70 trpA605 rpsL171 gyrA19 recB21 sbcB15 sbcC250* λ*clnd*$^-$] [14]; *E. coli* N4830-1 [F$^-$ *galK8 thi1 thr1 leuB6 lacY1 fhuA21 supE44 rfbD1 mcrA1 his ilv* Δ(*hem*F-*esp*) Δ(bio-*uvrB*) λ[ΔBam

Table 1. *E. coli* strains assayed for OmpP activity.

E. coli strain	Subclass	OmpP activity	Reference
DH5αF′I^q	K	+	[10]
CJ236	K	+	[11]
JM109	K	+	[12]
UH300	K	+	[13]
UH300Δ*ompA*	K	+	U. Henning
UH300Δ*ompC*	K	+	U. Henning
KL442	K	+	[14]
N4830-1	K	+	[15]
BL21(DE3)	B	–	[16]

Δ(*cro-att*R) N$^+$ *c*I857]] [15]; *E. coli* BL21(DE3), an *E. coli* B strain [F$^-$ *ompT hsdS*$_B$ (r$_B^-$m$_B^-$)] with a λ prophage carrying the T7 RNA polymerase gene [16] (Table 1).

Eukaryotic precursor proteins (protein data base accession numbers in parentheses): yeast F^1-ATPase β-subunit precursor (pF^{1}β; M12082); human methylmalonyl-CoA mutase precursor (pMUT; P22033); rat ornithine transcarbamylase precursor (pOTC; M11266); rat malate dehydrogenase precursor (rat pMDH; P04636); water-melon glyoxysomal malate dehydrogenase precursor (pgMDH; P19446); water-melon mitochondrial malate dehydrogenase precursor (water-melon pmMDH; P17783); *Neurospora* ubiquinol-cytochrome *c* reductase iron–sulfur subunit precursor (pFe/S; X02472).

Chromatographic resins: DEAE Bio-Gel Agarose (100–200 mesh) (BioRad, Hercules, CA); Benzamidine Sepharose 6B (Pharmacia); SP-Sepharose (Pharmacia).

Filtration media: Centriprep-10 concentrators (Amicon, Beverly, MA, USA).

Activity assay

In a typical assay to measure peptidase activity, we used pF^{1}β from *Saccharomyces cerevisiae*, which was synthesized by *in-vitro* translation in a rabbit reticulocyte lysate containing [^{35}S]methionine [17]. The radiolabeled product of the translation reaction was incubated for 1 h at 27 °C, together with the pure enzyme or a crude extract. All other conditions were as described previously [17]. The reaction product was then analyzed directly on SDS/PAGE.

Protein purification

All steps, if not specified otherwise, were conducted at 4 °C. Five hundred millilitres of LB phosphate medium (1% tryptone/0.5% yeast extract/0.5% NaCl/60 mM K-PO$_4$, pH 7.5) were inoculated with 5 mL of an overnight culture of *E. coli* DH5α F′I^q and shaken for 15 h at 37 °C. Cells were harvested at D_{600} = 4.6 by 10 min centrifugation at 3300 ***g***, washed in 75 mL buffer A (5 mM Tris/Cl, pH 7.5 at 4 °C) and resuspended in 40 mL buffer A.

For disruption, the cell suspension was filled in two SS34 tubes and fresh 0.2 mM phenylmethanesulfonyl fluoride solution in ethanol added to a final concentration of 0.2 μM. The suspension was sonicated three times for 30 s with 1 min cooling periods. Cell debris were removed by 40 min centrifugation at 31 000 ***g*** which yielded 40 mL crude extract in the supernatant.

The crude extract was loaded directly on a 40-mL DEAE-Biogel Agarose column (2.5 × 4 cm) equilibrated with buffer A, washed with one column volume of buffer A, and the flow-through and wash fractions collected, yielding 80 mL solution. The combined flow-through and wash fractions were then applied to a 10-mL column of Benzamidine Sepharose 6B (1.5 × 5.6 cm) equilibrated with buffer A, and washed with 20 mL of the same buffer. Again, flow-through and wash fractions were collected and combined. Ammonium sulfate was then added to a final saturation of 30% and stirred for 2 h at room temperature. The precipitate was sedimented by centrifugation for 30 min at 16 300 ***g***. The supernatant was discarded and the pellets dissolved in a total of 8 mL buffer B (50 mM Tris-Cl, pH 7.5 at 4 °C/0.1% Triton X-100) yielding a solution with a concentration of approximately 0.1 M ammonium sulfate, as determined by conductometry. To reduce the ion strength of this solution prior to the next step, the solution was diluted 20-fold with buffer B, which brought the ammonium sulfate concentration down to 5 mM. The diluted solution was heated to 60 °C and stirred at this temperature for 40 min, yielding an opaque solution. This was followed by cooling to 10 °C. The sample was then loaded on a 8-mL DEAE-Biogel Agarose column (1 × 10 cm) equilibrated with buffer B. After washing the column with 20 mL of buffer B, a gradient from 0.0 M to 0.5 M NaCl in 20 column volumes was applied. Fractions were assayed for peptidase activity, and those with the highest activity (75–125 mM NaCl) were pooled. The solution was then concentrated by Centriprep-10 concentrators, and re-equilibrated to buffer C (25 mM Na-Mes, pH 6.0 at 4 °C/0.1% Triton X-100). This solution was loaded on a 2.5-mL SP-Sepharose column (1 × 3 cm) equilibrated with buffer C, and washed with 7 mL buffer C. Flow-through and wash fractions were collected, yielding a solution of 22 mL, which was applied to a 2.5-mL Benzamidine Sepharose 6B column (1 × 3 cm) equilibrated with buffer C. The column was washed with additional 7 mL of the same buffer, and eluted by a gradient from 0.0 to 0.5 M NaCl in 20 column volumes. Fractions were assayed for activity, and active fractions (65–165 mM NaCl) were pooled.

The solution containing peptidase activity was concentrated using a Centriprep-10 concentrator, and re-equilibrated with buffer B. In order to remove contaminating, nonprotein components, the solution was loaded on a DEAE-Biogel agarose column (1 × 6.4 cm) equilibrated with buffer B, washed with 13 mL of the same buffer, and the protein eluted with 10 mL buffer B/250 mM NaCl. The eluted protein was concentrated further by Centriprep-10 concentrators, desalted and reconcentrated to a final volume of 1.2 mL.

Extraction of OmpP from *E. coli* DH5α F′I^q cell envelopes

Preparation of *E. coli* DH5α F′I^q cell envelopes was accomplished by centrifuging 1.5 mL *E. coli* DH5α F′I^q crude extract for 10 min at 4 °C and 16 000 ***g***. The supernatant was discarded and the cell envelopes in the pellet were solubilized in 1.5 mL HD buffer 10 mM Hepes, pH 7.4/1 mM dithiothreitol) containing 0.1% Triton X-100. After 10 min at room temperature the sample was centrifuged again for 10 min at 4 °C and 16 000 ***g***, and the supernatant assayed for OmpP activity.

Radiosequencing

Radiolabeled precursors were synthesized in a rabbit reticulocyte lysate containing 200 μCi of [^{3}H]leucine or 70° μCi of [^{3}H]arginine. Translation mixtures with a total volume of 100 μl were then incubated with samples of purified protease as required, under the conditions described above. The products of the processing reaction were separated on SDS/PAGE and

electroblotted onto a poly(vinylidene difluoride) membrane. The bands corresponding to the products of interest were localized, excised, and sequenced directly on a sequencer (model 470 A; Applied Biosystems, Inc.) as described [18]. The fractions obtained by protein sequencing were collected separately and their radioactivity scored.

RESULTS

Peptidase purification

We assayed crude extracts of several *E. coli* strains for the newly detected specific peptidase activity (see Materials and methods) and found its presence in a range of *E. coli* K strains, but not in *E. coli* B strain BL21(DE3) (Table 1). We then optimized the conditions for production of this peptidase in *E. coli* DH5αF′I^q. We found that addition of 0.25% glycerol to the LB medium impaired processing activity in *E. coli* DH5αF′I^q crude extracts slightly, suggesting production of less enzyme. Addition of 0.4% glucose to the LB medium used for cultivation reduced activity in crude extracts to about 10% of the activity found in crude extracts of bacteria grown in LB medium only.

Specific peptidase activity was detectable in *E. coli* DH5αF′I^q both in crude extracts and in Triton X-100 extracts of cell envelopes; (for peptidase purification from crude extracts and extraction of cell envelopes see Materials and methods). Practical reasons led us to establish a purification procedure from crude extracts, which is summarized in Table 2. Analysis of the fractions of the last Benzamidine Sepharose 6B column on SDS/PAGE (not shown) yielded only one protein band whose distribution correlated to the distribution of peptidase activity, therefore confirming the assignment of the new peptidase activity to the isolated protein band. We determined the first 22 N-terminal amino acids by protein sequencing. The resulting sequence matched exactly the 22 N-terminal amino acids of mature *E. coli* outer membrane protein OmpP reported recently [1].

Confirmation of the assignment of the newly found *E. coli* peptidase activity to OmpP by Western blot

To confirm the identity of the peptidase activity and OmpP further, we performed Western blot analysis using an antibody raised against OmpP in rabbits (courtesy of Dr Henning, Tübingen, Germany). The antibody bound strongly to a sample of the purified protease, weakly to crude extracts of *E. coli* DH5αF′I^q, and did not bind to crude extracts of *E. coli* BL21(DE3), where no processing of mitochondrial precursor proteins was observed (Fig. 1). This is consistent with results of previous studies, where OmpP (then called OmpX) was found to be absent in *E. coli* B [19].

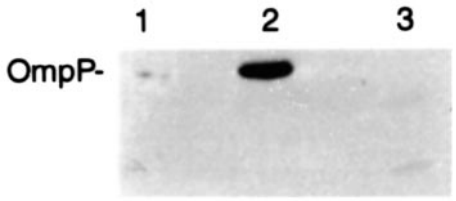

Fig. 1. Detection of OmpP by anti-OmpP antibodies in *E. coli* crude extracts and a purified OmpP sample. Antigen recognition is strongest in the purified OmpP sample (lane 2), weak in *E. coli* DH5αF′I^q (lane 1), and absent in the *E. coli* BL21(DE3) crude extract (lane 3).

Determination of the cleavage specificity

OmpP was originally described as a protease cleaving peptide bonds between two adjacent basic amino-acid residues on the fusion protein consisting of CtxB (B subunit of the cholera toxin of *V. cholerae*) and IgAβ (β domain of the IgA protease of *N. gonorrhoeae*), as substrate [1]. Our studies of the OmpP peptidase activity show further that OmpP produces cleavage patterns close to the specificity of rat mitochondrial processing peptidase (MPP) (Fig. 2). Cleavage specificity of the OmpP preparation was determined by radiosequencing of three mitochondrial precursor proteins (mitochondrial leader peptides are reviewed in [20]).

First, [^{3}H]leucine labeled *Neurospora* pFe/S was cleaved by OmpP and radiosequenced. OmpP cleaved mainly between R20 and A21, to a lesser extent between R23 and A24. The main OmpP cleavage position is followed by the three amino-acid motif AVR. Rat MPP cleavage on *Neurospora* pFe/S occurs between A24 and L25 [20] (Fig. 3a).

Second, [^{3}H]leucine-labeled rat pOTC was cleaved by OmpP and then radiosequenced. Cleavage by OmpP occurred mainly between A13 and L14, to a lesser extent between A12 and A13. This difference in the extent of cleavage between both positions suggests different kinetics of cleavage at each position, or partial degradation of the N-terminal A13 of the cleavage product. A cleavage position between A12 and A13 is followed by the three amino-acid motif ALR. Comparison with water-melon mitochondrial pMDH cleavage (Fig. 2, panel VI, lane 2 and Fig. 3a) shows that rat MPP cleavage position on rat pOTC is presumably between K16 and A17 not, as under most experimental conditions, between N24 and F25. This is consistent with a rat MPP cleavage position at −2 of R15, which might serve in some cases as a substitute to the usually recognized R23 (for pOTC processing see [21]).

Table 2. Purification of OmpP. One Unit (U) of the peptidase activity is defined as the amount of enzyme that catalyzes the conversion of 50% of *in-vitro* translated yeast pF^{1}ß in 10 µl reaction mixture to its mature form in 1 h at 27 °C.

Purification step	Total protein (µg)	Total activity (U)	Specific activity (U·µg^{-1})	Activity yield (%)	Enrichment factor
Crude extract	360 000	10 000	0.03	100	1.0
1st DEAE-Biogel	27 000	4 500	0.16	45	5.3
1st Benzamidine Sepharose	26 000	4 300	0.16	43	5.3
Ammonium sulfate precipitation and heat treatment	420	3 200	7.6	32	253.3
2nd DEAE-Biogel	240	3 000	12.5	30	416.6
SP-Sepharose	56	1 700	30.4	17	1013.3
2nd Benzamidine Sepharose	31	1 560	50.5	15.6	1683.3
3rd DEAE-Biogel	24	1 500	62.5	15	2083.3

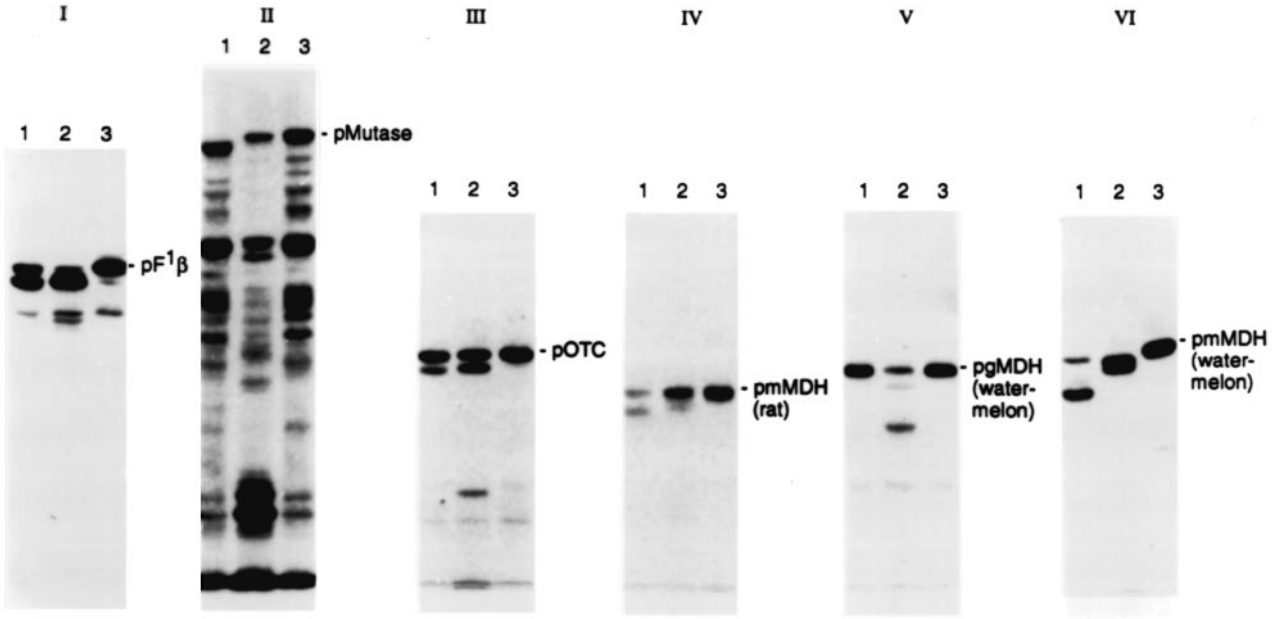

Fig. 2. Comparison of OmpP and rat MPP cleavage patterns on mitochondrial and glyoxysomal precursor substrates. Lanes 1, processing by rat MPP; lanes 2, processing by *E. coli* peptidase OmpP; lanes 3, unprocessed translation product; p, precursor; m, mitochondrial; g, glyoxysomal; rat MPP was purified from rat liver according to [30].

Third, [^{3}H]arginine-labeled water-melon mitochondrial pMDH was cleaved by OmpP and radiosequenced. OmpP cleavage was mainly between S8 and V9. Additionally some degradation of M1 was observed. In this case, the cleavage of OmpP is within a SVR motif, one residue downstream of the position expected from comparison with the *Neurospora* pFe/S and rat pOTC radiosequencing results. This includes the possibility of OmpP cleavage between R7 and S8 with considerable degradation of the N-terminal S8 of the cleavage product. Rat MPP cleavage position on water-melon mitochondrial pMDH is, by analogy with rat mitochondrial pMDH [20], expected to occur between S26 and F27 (For MPP cleavage pattern on water-melon mitochondrial pMDH see also Fig. 2, panel VI, lane 1).

As a general rule, OmpP seems to cleave before a three amino-acid motif carrying a residue with a small aliphatic side chain like alanine or serine in the first position, a large hydrophobic amino acid, such as leucine or valine, in the second position, and in the third position an arginine residue. There is a high tendency to degrade the N-terminal amino acid of a substrate as an additional step. The most abundant form of this putative recognition sequence in those mitochondrial precursor proteins investigated is the motif AhR (where h is a large hydrophobic residue), but the requirement for a higher-order structural motif for correct recognition by OmpP is also clear, as cleavage of water-melon mitochondrial pMDH does not appear to obey this rule exactly, and yeast pF^{1}β, which is cleaved very well by OmpP, does not carry an AhR motif in its signal peptide (Fig. 3b). Judged by the mobility on SDS/PAGE (Fig. 2, panel I, lanes 1–3), rat MPP and OmpP cleave yeast pF^{1}β at the same position, between K19 and Q20. This exceptional cleavage of yeast pF^{1}β is still more interesting, as yeast pF^{1}β belongs to a group of mitochondrial precursors which do not obey the general recognition rule suggested for rat MPP cleavage either [20].

Other mitochondrial precursor sequences, studied experimentally for OmpP cleavage in comparison with rat MPP cleavage on SDS/PAGE, but not by radiosequencing, are listed in Fig. 3b. Positions of AhR motifs and rat MPP cleavage sites are indicated. In comparison with Fig. 2, where OmpP and rat MPP cleavage products of most of these precursors are aligned, it is clear that size differences between OmpP and rat MPP cleavage products are well accounted for by the AhR cleavage motif rule.

As expected, human pMUT shows no processing by OmpP (Fig. 2, panel II, lane 2), whereas processing by rat MPP leads to removal of 32 N-terminal amino-acid residues (Fig. 2, panel II, lane 1 vs. lane 3). This is consistent with the absence of an AhR motif in that region, as the only AhR motif is located within residues 1–3 (MLR). Another mitochondrial precursor, rat pMDH, is cleaved by MPP to a product slightly smaller than the OmpP cleavage product (Fig. 2, panel IV). An ALR motif in this precursor is at positions 12–14, whereas rat MPP cleaves between S16 and F17. Different patterns of cleavage are obtained with water-melon glyoxysomal pMDH, which is not processed by the mitochondrial processing peptidase MPP. In contrast, precursor processing occurs with OmpP, yielding a mature product about 30 amino acids shorter. (Additionally the far shorter product of a internal protein cleavage is visible; Fig. 2, panel V). Again, this is in accordance with an ALR motif at position 30–32 (Fig. 3b). Also, rat dimethylglycine dehydrogenase precursor (pMe_2GlyDH) is cleaved with OmpP to a product only a few amino acids shorter than the precursor (data not shown). Two ALR motifs are present at positions 6–8 and 13–15. The putative rat MPP cleavage position is between A43 and E44 ([22]; Fig. 3b). The obtained results show, however, that despite those similarities, cleavage products generated by OmpP do not exactly match mature protein products generated by rat MPP.

Effect of divalent cations on enzyme activity

Determining the dependency on divalent cations may provide useful information about the protease family into which a given peptidase has to be classified. Rat MPP is known to be a member

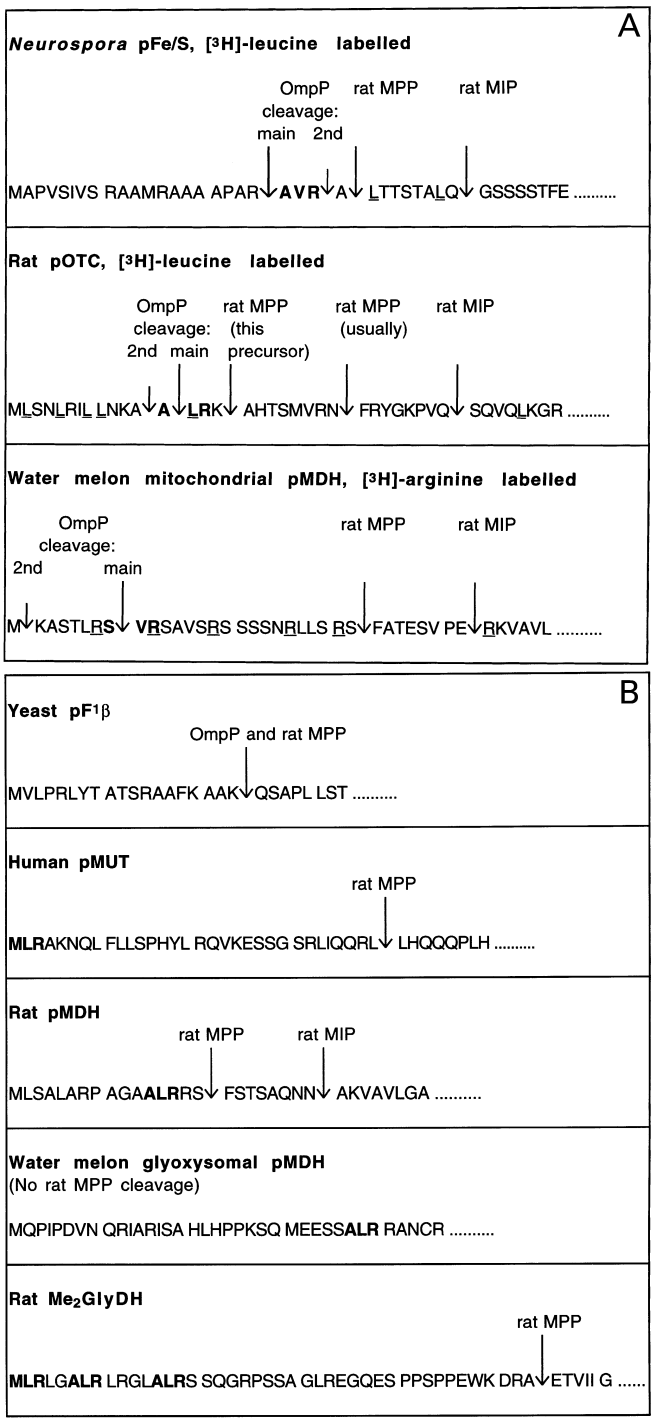

Fig. 3. Leader peptides of selected mitochondrial and glyoxysomal precursor proteins. (A) Leader peptides of three mitochondrial precursor proteins used for determination of the OmpP cleavage position by radiosequencing. Cleavage positions of OmpP, rat MPP and rat mitochondrial intermediate peptidase (MIP) are indicated by arrows; the three amino-acid motifs serving as putative recognition sequences for OmpP are shown in bold type; amino acids which were labeled radioactively are underlined. (B) Leader peptides of four mitochondrial and one glyoxysomal precursors used for comparison of OmpP and rat MPP cleavage patterns. As the OmpP cleavage positions in these instances are not confirmed by radiosequencing, only the three amino-acid motifs serving as putative OmpP recognition sequences are shown in bold type; rat MPP and rat MIP cleavage positons are indicated by arrows.

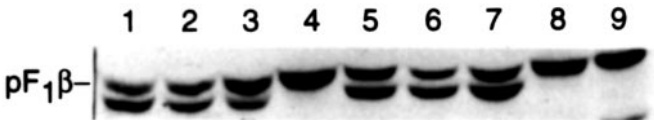

Fig. 4. OmpP and rat MPP activity at different Zn^{2+} concentrations. Lanes 1–4, *E. coli* crude extracts containing OmpP; lanes 5–8, partially purified ratMPP; reaction mixtures were adjusted to the following Zn^{2+} concentrations: 0 mM (lanes 1 and 5), 0.1 mM (lanes 2 and 6), 1.0 mM (lanes 3 and 7), 10.0 mM (lanes 4 and 8); unprocessed precursor (lane 9); enzyme activities were determined with yeast $pF^1\beta$ as substrate.

of a metalloprotease family [23,24], with reaction optimum in the presence of 1 mM Mn^{2+}, whereas OmpP is, by its 87% identity to the *E. coli* peptidase OmpT, probably a member of the serine protease family [1]. Members of the two families behave differently in the presence or absence of divalent metal cations; this can be shown by comparing rat MPP and OmpP at different levels of divalent metal ions. Figure 4 shows OmpP and rat MPP activities at different Zn^{2+} concentrations (other divalent metal ions i.e. Mg^{2+}, Mn^{2+}, Co^{2+}, and Ca^{2+} were also evaluated and give similar results; data not shown). Whereas OmpP is most active in absence of Zn^{2+} ions, activity of rat MPP reaches a maximum at about 1 mM Zn^{2+} and drops only at extreme Zn^{2+} concentrations. This confirms that OmpP is not a member of a metal ion-dependent protease family, and that a similar function is executed by two enzymes belonging to different protease families.

pH dependency of the enzyme activity

Whereas MPP, as an enzyme of the mitochondrial matrix, has its maximum activity at neutral to moderate basic pH values [25,26], OmpP activity is stable over a much broader pH range. By adjusting OmpP-containing *E. coli* DH5αF′I^q crude extracts to pH values between 6.4 and 9.2, leaving them 10 min on ice, and assaying them at the adjusted pH values, we found no decline in OmpP activity from the pH usually used, pH 7.4, towards pH 6.4 or pH 9.2 extremes. It is possible that the limiting factor at more extreme pH values is the stability of the used precursor proteins, which could undergo conformational alterations during the assay, therefore altering the structural patterns necessary for recognition by the peptidase. Thus, we adjusted another OmpP containing crude extract to pH 3.4 and incubated on ice for 10 min. As this resulted in the precipitation of most proteins, we separated insoluble and soluble proteins by 5 min centrifugation at 16 000 ***g***, re-adjusted both fractions to pH 7.4 (which led to the precipitated proteins being re-dissolved), and performed activity assays with both fractions. The result showed no OmpP activity in the pH 3.4 supernatant fraction, but the pH 3.4 precipitate fraction was fully active,

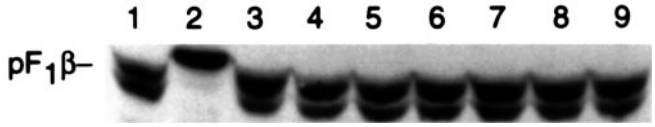

Fig. 5. Effect of pH on OmpP activity. *E. coli* DH5αF′I^q crude extracts were adjusted to different pH and OmpP assayed with yeast $pF^1\beta$ as substrate: pH 6.4 (lane 3); pH 6.9 (lane 4); pH 7.4 (lane 5); pH 7.9 (lane 6); pH 8.4 (lane 7); pH 8.9 (lane 8); pH 9.2 (lane 9); the pH 3.4 sample was separated into pellet (lane 1) and supernatant fractions (lane 2); OmpP is fully active within the assayed range from pH 3.4 (pellet) to pH 9.2.

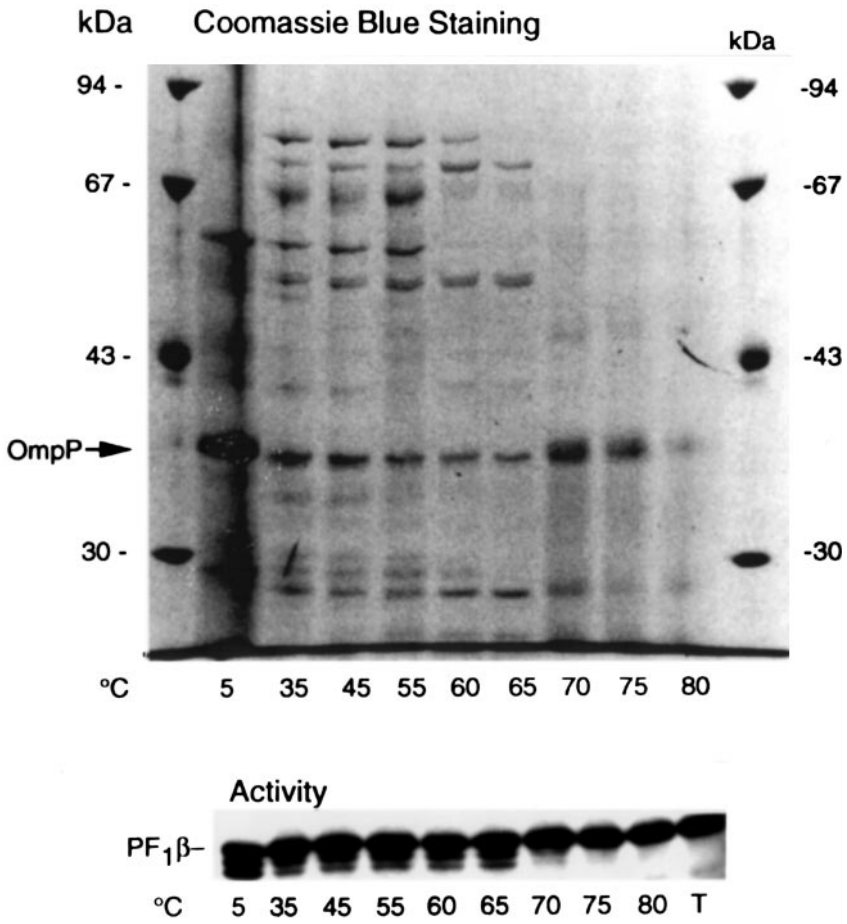

Fig. 6. Stability of OmpP activity at different temperatures. OmpP protein (upper panel) and OmpP activity (lower panel) are shown after 30 min incubation of OmpP samples at the indicated teperatures. Processing reaction is described in Materials and methods. OmpP activity is not affected if the enzyme is incubated previously in a temperature range from 5 °C to 70 °C.

showing no difference in its activity to samples incubated at pH 7.4 at all times (Fig. 5).

Temperature range of the enzyme activity

Temperature stability of the OmpP activity was assayed by splitting a partially purified OmpP solution into 20 equal parts and incubating them each at one of 20 different temperatures, the lowest temperature at 5 °C, the other samples at temperatures up to 100 °C, in 5 °C steps . The temperatures were kept accurate within a range of 2 °C for 30 min. Then the heat-treated solutions were assayed for OmpP activity at 27 °C (Fig. 6). OmpP activity was detectable in OmpP samples that were incubated up to 75 °C, setting this temperature as an upper limit on enzymatic activity of the peptidase, while maximum OmpP activity was obtained at temperatures between 20° and 35 °C. We therefore chose as standard conditions for assaying OmpP an incubation at 27 °C for 1 h, similar to those conditions used for activity assays of MPP. There are additional features of OmpP related to the temperature. When *E. coli* crude extracts were kept at 4 °C, the peptidase was not able to bind to any of the chromatography resins we investigated. This included anion-exchange resins (DEAE Biogel agarose, Mono Q, Q-Poros), cation-exchange resins (CM cellulose, CM 52, SP-Sepharose, Mono S, S-Poros), hydrophobic resin (alkyl Superose), and affinity resin (benzamidine Sepharose). In case of anion-exchange resins and benzamidine Sepharose, 30 min warming of OmpP-containing solutions beyond room temperature led to stable binding of OmpP without impairing enzyme activity. This process could not be reversed by cooling the samples below room temperature, suggesting an alteration in OmpP conformation or in the quarternary structure of an originally existing protein complex.

DISCUSSION

In this work, a new OmpP cleavage specificity was established that has some similarities with that of rat MPP. The consensus sequence for OmpP recognition and cleavage found in most precursor proteins contains an amino-acid residue with a small aliphatic side chain at position +1, a large hydrophobic amino-acid residue at position +2, and an arginine residue at position +3 relative to the cleavage site. Additional structural features of the substrates seem to be required for proper cleavage. Rat MPP also has a three amino-acid motif with amino acids of similar chemical characteristics crucial for recognition of most of its protein substrates: an arginine residue at position −2, a hydrophobic residue at position +1, and a serine, threonine or glycine residue at position +4 relative to the cleaved peptide bond [20]. Structural requirements for MPP substrates include the arrangement of the signal peptide as an amphiphilic α-helix with a highly positively charged face and a hydrophobic face [27].

The finding that OmpP may function as a specific endopeptidase with the ability to cleave off leader peptides further extends the number of *E. coli* processing peptidases. To date there are two major leader peptidases known in *E. coli*, signal peptidase I and signal peptidase II. Whereas most exported *E. coli* protein precursors are processed by signal peptidase I [28], signal peptidase II is responsible for cleavage

of signal peptides of lipoproteins directed to the cell membrane. Signal peptidase I has a cleavage specificity which follows the −3, −1 rule of von Heijne, which requires larger polar or aromatic residues at position −2, whereas small, neutral amino-acid residues are found at position −3 and −1 from the cleavage site [29].

The similarity of cleavage specificities between a bacterial outer membrane peptidase and a mitochondrial processing peptidase is still more surprising as both enzymes belong to totally different peptidase families. Rat MPP is a member of the insulinase superfamily, a subdivision of the metallopeptidase class of endopeptidases [23], whereas OmpP shows all characteristics of a serine protease, even though inhibition by some serine protease inhibitors, like phenylmethanesulfonyl fluoride, is limited. It is probable that cleavage similarities between *E. coli* OmpP and rat MPP are the result of a co-evolution of different endopeptidase families in order to adapt to common sequential and structural features of signal peptides.

The characterization of OmpP as a specific leader peptidase might also lead to further insights into the role of OmpT. As both proteins probably evolved from a common ancestral protein and both show proteolytic action [1], it can be assumed that both proteins still have similar physiological functions today. Therefore it might be possible that OmpP has the ability to cleave certain precursor proteins specifically as well.

From the same reason, further consideration should be given to find OmpP-like proteins in bacterial hosts other than *E. coli*. Screening of different bacterial strains with a range of precursor proteins addressed to different eukaryotic cell compartments could yield new, unknown peptidase specificities.

In addition to OmpP, those *E. coli* proteins controlling OmpP function could be of interest. During OmpP purification we found a strong increase in specific activity after application of ammonium sulfate precipitation followed by heat treatment at 60 °C (Table 2). This increase, in combination with a different binding behavior of the enzyme on chromatographic resins before and after the heat treatment, suggests the irreversible dissociation of a protein complex and therefore a functioning of the enzyme in close contact with other proteins. Although we can not fully exclude the possibility that small amounts of a protein cofactor are necessary for enzyme function, prokaryotic signal peptidases are known to act as monomers in contrast to eukaryotic signal peptidases, which often appear in protein complexes. So far, we have not been able to identify any cofactor. Another way to control OmpP activity could be accomplished by a specific inhibitor. Increase of OmpP activity following heat treatment could be the result of an inactivation of such an inhibitor.

The present purification and characterization of OmpP may be a way to extend our knowledge about structure and function of bacterial as well as eukaryotic endopeptidases. The similar specificities of *E. coli* OmpP and rat MPP in combination with their classification into different endopeptidase families could be a further step to identify crucial structural elements for specific recognition of amino-acid patterns in substrate proteins.

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REFERENCES

1. Kaufmann, A., Stierhof, Y.-D. & Henning, U. (1994) New outer membrane-associated protease of *Escherichia coli* K-12. *J. Bacteriol.* **176**, 359–367.
2. Maurizi, M.R. (1992) Proteases and protein degradation in *Escherichia coli*. *Experientia* **48**, 178–201.
3. Zwizinski, C., Date, T. & Wickner, W. (1981) Leader peptidase is found in both the inner and outer membranes of *Escherichia coli*. *J. Biol. Chem.* **256**, 3593–3597.
4. Tokunaga, M., Loranger, J.M., Chang, S.Y., Regue, M., Chang, S. & Wu, H.C. (1985) Identification of prolipoprotein signal peptidase and genomic organization of the *Isp* gene in *Escherichia coli*. *J. Biol. Chem.* **260**, 5610–5616.
5. Pacaud, M. (1982) Purification and characterization of two novel proteolytic enzymes in membranes of *Escherichia coli*. *J. Biol. Chem.* **257**, 4333–4339.
6. Grodberg, J. & Dunn, J.J. (1988) *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J. Bacteriol.* **170**, 1245–1253.
7. Sugimura, K. & Nishihara, T. (1988) Purification, characterization, and primary structure of *Escherichia coli* protease VII with specificity for paired basic residues: identity of protease VII and OmpT. *J. Bacteriol.* **170**, 5625–5632.
8. Hollifield, W.C. Jr, Fiss, E.H. & Nielands, J.B. (1978) Modification of ferric enterobactin receptor protein from the outer membrane of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **83**, 739–746.
9. Baneyx, F. & Georgiou, G. (1990) *In vivo* degradation of secreted fusion proteins by *Escherichia coli* outer membrane protease OmpT. *J. Bacteriol.* **172**, 491–494.
10. Woodcock, D.M., Crowther, P.J., Doherty, J., Jefferson, S., DeCruz, E., Noyer-Weidner, M., Smith, S.S., Michael, M.Z. & Graham, M.W. (1989) Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* **17**, 3469–3478.
11. Kunkel, T.A., Roberts, J.D. & Zabour, R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.
12. Yanisch-Perron, C., Viera, J. & Messing, J. (1985) Improved M13 phage cloning vector and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 224–228.
13. MacIntyre, S., Mutschler, B. & Henning, U. (1991) Requirement of the SecB chaperone for export of a non-secretory polypeptide in *Escherichia coli*. *Mol. Gen. Genet.* **227**, 224–228.
14. Horwich, A.L., Low, K.B., Fenton, W.A., Hirshfield, I.N. & Furtak, K. (1993) Folding *in vivo* of bacterial cytoplasmatic proteins: role of GroEL. *Cell* **74**, 909–917.
15. Gottesman, M.E., Adhya, S. & Das, A. (1980) Transcription antitermination by bacteriophage lambda N gene product. *J. Mol. Biol.* **140**, 57–75.
16. Studier, F.W., Rosenberg, A.H., Dunn, J.J. & Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60–89.
17. Striebel, H.-M., Rysavy, P., Adamec, J., Spizek, J. & Kalousek, F. (1996) Mutational analysis of both subunits from rat mitochondrial processing peptidase. *Arch. Biochem. Biophys.* **335**, 211–218.
18. Matsudaira, P. (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**, 10035–10038.
19. Drexler, K., Dannull, J., Hindennach, I., Mutschler, B. & Henning, U. (1991) Single mutations in a gene for a tail fiber component of an *Escherichia coli* phage can cause an extension from a protein to a carbohydrate as receptor. *J. Mol. Biol.* **219**, 655–663.
20. Hendrick, J.P., Hodges, P.E. & Rosenberg, L.E. (1989) Survey of amino-terminal proteolytic cleavage sites in mitochondrial precursor proteins: Leader peptides cleaved by two matrix proteases share a three-amino acid motif. *Proc. Natl Acad. Sci. USA* **86**, 4056–4060.
21. Horwich, A.L., Kalousek, F., Fenton, W.A., Pollock, R.A. & Rosenberg, L.E. (1986) Targeting of pre-ornithine transcarbamylase

to mitochondria: definition of critical regions and residues in the leader peptide. *Cell* **44**, 451–459.

22. Lang, H., Polster, M. & Brandsch, R. (1991) Rat liver dimethylglycine dehydrogenase. Flavinylation of the enzyme in hepatocytes in primary culture and characterization of a cDNA clone. *Eur. J. Biochem.* **198**, 793–799.
23. Rawlings, N.D. & Barrett, A.J. (1991) Homologues of insulinase, a new superfamily of metallopeptidases. *Biochem. J.* **275**, 389–391.
24. Becker, A.B. & Roth, R.A. (1992) An unusual active site identified in a family of zinc metalloendopeptidases. *Proc. Natl Acad. Sci. USA* **89**, 3835–3839.
25. Kalousek, F., Hendrick, J.P. & Rosenberg, L.E. (1988) Two mitochondrial matrix proteases act sequentially in the processing of mammalian matrix enzymes. *Proc. Natl Acad. Sci. USA* **85**, 7536–7540.
26. Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. & Neupert, W. (1988) Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. *Cell* **53**, 795–806.
27. Kleiber, J., Kalousek, F., Swaroop, M. & Rosenberg, L.E. (1990) The general mitochondrial processing peptidase from rat liver: structural characterization of the catalytic subunit. *Proc. Natl Acad. Sci. USA* **87**, 7978–7982.
28. Dalbey, R.E. (1991) Leader peptidase. *Mol. Microbiol.* **5**, 2855–2860.
29. Dev, I.K. & Ray, P.H. (1990) Signal peptidases and signal peptide hydrolases. *J. Bioenerg. Biomembr.* **22**, 271–290.
30. Isaya, G., Kalousek, F., Fenton, W.A. & Rosenberg, L.E. (1991) Cleavage of precursors by the mitochondrial processing peptidase requires a compatible mature protein or an intermediate octapeptide. *J. Cell Biol.* **113**, 65–76.