FEBS Letters 505 (2001) 426–430 FEBS 25262

Identification of essential acidic residues of outer membrane protease OmpT supports a novel active site

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Received 8 August 2001; accepted 23 August 2001

First published online 6 September 2001

Edited by Maurice Montal

Abstract ***Escherichia coli*** **outer membrane protease OmpT has previously been classified as a serine protease with Ser^{99} and His^{212} as active site residues. The recently solved X-ray structure of the enzyme was inconsistent with this classification, and the involvement of a nucleophilic water molecule was proposed. Here, we substituted all conserved aspartate and glutamate residues by alanines and measured the residual enzymatic activities of the variants. Our results support the involvement of a nucleophilic water molecule that is activated by the Asp^{210}/His^{212} catalytic dyad. Activity is also strongly dependent on Asp^{83} and Asp^{85}. Both may function in binding of the water molecule and/or oxyanion stabilization. The proposed mechanism implies a novel proteolytic catalytic site.**

Key words: Active site; Asp/His dyad; Protease; OmpT; Outer membrane protein

1. Introduction

Escherichia coli OmpT (EC 3.4.21.87) is a 33.5 kDa outer membrane protease that cleaves peptides and proteins preferentially between two consecutive basic amino acids [1–5]. The enzyme has been suggested to be involved in urinary tract disease [6], in DNA excision repair [7] and in the breakdown of antimicrobial peptides [8], but its actual biological function remains to be elucidated. OmpT is a member of the omptin family that includes the proteases PgtE of *Salmonella typhimurium* (mature part 49% identical to OmpT) [9], Pla of *Yersinia pestis* (50% identical) [10], OmpP of *E. coli* (72% identical) [11] and SopA of *Shigella flexneri* (60% identical) [12]. As OmpT, Pla and OmpP lack cysteine residues, the omptins do not belong to the class of cysteine proteases. The omptin family appears to constitute a novel class of proteases, since the amino acids conserved within the omptins are not part of known active site consensus sequences found in serine proteases, aspartic proteases or metalloproteases [13]. This is confirmed by the observation that OmpT activity is not or only slightly inhibited by commonly used class-specific protease inhibitors [1,14,15]. Significant inhibition of OmpT activity has been observed only at high concentrations of the serine protease inhibitors diisopropylfluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF) [1]. Members of the omptin family have been classified as serine proteases mainly based on these observations [16], even though several experiments have been reported where DFP and PMSF did not affect the proteolytic activity of OmpT [14,15,17].

In serine proteases, the scissile peptide bond is attacked by a nucleophilic hydroxyl of the catalytic serine that is usually activated by a histidine residue [13]. To identify the putative active site serine and histidine of OmpT, we previously substituted all conserved serines and histidines by site-directed mutagenesis and concluded that Ser^{99} and His^{212} are essential active site residues [18]. The activities of H212A, H212N and H212Q OmpT were reduced by four orders of magnitude. However, the mutation S99A in OmpT reduced activity only 500-fold, where at least a 10 000-fold reduced activity was expected (e.g. [19]). It was therefore emphasized that a role for Ser^{99} other than performing the nucleophilic attack should not be excluded. To further examine the active site mechanism, we wished to study the possible involvement of residues other than serine and histidine. In the classical serine protease triad, a negatively charged aspartate residue is thought to stabilize the transient positive charge on the histidine during catalysis [13]. Recently, it was shown that a glutamate is able to perform this function as well [20]. Conserved acidic residues are thus obvious candidates for being an active site residue of OmpT. Furthermore, at least one aspartate or glutamate residue is expected to be involved in substrate binding, since OmpT specifically cleaves peptides between two positively charged amino acids [5]. OmpT contains 36 acidic residues, of which six aspartates (at positions 43, 83, 85, 97, 208 and 210) and five glutamates (at positions 27, 111, 136, 193 and 250) are fully conserved within the omptin family (Fig. 1). Using the same approach as described for the identification of Ser^{99} and His^{212} [18], these 11 residues were replaced by alanines and the residual activities of the resulting variants were measured. In parallel with these mutagenesis studies, we solved the X-ray structure of OmpT, which revealed a puta-

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Abbreviations: Abz, *o*-aminobenzoyl; Dap(dnp), *N*-β-dinitrophenyl-L-diaminopropionic acid; DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonyl fluoride; Triton X-100, polyethyleneglycol tert-octylphenyl ether; Tween 20, polyoxyethylene sorbitanmonolaurate

0014-5793/01/$20.00
PII: S0014-5793(01)02863-0

tive active site cleft at the top of the extracellular region [21]. Ser^{99} and His^{212} are both located in this cleft, but they are separated by 9 Å. The concerted action of Ser^{99} and His^{212} was seriously questioned and an alternative mechanism was proposed, involving an Asp^{210}/His^{212} catalytic dyad with a water molecule acting as nucleophile [21].

Here, we present the mutagenesis data of the conserved aspartate and glutamate residues and discuss their importance for the proteolytic activity of OmpT. The results support the involvement of an Asp^{210}/His^{212} catalytic dyad, suggesting that OmpT would be the first protease exhibiting this type of catalytic mechanism.

2. Materials and methods

2.1. Materials

DNA restriction enzymes were purchased from New England Biolabs. Oligonucleotides were bought from Amersham Pharmacia Biotech. Polyethyleneglycol tert-octylphenyl ether (Triton X-100) was obtained from Serva and polyoxyethylene sorbitanmonolaurate (Tween 20) was obtained from Bio-Rad. The fluorogenic peptide substrate Abz-Ala-Arg-Arg-Ala-Dap(dnp)-Gly (Abz = *o*-aminobenzoyl; Dap(dnp) = *N*-β-dinitrophenyl-L-diaminopropionic acid) was a kind gift of Mr. Ruud C. Cox (Utrecht University). Anti-OmpT monoclonal antibodies were obtained as described before [18]. Goat anti-mouse IgG alkaline phosphatase conjugate was purchased from Promega. All other chemicals were of analytical grade.

2.2. Bacterial strains and plasmids

E. coli K-12 strain DH5α [22] was used in the cloning procedures. *E. coli* strain BL21(DE3) [23] lacks the *ompT* gene and was used for expression. Plasmids pND9 and pND10 [4] are derivatives of pUC19 [24], containing the *ompT* gene, including its own promoter sequence oriented clockwise and counter-clockwise with the *lac* promoter, respectively.

All mutations were introduced using PCR according to the manufacturer's protocol provided with the QuikChange site-directed mutagenesis kit (Stratagene). The *ompT* gene contains only a few unique restriction sites. Therefore, four additional unique restriction sites were introduced to facilitate subcloning of introduced mutations. The sites *Xho*I (at Ser^{89} of mature OmpT), *Sac*II (at Arg^{144}), *Age*I (at Arg^{222}) and *Eag*I (at Ala^{291}) were introduced as silent mutations in plasmids pND10 and pND9, resulting in plasmids pRAK22 and pRAK23, respectively. Mutations resulting in the substitution of Asp or Glu by Ala were introduced individually into pRAK22. To facilitate the screening for introduced mutations, silent mutations resulting in the introduction or deletion of restriction sites were made concomitantly with the amino acid mutations. After verification of the correctly introduced mutations by DNA sequencing, all mutations were subcloned into pRAK23 using the newly introduced restriction sites. The sequences of the relevant parts of the constructed plasmids were checked by restriction enzyme analysis and DNA sequencing.

2.3. Expression, isolation and analysis of OmpT variants

After transformation by the various plasmids, BL21(DE3) cells were grown overnight in 10 ml LB medium [25] with 50 μg/ml ampicillin at 37°C. Membrane fractions containing OmpT were isolated from the bacteria, as described before [18], and solubilized in 200 μl buffer A (0.1% Triton X-100, 50 mM Tris, pH 7.5). The total amount of protein in the isolated membrane fractions was determined using the Bio-Rad protein assay with bovine serum albumin as a reference. Samples of membrane fractions corresponding to equal amounts of total protein were analyzed by Western blotting using anti-OmpT monoclonal antibodies, as described before [18].

2.4. Enzymatic activity assays

Samples of membrane fractions were diluted in buffer A to appropriate concentrations (varying from 0.5 μg to 0.2 mg of total protein per ml) prior to OmpT activity measurements. The internally quenched fluorogenic substrate Abz-Ala-Arg-Arg-Ala-Dap(dnp)-Gly was used in a fluorimetric activity assay, as described previously [4]. Assay conditions were 5 μM substrate, 1 mM Tween 20, 5 mM EDTA, 10 mM Tris, pH 8.3. Activity was measured in a fluorimeter using excitation and emission wavelengths of 325 and 430 nm, respectively. After recording the initial linear increase in fluorescence, 20 μg of trypsin was added to determine the fluorescence of completely hydrolyzed substrate, which enabled quantification of the inner filter effect and allowed for the calculation of the activity in enzymatic units (U = μmol substrate converted per min).

3. Results

3.1. Expression of OmpT

Single mutant proteins of OmpT were constructed with the conserved acidic residues replaced by alanines. BL21(DE3) cells expressing either these alanine variants or wild-type OmpT were grown in triplicate and membrane fractions were isolated. Cells lacking a plasmid with the gene for OmpT were used as a control. The fact that isolated membrane fractions were used instead of purified protein had two important consequences. An important advantage of using outer membrane fractions was that all samples contained endogenous lipopolysaccharide, which has been shown to be required for OmpT activity [4]. Unfortunately the concentration of OmpT in the samples could not be accurately determined. Therefore, concentrations of total protein were determined for each sample and used as a measure for the amount of OmpT. We previously validated this method for serine and histidine variants of OmpT by showing that the expression levels of these variants were comparable to that of wild-type OmpT [18]. To check whether this was also true for the aspartate and glutamate variants, SDS–PAGE and Western blot analysis were performed with all samples containing equal amounts of total protein. As expected, the expression levels of the variants and wild-type OmpT were comparable within a factor of three (data not shown). These differences are acceptable in our studies, since activities reduced by at least two orders of magnitude are expected when essential residues are replaced. SDS–PAGE and Western blotting were also used to check the correct folding of the variants. At room temper-

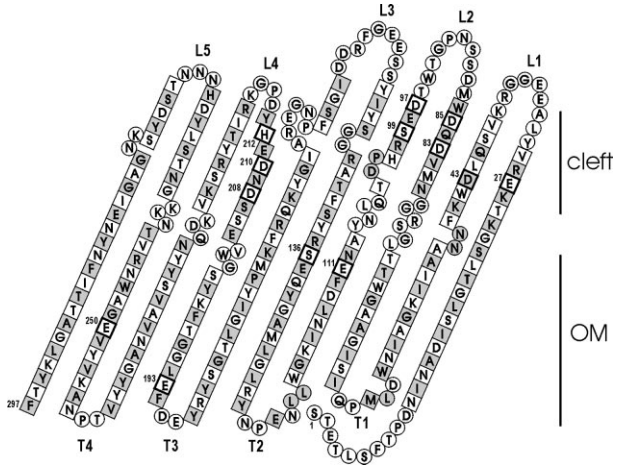

Fig. 1. Topology of OmpT (adapted from [21]). Numbers refer to amino acid positions in mature OmpT. Residues in β-strands are shown in squares; other residues are in circles. β-Strand residues pointing to the protein exterior are in shaded squares. Conserved acidic residues, as well as Ser^{99} and His^{212}, are in squares with dark edges. The approximate positions of the outer membrane (OM) and the putative active site cleft of OmpT are indicated. The N-terminus and the short turns (T1–T4) are localized in the periplasm, and the large loops (L1–L5) face the extracellular side of the membrane.

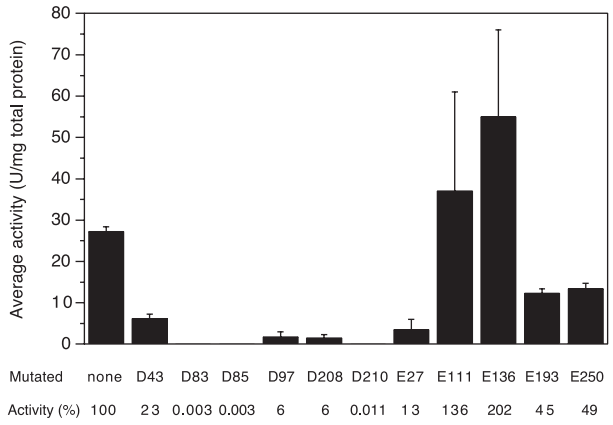

Fig. 2. Enzymatic activities of OmpT and its variants as measured in a fluorimetric assay. Average absolute activities of three independent measurements (black bars) and the corresponding relative activities compared to wild-type OmpT (percentages) were determined for each variant. See the text for experimental details.

ature, the presence of SDS is not sufficient to unfold the β-barrel conformation of OmpT. As a consequence, native OmpT has a more compact shape than the heat-denatured protein and runs faster on SDS–PAGE gel [4,18]. This difference in migration rate between folded and unfolded protein is known as heat-modifiability, which is generally observed for outer membrane proteins [26]. The Western blots showed that all of the 11 active site variants displayed this effect (data not shown), indicating their proper folding and assembly in the outer membrane.

3.2. Activity measurements of OmpT variants

The enzymatic activity of OmpT in the isolated membrane fractions was determined using a fluorimetric assay. For all variants, membrane fractions of three independent cell cultures were measured. The average activities are shown in Fig. 2. A large spread in the activities was observed for several variants, which was at least partially due to variations in expression levels as mentioned above. Variants E111A, E136A, E193A and E250A displayed enzymatic activities that were comparable to wild-type within a factor of two. In contrast, variant E27A, as well as all aspartate variants, showed significantly reduced activities compared to wild-type OmpT. Specific activities of the variants E27A, D43A, D97A and D208A were reduced 4–17-fold. The strongest decrease was observed for variants D83A, D85A and D210A, exhibiting at least 10 000-fold lower activity than wild-type OmpT.

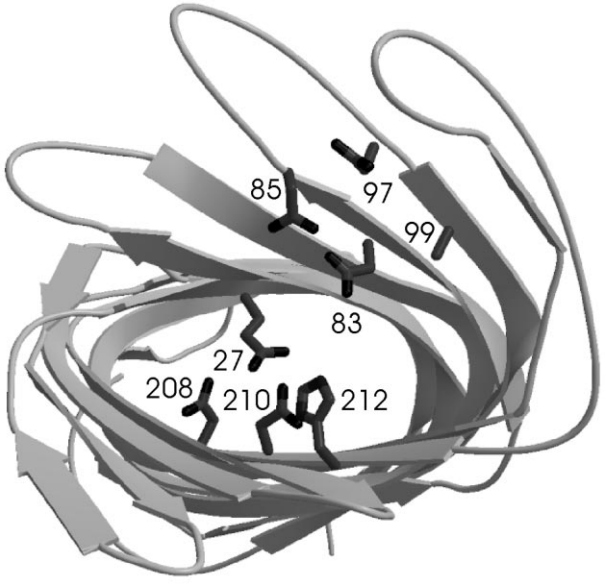

Fig. 3. Active site cleft of OmpT (adapted from [21]). OmpT (ribbon presentation) is viewed parallel to the barrel axis from the extracellular side of the outer membrane. The conserved acidic residues Glu[27], Asp[83], Asp[85], Asp[97], Asp[208] and Asp[210], as well as Ser[99] (Ala[99] in the crystallized protein) and His[212], are represented as stick model.

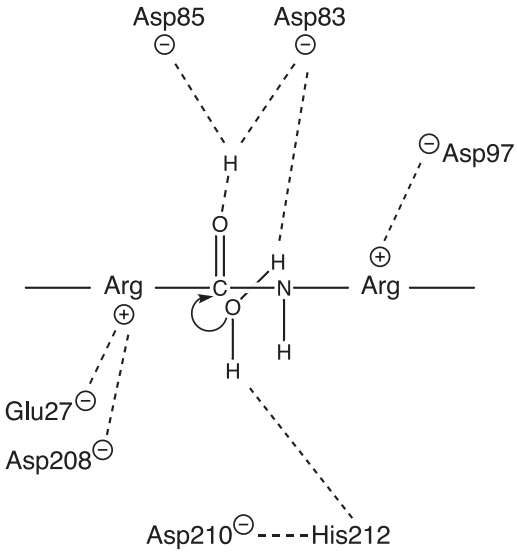

Fig. 4. Schematic two-dimensional model of a peptide in the active site of OmpT. Hydrogen bonds and ionic interactions are indicated by dashed lines. The curved arrow represents the nucleophilic attack on the carbonyl carbon of the scissile peptide bond.

4. Discussion

The experimental basis for the current classification of OmpT as a serine protease is weak, as pointed out in Section 1. To investigate the nature of the active site, we studied the involvement of acidic residues in substrate binding and/or hydrolysis by constructing single mutant proteins of OmpT. Substitution of the conserved residues Asp[43], Glu[111], Glu[136], Glu[193] or Glu[250] by alanines did not lead to severe reductions in proteolytic activity (Fig. 2). Not surprisingly, the side chain of none of these residues is located in the putative active site cleft (Fig. 1) [21]. Deleting either of the remaining six conserved acidic residues, which are all located inside the highly negatively charged cleft (Fig. 3) [21], resulted in at least sevenfold lower activity. OmpT preferentially cleaves peptides between two basic amino acids [5], therefore the anionic nature of the cleft is probably a major determinant of its substrate specificity. More specifically, we proposed earlier, based on the crystal structure of OmpT [21], that Glu[27] and Asp[208] may define the high specificity of OmpT for Arg or Lys at position P1 in the substrate (nomenclature as in reference [27]). The mutagenesis data reported here are in good agreement with this proposal. For the P1′ position, the specificity was less exclusive, but a positively charged amino acid was

preferred there as well [5], likely due to interaction with an anionic residue of OmpT. Assuming that the substrate has an extended conformation and that the P1 side chain points towards Glu^{27} and Asp^{208}, the P1′ chain would be located close to Asp^{97}. D97A OmpT displayed only 6% residual activity, therefore we propose that Asp^{97} is responsible for the observed P1′ specificity. A schematic model of a peptide in the active site is shown in Fig. 4.

Substituting the aspartate at position 83, 85 or 210 reduced activity at least 10 000-fold, suggesting that these residues may participate directly in enzymatic catalysis. The absolute requirement for more than one aspartate is reminiscent of the aspartic proteases, in which two aspartates activate a nucleophilic water molecule [13]. The relative orientation of Asp^{83} and Asp^{85} is in fact similar to that in aspartic proteases [21]. However, OmpT does not contain the D(T/S)G consensus sequence of aspartic proteases and it is not active at acidic pH [4], while the overall fold is very different from aspartic proteases [13]. A possible role for the Asp^{83}/Asp^{85} couple is discussed below. The X-ray structure of OmpT showed that the relative orientation of Asp^{210} and His^{212} is similar to that in the classical Ser/His/Asp triad and that there is no density at the position where a serine would be expected (Fig. 3) [21]. Based on this information, we recently postulated that OmpT has an Asp^{210}/His^{212} catalytic dyad with a water molecule acting as nucleophile [21]. The observation that Asp^{210} is essential for activity supports this hypothesis. The existence of an Asp/His catalytic dyad has been proposed for several enzymes catalyzing hydrolytic reactions, including phospholipase A2 [28], phosphoesterases [29], endonucleases [30], endocellulases [31] and haloalkane dehalogenase [32]. Interestingly, it has never been observed in proteases, implying that OmpT would be the first example of a protease using this type of catalytic mechanism. As amide bonds are relatively stable, other factors are expected to facilitate peptide cleavage by activated water. Presumably the Asp^{83}/Asp^{85} couple plays a role in the catalytic mechanism, for example by coordinating the nucleophilic water molecule as proposed before [21]. In this way, Asp^{83} and Asp^{85} would be indirectly involved in activation of the water molecule, a mechanism that is different from the direct activation of water by aspartates in aspartic proteases. Alternatively or in addition, a proton shared by the carboxyl moieties of Asp^{83} and Asp^{85} might stabilize the oxyanion intermediate during the reaction. In agreement with these hypotheses, the mutagenesis experiments show that both amino acids are required for activity. Further insight in the role of the essential residues Asp^{83}, Asp^{85} and Asp^{210} might be obtained by determining the kinetic parameters k_{cat} and K_M of the corresponding OmpT variants. Unfortunately though, the residual activities of D83A, D85A and D210A OmpT were very close to the detection limit of our assay, making it impossible to obtain reliable Michaelis–Menten curves for these variants. The preferred route to detailed elucidation of the active site mechanism would be to determine the X-ray structure of OmpT in the presence of a peptide substrate. Attempts to crystallize OmpT in complex with a substrate analogue are currently under way.

Prior to submission of this paper, we became aware of a very recent publication on omptin Pla of *Y. pestis* by Kukkonen et al. [33], who observed proteolytic importance for Pla residues corresponding to Ser^{99}, His^{101}, His^{212}, Asp^{83}, Asp^{85} and Asp^{210} in OmpT. They could not settle the exact roles of these residues, but hypothesized that Pla may contain a Ser^{99}/His^{212}/Asp^{210} triad (OmpT numbering), whereas Asp^{83}, Asp^{85} and His^{101} were proposed to be involved in substrate interaction [33]. Now that we have solved the crystal structure of OmpT and generated mutagenesis data on all conserved Ser, His, Asp and Glu residues in OmpT, we have a sound basis for our novel active site model, which does not involve a nucleophilic serine. The fact that the proposed catalytic residues Asp^{83}, Asp^{85}, Asp^{210} and His^{212} of OmpT have proteolytically important counterparts in Pla, indicates that all omptins likely use the same catalytic mechanism.

Acknowledgements: The authors wish to thank Mr. Ruud C. Cox for synthesis of the fluorogenic substrate. This research has been financially supported by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO).

References

[1] Sugimura, K. and Nishihara, T. (1988) J. Bacteriol. 170, 5625–5632.
[2] Grodberg, J., Lundrigan, M.D., Toledo, D.L., Mangel, W.F. and Dunn, J.J. (1988) Nucleic Acids Res. 16, 1209.
[3] Mangel, W.F., Toledo, D.L., Brown, M.T., Worzalla, K., Lee, M. and Dunn, J.J. (1994) Methods Enzymol. 244, 384–399.
[4] Kramer, R.A., Zandwijken, D., Egmond, M.R. and Dekker, N. (2000) Eur. J. Biochem. 267, 885–893.
[5] Dekker, N., Cox, R.C., Kramer, R.A. and Egmond, M.R. (2001) Biochemistry 40, 1694–1701.
[6] Webb, R.M. and Lundrigan, M.D. (1996) Med. Microbiol. Lett. 5, 8–14.
[7] Sedliakova, M., Masek, F., Slezarikova, V. and Pirsel, M. (1997) J. Photochem. Photobiol. B 41, 245–248.
[8] Stumpe, S., Schmid, R., Stephens, D.L., Georgiou, G. and Bakker, E.P. (1998) J. Bacteriol. 180, 4002–4006.
[9] Yu, G.Q. and Hong, J.S. (1986) Gene 45, 51–57.
[10] Sodeinde, O.A. and Goguen, J.D. (1989) Infect. Immun. 57, 1517–1523.
[11] Kaufmann, A., Stierhof, Y.D. and Henning, U. (1994) J. Bacteriol. 176, 359–367.
[12] Egile, C., d'Hauteville, H., Parsot, C. and Sansonetti, P.J. (1997) Mol. Micobiol. 23, 1063–1073.
[13] Barrett, A.J., Rawlings, N.D. and Woessner, J.F. (1998) Handbook of Proteolytic Enzymes, Academic Press, San Diego, CA.
[14] White, C.B., Chen, Q., Kenyon, G.L. and Babbitt, P.C. (1995) J. Biol. Chem. 270, 12990–12994.
[15] Sugimura, K. and Higashi, N. (1988) J. Bacteriol. 170, 3650–3654.
[16] Rawlings, N.D. and Barrett, A.J. (1994) Methods Enzymol. 244, 19–61.
[17] Hollifield Jr., W.C., Fiss, E.H. and Neilands, J.B. (1978) Biochem. Biophys. Res. Commun. 83, 739–746.
[18] Kramer, R.A., Dekker, N. and Egmond, M.R. (2000) FEBS Lett. 468, 220–224.
[19] Carter, P. and Wells, J.A. (1988) Nature 332, 564–568.
[20] Hakansson, K., Wang, A.H.-J. and Miller, C.G. (2000) Proc. Natl. Acad. Sci. USA 97, 14097–14102.
[21] Vandeputte-Rutten, L., Kramer, R.A., Kroon, J., Dekker, N., Egmond, M.R. and Gros, P. (2001) EMBO J., in press.
[22] Hanahan, D. (1983) J. Mol. Biol. 166, 557–580.
[23] Studier, F.W. and Moffatt, B.A. (1986) J. Mol. Biol. 189, 113–130.
[24] Yanisch Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103–119.
[25] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[26] Heller, K.B. (1978) J. Bacteriol. 134, 1181–1183.
[27] Schechter, I. and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162.
[28] Yuan, C. and Tsai, M. (1999) Biochim. Biophys. Acta 1441, 215–222.

[29] Knofel, T. and Strater, N. (1999) Nat. Struct. Biol. 6, 448–453.
[30] Gorman, M.A., Morera, S., Rothwell, D.G., de La Fortelle, E., Mol, C.D., Tainer, J.A., Hickson, I.D. and Freemont, P.S. (1997) EMBO J. 16, 6548–6558.
[31] Russell, R.B. (1998) J. Mol. Biol. 279, 1211–1227.
[32] Pries, F., Kingma, J., Krooshof, G.H., Jeronimus Stratingh, C.M., Bruins, A.P. and Janssen, D.B. (1995) J. Biol. Chem. 270, 10405–10411.
[33] Kukkonen, M., Lahteenmaki, K., Suomalainen, M., Kalkkinen, N., Emody, L., Lang, H. and Korhonen, T.K. (2001) Mol. Micobiol. 40, 1097–1111.