CHAPITRE 3: Relationship between propeptide pH unfolding and inhibitory ability during proDer p 1 activation mechanism

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3.1 Abstract

The major allergen Der p 1 of the house dust mite Dermatophagoides pteronyssinus is a papain-like cysteine protease (CA1) produced as an inactive precursor and associated with allergic diseases. The propeptide of Der p 1 exhibits a specific fold which makes it unique in the CA1 propeptide family. In this study, we investigate the activation steps involved in the maturation of the recombinant proDer p 1 expressed in Pichia pastoris and the interaction of the full length and truncated soluble propeptides with its parent enzyme in terms of activity inhibition and Biacore interaction analysis. According to our results, the activation of proDer p 1 is a multistep mechanism which is characterized by at least two intermediates. The propeptide strongly inhibits unglycosylated and glycosylated recombinant Der p 1 (K_D= 7 nM) at neutral pH. This inhibition is pH dependent. It decreases from pH 7 to pH 4 and can be related to conformational changes of the propeptide characterized by an increase of its flexibility and formation of a molten globule state. Our results indicate that activation of the zymogen at pH 4 is a compromise between activity preservation and propeptide unfolding.
3.2 Introduction
House dust mite allergens are commonly associated with allergic diseases such as asthma, perennial rhinitis and atopic dermatitis. Der p 1 is a major allergen from *Dermatophagoides pteronyssinus* as more than 80% of the house dust mite sensitized population produce large amounts of Der p 1 specific IgE antibodies (Chapman *et al.* 1983; Platts-Mills and Chapman 1987). It is a 25 kDa cysteine protease belonging to the papain-like protease family (CA1), which includes cathepsins K, L and B. Like most secreted cysteine proteases, Der p 1 is synthesized as a 34 kDa inactive precursor called proDer p 1, consisting of a cysteine protease domain (222 residues) and a N-terminal propeptide (80 residues), which blocks the proteolytic activity. Each domain contains one potential N-glycosylation site in positions 16p for the propeptide and 52 for Der p 1 (Chua *et al.* 1988; Chua *et al.* 1993). The propeptide might also function as an intramolecular chaperone, which ensures correct folding and targeting of the enzyme through the secretory pathway.

The proteolytic activity of Der p 1 has been reported to be an additional factor for the allergic pathogenesis (Kikuchi *et al.* 2006). Several reports indicate that Der p 1 is able to increase the bronchial permeability by disruption of the intercellular tight junctions and cleavage of the α1-antitrypsin serine protease inhibitor (Wan *et al.* 1999; Brown *et al.* 2003). Furthermore, Der p 1 is capable of cleaving receptors including the IgE low affinity receptor (CD23) present at the surface of plasmocyte cells, the α-subunit of the IL-2 receptor (CD25) of T-cells and the CD40 of dendritic cells (Schulz *et al.* 1997; Schulz *et al.* 1998; Shakib *et al.* 1998; Kikuchi *et al.* 2006; Furmonaviciene *et al.* 2007). All these processes favor the development of the allergic response, and thus the development of specific Der p 1 inhibitors is of a considerable interest. Furthermore, the comprehension of the proDer p 1 activation process has a considerable interest in the development of efficient recombinant mature allergen production systems thus essential for the screening of hypoallergenic vaccine.

Like most cysteine proteases, proDer p 1 is activated under low pH conditions (Menard *et al.* 1998; Jacquet *et al.* 2002; Takai *et al.* 2002). Despite several attempts, *in vitro* demonstration of the activation of proDer p 1 remains partly unsuccessful. For example, purified recombinant proforms of Der p 1 expressed in mammalian CHO and insect *Drosophila* cells could be converted into mature forms by incubation at pH 4, but did not exhibit enzymatic activity (Jacquet *et al.* 2000; Massaer *et al.* 2001). More recently, however, several groups have succeeded in converting proDer p 1 expressed in *Pichia pastoris*, into mature, enzymatically active forms, with IgE reactivity (Jacquet *et al.* 2002; Takai *et al.* 2002). Finally, several experiments performed with proDer p 1 mutants lacking the N-glycosylation sites, or with endoglycosylase H-pretreated proDer p 1, indicated that the glycosylation of recombinant zymogen expressed in *Pichia pastoris* can impair the maturation of the allergen (van Oort *et al.* 2002; Takai *et al.* 2006).

Analysis of the X-ray structure of the zymogen form of the allergen reveals that the propeptide of Der p 1 adopts a new fold within the CA1 protease family (Meno *et al.* 2005) (Figure 1). This fold is characterized by the presence of an N-terminal globular domain formed by the association of three amphipathic α-helices maintained together by aromatic and hydrogen bond interactions. This domain, despite tertiary structure similarities, shows little or no conservation of the consensus residues characteristic of the cathepsin L-like propeptide subfamily (Karrer *et al.* 1993). Moreover, the
presence of a fourth α-helix instead of a long terminal coil and a beta sheet at the C-terminal extremity makes it unique among the previously described CA1 propeptides. Based on Karrer’s classification, the Der p 1 propeptide could not be assigned to the cathepsin L or B subfamilies. Meno et al. (Meno et al. 2005) proposed that the Der p 1 propeptide belongs to a third subfamily characterized by a shorter propeptide with four α-helices and the absence of β-strands. Therefore, the study of the proDer p 1 activation mechanism could represents an interesting way to highlight the importance of the presence or absence of conserved propeptide motif and secondary structure elements during the activation of papain-like precursor.

In the last decade, the potent inhibitor ability of several propeptides towards their corresponding enzymes of the papain-like family has been extensively studied (Groves et al. 1996; Maubach et al. 1997; Billington et al. 2000; Wiederanders 2003). Some cathepsin propeptides have been shown to display a strong inhibition capacity at neutral pH, with values of the dissociation constant $K_D$ ranging from 0.12 nM for cathepsins L and B to 7.6 nM for cathepsins S and K. Interactions between the propeptides and the proteases are pH dependent: at neutral pH, slow binding interactions occur, whereas at acidic pH, weak or no interactions are observed (Maubach et al. 1997). For the cathepsin L propeptide, lack of inhibition has been associated with partial unfolding of the propeptide and formation of a molten globule state under acidic conditions (Jerala et al. 1998). Moreover, under these conditions, it has been demonstrated that the cathepsin S propeptide is slowly degraded by mature cathepsin L (Maubach et al. 1997).

To date, the interaction between mature Der p 1 and its propeptide has not been investigated, due to the difficulty to obtain correctly matured recombinant Der p 1 (rDer p 1). In order to characterize this interaction, we have studied the activation steps involved in the zymogen maturation mechanism. Furthermore, we have produced different recombinant forms of the Der p 1 propeptide (Figure 1) and analysed their interactions with mature glycosylated and unglycosylated rDer p 1 at different pH values, in terms of both activity inhibition and structural changes.

FIGURE 1: Schematic representation of the different forms of the Der p 1 propeptide and location of the cleavage sites identified during zymogen maturation. A: Overall structure of the proDer p 1 zymogen showing the unique propeptide folding (1XKG). The propeptide is coloured in black, catalytic site in red, whereas the structure of the mature protease is shown in green. B: 1) Sequence of the full length Der p 1 propeptide. The numbering of the propeptide is labelled with the letter p. Position 1 corresponds to the N-terminal amino acid of the mature Der p 1. Arrows a, b, c and d indicate the positions of the different cleavage sites of the propeptide during the maturation of the N52Q proDer p 1 at pH 4. The α-helices are highlighted in grey. The position (16p) of the potential N-glycosylation site of the propeptide is indicating by *. 2) Amino acid sequences of the His tag recombinant forms of the complete, C and N -truncated propeptides.
3.3 Materials and methods

3.3.1 Construction of the unglycosylated proDer p 1 expression vector
The (N52Q), (N16pQ) and (N16pQ/N52Q) unglycosylated proDer p 1 mutants (Der p 1 numbering) were constructed in two steps by overlapping PCR from pNIV4878 (Massaer et al. 2001). The sequences of the primers used in this study are detailed in Table I. The amplified fragment was cloned into the pCR2.1 TOPO cloning vector (Invitrogen, Groeningen, the Netherlands) and the presence of both mutations of interest was verified by DNA sequencing. The fragments containing the proDer p 1 coding sequence, isolated by digestion of the recombinant pCR2.1 TOPO with StuI-AvrII, were subsequently cloned into the pPIC9K expression vector (Invitrogen) previously restricted by SnaB1-AvrII. The resulting expression vectors contain the proDer p 1 cassette with one or two mutations downstream of the S. cerevisiae α-factor.

3.3.2 Expression of the proDer p 1 proforms in Pichia pastoris
The recombinant expression vectors were introduced into P. pastoris SMD1168 (Invitrogen) using the electroporation transforming method. Transformants were first selected for histidinol dehydrogenase (His+), then for increasing geneticin (G418) resistance (0.25 to 3 mg/ml). Selected clones were grown at 30 °C in 200 ml of Yeast Glycerol Buffered Media (BMGY) to an A600 value of 2 to 6. This preculture was then transferred into 3.5 liters of fermentation minimal medium and cultured for 24 hours at 30 °C pH 6. Production of the recombinant proDer p 1 was then induced by addition of methanol over four days (final concentration 0.5%). The methanol feed rate was regulated by following the dissolved oxygen level (30%). The supernatant was finally obtained by centrifugation of the culture at 13000 g during 20 min.

3.3.3 Purification and maturation of the proDer p 1 proforms
The yeast culture medium containing the recombinant proDer p 1 was dialyzed during 24h against 20 mM Tris-HCl buffer pH 8.5 (starting buffer). The medium was then loaded onto a Q-HP sepharose column (60 ml) (Amersham Biosciences, GE Healthcare, Uppsala, Sweden), equilibrated with the starting buffer. The column was washed with the same buffer to eliminate the non-absorbed contaminants and the bound proteins were eluted with a linear NaCl gradient (0-0.3 M). The fractions containing proDer p 1 were pooled and concentrated by ultrafiltration (cut off: 10 kDa). A homogeneous solution of proDer p 1 could be obtained by gel filtration on a Sephacryl-100HR column (120 ml) (Amersham), equilibrated in 10 mM Na2HPO4/KH2PO4 buffer, pH 7.4 in the presence of 150 mM NaCl (PBS). The concentration of proDer p 1 was estimated by BCA assay (Pierce, Rockford, USA).

3.3.4 proDer p 1 maturation assay
The maturation was performed as previously described (Jacquet et al. 2002), with the following modifications: the purified proDer p 1 was dialyzed against 100 mM acetate buffer at pH 4 during 72 hours at 4 °C (Jacquet et al. 2002; Takai et al. 2002). For the N52Q proform, maturation was performed at 37°C in polybuffer (mix of 50 mM Tris, phosphate, citrate, acetate and KCl, adjusted to choosen pH with HCl) pH 4, with or without addition of 1 mM DTT and 1 mM EDTA. The maturation was followed by SDS-PAGE analysis, confirmed by N-terminal sequencing and enzymatic activity measurements.
3.3.5 Construction of the full length and truncated propeptide expression vectors

The DNA corresponding to the full length (Arg1p-Glu80p), to the N-terminal (Tyr19p-Glu80p) and C-terminal (Arg1p-Arg60p) truncated forms of the Der p 1 propeptide together with a N-terminal sequence encoding for six histidines (His-tag), were constructed by PCR using pNIV4878 as template (Jacquet et al. 2002). The primers used to amplify the propeptide sequences were obtained from Eurogentec (Seraing, Belgium) (Table I). The PCR products were cloned into pGEM-T Easy (Promega, Madison, USA). The DNA fragments encoding the propeptide were sequenced to verify the correct amplification and the presence of the histidine tag. The propeptide coding cassettes were digested with NdeI and EcoRI restriction enzymes and cloned into the pET-22b(+) (Novagen, Oxon, UK), previously restricted with the same enzymes.

Table I. Nucleotidic sequences of the primers.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProNdeIUP</td>
<td>ATCCATATGCACTACACCACCCACCACGCGAGCTCCATTAAAGACCTTCG</td>
</tr>
<tr>
<td>ProNdeI-18UP</td>
<td>ATCCATATGCACTACACCACCCACCACATGCACTCCCTCGAGGAGGAGGAGG</td>
</tr>
<tr>
<td>ProEcoRIDO</td>
<td>TCGCGAATTCTACTCCCGTTAGATCAAACCTGTCG</td>
</tr>
<tr>
<td>ProEcoRI-20DO</td>
<td>TCGCGAATTCTACTCCCGTTACTGAACCTGTCAAAGACAGGTCG</td>
</tr>
<tr>
<td>N52QUP</td>
<td>CCTCGCGTATCGCAACAGAGGCGTCAATGGAC</td>
</tr>
<tr>
<td>N52QDO</td>
<td>GGTCAAGGCTCTGGTGACGCGTGAGAGGAGAGGAG</td>
</tr>
<tr>
<td>N16pQUP</td>
<td>ATCAAAGAAGGCTCTCCAGAGGAGCTAGT</td>
</tr>
<tr>
<td>N16pQDO</td>
<td>AGGTGGCATAGCTCTTCTGGAAGGCTTTCTTGTAT</td>
</tr>
</tbody>
</table>

The bold types indicate the introduced restriction sites.

3.3.6 Expression and purification of the propeptides

Recombinant expression vectors were transformed into E. coli BL21 (DE3) pLys cells (Novagen). Transformants were selected on Luria-Bertani (LB) agar plates containing ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml). The expression was initiated by a (1/50) dilution of an overnight subculture in 2 liters of LB medium containing ampicillin (100 µg/ml) and chloramphenicol (30 µg/ml), until an A_600 value of 0.5 to 1 was reached. The propeptide expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Immunosource, Halle-Zoersel, Belgium) to a final concentration of 1mM in the medium. After 6 hours of incubation at 25 °C, the cells were collected by centrifugation. The pellet was resuspended in PBS pH 7.4 and then lysed with the help of a desintegrator (Constant Systems, Daventry, UK). The lysate was centrifuged for 20 min at 12000 g and the pellet was discarded. The supernatant was applied onto a Ni-PDC column (10 ml) (Affiland, Liège, Belgium) equilibrated in PBS buffer. The column was washed with 10 volumes of PBS buffer and PBS added with 2 M NaCl. The propeptide was eluted with a linear imidazole (20-150 mM) gradient. Fractions enriched in propeptide were pooled and dialyzed against 20 mM Tris buffer, pH 8 (TB). Further purification was achieved by anion-exchange chromatography on a MonoQ column (Amersham Biosciences) equilibrated with TB buffer. The elution was performed with a linear NaCl (0-600 mM) gradient. Fractions containing the pure propeptide were pooled and dialyzed overnight against PBS. The homogeneity of the preparation was assessed by SDS-PAGE, N-terminal sequencing and mass spectrometry.

The concentration of the purified propeptide was determined from the A_280 value, using the calculated extinction coefficient value of 3840 M^-1 cm^-1, and confirmed by a BCA assay (Pierce), using bovine
serum albumin as standard. Purified soluble propeptides and processed proDer p 1 were sequenced in an Applied Biosystems Procise 492 sequencer (Applied Biosystems), based on Edman degradation.

### 3.3.7 Inhibition of the recombinant Der p 1 by the propeptide

The proteolytic activity of rDer p 1 was estimated in the presence of various propeptide concentrations, by following the increase in fluorescence emission resulting from the liberation of the 7-amino-4-methylcoumarin (AMC) group upon hydrolysis of the substrate Boc-Gln-Ala-Arg-AMC (Boc: N-tert-butoxy-carbonyl) (Bachem, Budendorf, Switzerland). The measured fluorescence intensity could be converted into product concentration, with the help of an AMC (0-0.6 µM) standard curve. Measurements were performed with a LS 50 B fluorimeter (Perkin Elmer, Wellesley, USA), using excitation and emission wavelengths of 380 nm and 460 nm, respectively. Enzymatic reactions were initiated by addition of active rDer p 1 (5 nM) to substrate solutions containing various concentrations of propeptide. In all cases, the substrate concentration (40 or 160 µM) was below the $K_m$ value (280 µM) (Schulz et al. 1998) and the propeptide concentration (20-120 nM) was larger than the enzyme concentration. Under these conditions, it was possible to determine the value of the pseudo-first order rate constant ($k_{obs}$) characteristic of the complex formation, and the individual kinetic rate constants for association ($k_{on}$) and dissociation ($k_{off}$) respectively. All experiments were carried out at 25 °C, between pH 9 and 3, in 50 mM polybuffer containing 1mM DTT and 1 mM EDTA, using a 1 ml stirred cell. The $k_{obs}$ value was estimated from the non-linear part of the product formation (P) time-course, using the Grafit software (Erithacus Software Ltd.). Thus, for each propeptide concentration, the apparent first order rate constant ($k_{obs}$) was determined by fitting the decrease of the initial rate of the reaction ($v_0$) to its steady state rate ($v_s$), according to equation 1, where $k_{obs}$ depends on the propeptide concentration ($I$) according to equation 2 and considering equation 3 (Morrison and Walsh 1988):

$$ p = v_s t + (v_0 - v_s) \frac{1 - e^{-k_{off}}}{k_{obs}} $$ (Eq. 1)

$$ k_{obs} = k_{eff} + k_{on} \left( \frac{K_m}{K_m + [S]} \right) I $$ (Eq. 2)

$$ K_{D_{init}} = \frac{k_{eff}}{k_{on}} $$ (Eq. 3)

According to model 1, the overall $K_D$ ($K_{DGlocal}$) value was determined by linear regression of the first order rate constant ($k_{obs}$) as a function of the propeptide concentration, according to equation 4:

**Model 1:**

$$ E + I \overset{k_{off}}{\rightleftharpoons} E + P $$

$$ k_{obs} = k_{off} (1 + \left( \frac{I K_m}{K_{D_{init}} (K_m + [S])} \right) $$ (Eq. 4)

The value of the initial dissociation constant ($K_{Dinit}$) corresponding to the instantaneous inhibitory capacity of the propeptide was estimated by fitting the calculated initial rates ($v_i$) to equation 5, using
the corresponding values of $V_{\text{m}}$ ($V_m$), $K_m$ and $[S]$ (Morrison and Walsh 1988):

$$
\frac{V_{\text{m}}}{V_i} = 1 + \frac{K_m}{[S]} + \frac{[I]K_m}{K_{D_{\text{free}}}[S]}
$$  
(Eq. 5)

The value of the final dissociation constant ($K_{D_{\text{final}}}$) was determined using equation 6, considering the calculated values of the overall and initial dissociation:

$$
K_{D_{\text{free}}} = \frac{K_{D_{\text{free}}}K_{D_{\text{total}}}}{K_{D_{\text{total}}} + 1}
$$  
(Eq. 6)

### 3.3.8 Surface plasmon resonance (BIAcore) measurements

The interaction between the full-length propeptide and recombinant unglycosylated rDer p 1 was analysed by surface plasmon resonance (SPR) using a BIAcoreX instrument (Biacore AB, Upplandsväst, Sweden). Both binding under equilibrium conditions and kinetics of binding were studied. Approximately 270 resonance units (RU) of full-length propeptide were immobilized on a carboxymethylated dextran-coated sensor chip CM5 (Biacore AB), using the amine coupling chemistry (EDC/NHS) according to the manufacturer instructions. The binding/regeneration cycles were performed at 25 °C in HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v surfactant P20 -Polysorbate-). Surface regeneration was achieved by injection of 5 µl of 6 M guanidinium chloride, at a flow rate of 10 µl/min. All measurements were repeated at least twice. In equilibrium binding experiments, unglycosylated rDer p 1 (128 nM) was incubated for 2 hours (ensuring that the equilibrium was reached) with increasing concentrations of full-length propeptide (6 to 1500 nM), and then 100 µl of the solution were injected on the CM5 surface coated with the propeptide, at a flow rate of 30 µl/min. The $K_D$ value was determined using the model of “Affinity in solution” from the BIAevaluation 4.1 software which, is based on equation 7:

$$
E_{\text{free}} = \frac{E - I - K_D}{2} + \sqrt{\frac{(I + E + K_D)^2}{4} - IE}
$$  
(Eq. 7)

where $E$, $E_{\text{free}}$, $I$ and $K_D$ are the total concentration of rDer p 1, the concentration of unbound rDer p 1, the total concentration of propeptide and $K_D$ the dissociation constant respectively.

For kinetic experiments, different concentrations of unglycosylated rDer p 1 were injected at a flow rate of 70 µl/min. At this flow rate, the mass transfer effects were found to be negligible. Kinetic rate constant values for association ($k_a$) and dissociation ($k_d$) (Table II) were obtained on the basis of two different models: a two-state reaction model (model 2) which assumes a conformational change, or a heterogeneous ligand model (model 3), provided with the BIAevaluation software (version 4.1) :

Model 2:

$$
K_A = \frac{k_{a2}}{k_{d2}} \times \left(1 + \frac{k_{a2}}{k_{d2}}\right)
$$

$$
K_D = \frac{1}{K_A}
$$  
(Eq. 8, 9)
pH dependence of propeptide inhibition

Where I and E represent the propeptide and rDer p 1, respectively, $k_{a1}$ and $k_{d1}$ are the kinetic rate constants for association and dissociation of the initially formed complex EI, and $k_{a2}$ and $k_{d2}$ are the kinetic rate constants for interconversion between EI and EI*. $K_A$ is the overall equilibrium association constant.

Model 3:

$$E + I \xrightarrow{k_{a1}} EI$$

$$K_{AI} = \frac{k_{a1}}{K_{d1}} = \frac{1}{K_{AI}} \quad \text{(Eq. 10, 11)}$$

$$E + I_1 \xrightarrow{k_{a2}, k_{d2}} EI_1$$

$$K_{A2} = \frac{k_{a2}}{K_{d2}} = \frac{1}{K_{A2}} \quad \text{(Eq. 12, 13)}$$

Where E, I, and I₂ represent rDer p 1 and two different propeptide conformations, respectively. The individual rate constants $k_a$ and $k_d$ correspond to the association and dissociation of the EI₁ and EI₂ complexes, respectively. $K_{A1}$ and $K_{A2}$ are the equilibrium association constants for each reaction.

3.3.9 Fluorescence measurements

All fluorescence experiments were performed with the help of a Perkin Elmer LS 50 B spectrofluorimeter, using a stirred cell with 1 cm pathlength. Propeptide intrinsic fluorescence and ANS-bound fluorescence emission spectra were recorded at 25 °C in 50 mM polybuffer, pH 2-9, using a scan speed of 280 nm per minute. The spectra were measured four times and averaged. Intrinsic fluorescence measurements were performed using a protein concentration of 5 µM, with an excitation wavelength of 280 nm and emission recorded from 285 to 400 nm. The binding of ANS (8-anilino-1-naphtalene-sulfonic acid) (Sigma, St Louis, USA) to the complete or truncated propeptides was measured using an excitation wavelength of 350 nm, and recording emission spectra from 400 to 600 nm. These were corrected for the background fluorescence of free ANS. The concentration of ANS (200 µM) was determined using the value of molar extinction coefficient of ANS at 350 nm (4950 M⁻¹ cm⁻¹), and the [ANS] / [Propeptide] ratio was about 40.

The dynamic quenching of fluorescence of tyrosine residues by acrylamide (ICN, Aurora, USA) was monitored with excitation and emission wavelengths of 280 nm and 305 nm, respectively, and using a 50 seconds integration time. Propeptides were incubated with increasing concentrations of a small quencher molecule (acrylamide) to probe the accessibility of the tyrosine residues within the protein. The decrease of fluorescence intensity arising from diffusive collisions between the quencher and the fluorophore reflects the ability of the quencher to penetrate the structure and can be viewed as an index of the protein flexibility (Lakowicz 1983). The solvent accessibility of tyrosine residues was estimated by linear fitting of the $(F_0/F)$ ratio where $F_0$ and F are the fluorescence in the absence and the presence of acrylamide, respectively, according to the modified Stern-Volmer equation (Equation 19), where $K_{SV}$ represents the Stern-Volmer constant for dynamic quenching and [Q] is the concentration of the quencher (i.e. acrylamide). The intrinsic protein fluorescence was corrected for the acrylamide inner filter effect ($f$), which was calculated using equation 20, with a molar extinction coefficient ($\varepsilon$) of 0.25 M⁻¹ cm⁻¹ for acrylamide at 280 nm.
\[ \frac{F}{F_0} = 1 + K_{sv} [Q] \]  
\[ f = 10^{-\frac{[Q]}{2}} \]  

(Eq. 19)  
(Eq. 20)

3.3.10 Circular dichroism measurements
Circular dichroism (CD) spectra were recorded using a Jasco J-810 CD spectropolarimeter (Jasco Inc., Easton, USA), equipped with a Peltier temperature controller. Far and near UV CD spectra were recorded at 25°C, in 1 and 10 mm pathlength cells, respectively. Five scans were averaged and corrected by baseline spectra subtraction. All experiments were performed in 10 mM polybuffer pH 2 to 7, using a propeptide final concentration of 25 µM and 100 µM for far and near UV CD experiments, respectively. Spectra were acquired at a scan speed of 20 nm per minute, with a 1 nm bandwidth and a 4 second integration time.

3.3.11 Computer simulation
The enzymatic experimental data were fitted on the two steps model (model 2) using the equation set (Eq. 14-18) within the Matlab software environment (version 5.3, MathWorks Inc.). The routine uses the \texttt{lsqcurvefit} function, which solves non-linear least squares problems. The differential equations of the model are solved by a low order Runge-Kutta method (\texttt{ode23} function), that only needs to know the solution at the preceding point.

As the values of the parameters slightly differ for the different initial concentrations of inhibitor, a unique set of values is chosen for the four parameters (the mean of the best fittings), and product concentration (P) is simulated using the equation system (solved by a low order Runge-Kutta method), in order to calculate the value of \(k_{\text{obs}}\). The software gives the concentration of the different species versus time.

\[ \frac{dE}{dt} = -k_1 \times E \times I + k_{-1} \times EI \]  
\[ \frac{dl}{dt} = -k_1 \times E \times I + k_{-1} \times EI \]  
\[ \frac{dEI}{dt} = k_1 \times E \times I + k_{-2} \times EI^* - (k_{-1} + k_2) \times EI \]  
\[ \frac{dEI^*}{dt} = k_2 \times EI - k_{-2} \times EI^* \]  
\[ \frac{dP}{dt} = 0.36 \times k_{\text{cat}} \times E \]  

Where 0.36 represents the ratio of \(\frac{[S]}{K_m + [S]}\)

3.3.12 Molecular modelling
The 3D coordinates of the initial structure of the propeptide were taken from the crystal structure of the complex between Derp1 and its propeptide (Meno \textit{et al.} 2005) and soaked in a 5 Å layer of water molecules. The geometry was first optimized with a convergence threshold of 0.02 kcal mol\(^{-1}\) Å\(^{-1}\), and then submitted to two dynamics simulations at 300 K during 100 ps and 1.1 ns respectively. The
chosen force field was Amber (Weiner 1981; Weiner 1986), the relative dielectric constant was set to 1 and the program used was Insight-Discover (Accelrys Software Inc., www.accelrys.com) running on a Pentium 4 3.4 GHz under Unix Debian.

3.4 Results

3.4.1 Production and maturation of purified proDer p 1 mutants

proDer p 1 mutants were constructed by overlapping PCR from the full-length proDer p 1 synthetic cDNA with optimized mammalian codon usage (Massaer et al. 2001). In these constructions, the potential N-glycosylation sites in the propeptide (N16p) or/and in the protease domain (N52) were eliminated by substitution of the asparagine by a glutamine residue (N16pQ, N52Q). WT proDer p 1 and the three mutants were successfully secreted by P. pastoris. Figure 2A indicates that the substitution of N16pQ in the proDer p 1 yielded a mature glycosylated rDer p 1. Therefore, the hyperglycosylation site of the zymogen is the N52. This conclusion is supported by the fact that the rDer p 1 obtained by activation of mutants N52Q or N16pQ/N52Q proDer p 1 are unglycosylated (Figure 2A). However, surprisingly, for the N16pQ and N16pQ/N52Q mutants, partial activation occurred during the production leading to the appearance of different forms. The N-terminal sequencing of the purified mutants revealed the presence of at least three forms, previously described by Takai et al. and Jacquet et al (Jacquet et al. 2002; Takai et al. 2002). The first one displayed the ATFE- sequence, which corresponds to the cleavage of the peptide bond between Tyr19p and Ala20p. This site is only three residues away from the propeptide N-glycosylation site and gives rise to the loss of the first alpha helix of the propeptide (site a, Figure 1). The two other forms showed the AETN- and TNA- sequences, which correspond to the mature allergen form with and without a two-residue extension, respectively. The ATFE- intermediate could readily be converted into the mature allergen, after incubation at pH 4. Such cleavages within the propeptide could not be observed for the WT proform produced under the same conditions, confirming previous observations that glycosylation of the propeptide might decrease the maturation ability of proDer p 1 (Takai et al. 2006).

FIGURE 2: Mutants glycosylation pattern and time-dependent processing of proDer p 1 N52Q zymogen at pH 4. A: Influence of glycosylation on the electrophoretic behaviour of proDer p 1 and Der p 1. Lane 1: Molecular mass marker. Lane 2: Glycosylated proDer p 1 WT. Lane 3: Glycosylated rDer p 1 yielded by the activation of N16pQ rproDer p 1. Lane 4: Unglycosylated rDer p 1 yielded from glycosylated N52Q rproDer p 1. Lane 5: Unglycosylated rDer p 1 yielded from glycosylated N16pQ/N52Q rproDer p 1. The data clearly show that the unglycosylated rDer p 1 (N52Q) migrate faster and as sharper bands due to the absence of the hyperglycosylation of N52 site. B: Identification of the different intermediates generated during proDer p 1 activation. Purified N52Q mutant was incubated at 25°C in polybuffer 50 mM pH 4 with or without 1 mM DTT and 1 mM EDTA. Samples were analyzed by SDS-PAGE (Coomassie Blue staining). Lanes 1 and 6: Molecular mass marker. Lanes 2 and 7 purified N52Q, i.e., time 0. Lanes 3 and 8: 2 hour incubation. Lanes 4 and 9: 8 hour incubation. Lanes 5 and 10; 24 h incubation time. The N-terminal sequences of A, B, C and D are RPSS-, ATFE-, SNGG- and AETNA-/TNA- respectively.
pH dependence of propeptide inhibition

With the proDer p 1 N52Q mutant four distinct bands (Figure 2B, lanes 2 and 7) were observed at the end of the purification. In order to determine whether these bands correspond to partially activated proDer p 1 or to a heterogeneous glycosylation at the N16p site, as proposed by Takai et al. (Takai et al. 2006), the proteins corresponding to the major bands (A and B) were analysed by N-terminal sequencing. This revealed the presence of the complete proform (RPSS-, band A) and the ATFE-intermediate (band B). The N52Q protein was submitted to the maturation assay under acidic conditions, in both the presence and absence of 1 mM DTT and 1 mM EDTA (Figure 2B). Upon incubation in acidic buffer, an increase of the intensity of band C, with an apparent molecular mass of 27 kDa, was observed. N-terminal sequencing of band C revealed the occurrence of one additional intermediate species, characterized by the SNGG-sequence, which corresponds to the cleavage of the peptide bond between residues Gln40p and Ser41p of the propeptide (site b, Figure 1). The evolution of the band intensities observed in the absence of DTT and EDTA (Figure 2B) clearly indicates that this form appears after the ATFE-intermediate. Finally, after a 24 hours incubation, form C evolved in the mature form of Der p 1 (band D). These results seem to confirm that proDer p 1 maturation is a multi-step mechanism, which leads to the formation of at least two intermediates. This process could be accelerated in the presence of DTT and EDTA (Figure 2B) suggesting that the catalytic cysteine may play a major role in the activation of the zymogen.

3.4.2 Production and purification of the full length and truncated propeptides

The proDer p 1 His-tagged full-length (Arg1p-Glu80p), N-truncated (Tyr19p-Glu80p) and C-truncated (Arg1p-Arg60p) propeptides were produced in E. coli as cytoplasmic soluble proteins. About 90 mg of purified proteins per liter of culture were obtained for the three propeptide forms. N-terminal sequencing of the three propeptides, revealed the MHHH-sequence, confirming the presence of the His-tag preceeded by the N-terminal methionine. The molecular mass of the full-length form was determined by mass spectrometry, which gave a value of 10227 Da, perfectly matching the value calculated from the amino acid sequence (10227.3 Da).

3.4.3 Activity inhibition and pH dependence

Our kinetic experiments indicated that the full length propeptide inhibits the proteolytic activity of both unglycosylated (N52Q) and glycosylated rDer p 1, with dissociation constant values (K_{DGlobal}) of 7 ± 1 nM (k_{off} = 0.008 ± 0.001 s^{-1}) and 9 ± 4 nM (k_{off} = 0.008 ± 0.003 s^{-1}) at pH 7, respectively. Progress curves of product formation in the presence of increasing concentrations of unglycosylated propeptide (from 0 to 120 nM) are shown in Figure 3A. These data highlight the slow binding interaction between the propeptide and rDer p 1, and allow calculation of the value of the pseudo-first order rate constant, k_{obs} at various propeptide concentrations. K_{D} values were computed based on model 1 using equation 4 (Figure 3B).

With both glycosylated and unglycosylated rDer p 1, identical K_{D} values within the error limit were obtained, indicating that glycosylation of the protease domain does not impair the propeptide association. This observation can be explained on the basis of the position of the N52 glycosylation site, which, in the structure of the zymogen, is situated on the opposite side to the enzyme active site and therefore, cannot interact with the propeptide during association.
pH dependence of propeptide inhibition

FIGURE 3: Inhibition of recombinant Der p 1 by its propeptide. Measurements were performed at 25°C in 50 mM polybuffer, pH 7. A: Time dependent inactivation of rDer p 1 (5 nM) by propeptide (25-100 nM). The concentration of Boc-Gln-Ala-Arg-AMC was 160 µM. Each curve was obtained in triplicate. B: Plot of $k_{obs}$ versus propeptide concentration. The $k_{obs}$ values were determined for the glycosylated (O) and unglycosylated (N52Q) (•) rDer p 1. Average values are given with SD.

Interestingly, no significant inhibition could be detected with truncated propeptides (data not shown). At the highest concentrations tested (5 µM), only a minor decrease (≤10%) of the initial activity could be monitored with the N-truncated propeptide, whereas no change was detected with the C-truncated form. These observations show the importance of both the C- and N-terminal domains of the propeptide for the inhibition process.

The activity of rDer p 1 and its interaction with the propeptide was shown to be pH dependent. The initial rates of product formation measured at different pH values are shown in Figure 4A. rDer p 1 is active at pH values ranging from 3.5 to 9, with maximal activity at pH ~6.5. At pH 4, rDer p 1 retains 15% of its maximal activity, which can be sufficient to ensure zymogen activation. Our results are in good agreements with the pH activity profile established by Shultz et al. (Schulz et al. 1998). A comparison of the steady state rates of product formation measured at different pH values, in the presence of increasing propeptide concentrations, highlights the pH dependence of the inhibition process (Figure 4 A, B). At pH 4, the enzyme remains fully active, even in the presence of high propeptide concentrations, whereas in the pH range 6-7 less than 20% of the enzyme activity is retained at propeptide concentration ≥ 40 nM.

FIGURE 4: pH dependence of the interaction between Der p 1 and its propeptide. Measurements were performed at 25°C in 50 mM polybuffer between pH 3 and 9. A: The activity of rDer p 1 N52Q (5 nM) at different pH and in the presence of propeptide (0 to 80 nM) was measured at the steady state of the hydrolysis rate of 40 µM Boc-Gln-Ala-Arg-AMC. Each experiment was done in triplicate. Average values are given with SD. B: Dependence of the residual activity (%) of rDer p 1 in presence of different propeptide concentrations.
3.4.4 Propeptide-Der p 1 association and dissociation by Surface Plasmon Resonance (SPR)

The affinity of the propeptide for rDer p 1 (N52Q) was first evaluated using a competition assays in solution, and a dissociation constant (K_D) value of 12 ± 2 nM could be determined (data not shown). This value indicates that the full length propeptide displays a high affinity for Der p 1. In addition, a kinetic analysis allowed calculation of the rate constant for both association (k_a) and dissociation (k_d).

A summary of the calculated rate and equilibrium constants is found in Table II.

Table II. Surface Plasmon Resonance (BIAcore) parameters

<table>
<thead>
<tr>
<th>Constants</th>
<th>Two states</th>
<th>Heterog. Lig.</th>
<th>Enz. inh.</th>
<th>Simul.</th>
</tr>
</thead>
<tbody>
<tr>
<td>k_on (M⁻¹s⁻¹x10⁶)</td>
<td>-</td>
<td>-</td>
<td>110 ± 2.3</td>
<td>-</td>
</tr>
<tr>
<td>k_off (s⁻¹x10⁻⁵)</td>
<td>-</td>
<td>-</td>
<td>7.8 ± 1.0</td>
<td>-</td>
</tr>
<tr>
<td>k_d1 (M⁻¹s⁻¹x10⁶)</td>
<td>10.1 ± 0.2</td>
<td>7.5 ± 0.02</td>
<td>-</td>
<td>7.5 ± 1.7</td>
</tr>
<tr>
<td>k_d2 (s⁻¹x10⁶)</td>
<td>7.6 ± 0.6</td>
<td>0.5 ± 0.006</td>
<td>-</td>
<td>1.8 ± 0.05</td>
</tr>
<tr>
<td>K_D (M x10⁻⁹)</td>
<td>4.3 ± 0.1</td>
<td>1.6 ± 0.001*</td>
<td>-</td>
<td>3.6 ± 0.05</td>
</tr>
<tr>
<td>K_D1 (M x10⁻⁹)</td>
<td>9.0 ± 0.1</td>
<td>123 ± 0.87</td>
<td>-</td>
<td>9.0 ± 0.4</td>
</tr>
<tr>
<td>K_D2 (M x10⁻⁹)</td>
<td>75.2 ± 4.5</td>
<td>-</td>
<td>36.7 ± 1.8</td>
<td>24 ± 6</td>
</tr>
<tr>
<td>K_D3 (M x10⁻⁹)</td>
<td>0.20 ± 0.01</td>
<td>-</td>
<td>0.23 ± 0.04</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td>K_D4 (M x10⁻⁹)</td>
<td>12.9 ± 0.2</td>
<td>-</td>
<td>7.0 ± 1.0</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>K_D5 (M x10⁻⁹)</td>
<td>-</td>
<td>7.2 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>K_D6 (M x10⁻⁹)</td>
<td>-</td>
<td>76.3 ± 1.2</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A global analysis of the association and dissociation data with several models revealed that the data could not be fitted to a simple 1:1 binding model (model 1), but are better described either by a model which accounts for conformational change (model 2), leading to the formation of a higher affinity complex (Figure 5A) or by a heterogeneous ligand model (model 3) (Figure 5B). The former model implies a 1:1 binding of rDer p 1 to the immobilized propeptide, followed by a conformational change of the complex, which can affect either the propeptide or rDer p 1 or even both. The value of the global affinity constant obtained by fitting the data to model 2 (K_D = 12.9 ± 0.2 nM) is not significantly different from the value obtained in solution. Alternatively, the heterogeneous ligand model yielded two K_D values of 7.2 nM and 76 nM.

FIGURE 5: Analysis by surface plasmon resonance spectroscopy of the binding of rDer p 1 to the full length propeptide immobilized on a Biacore CM5 chip. Different concentrations (0, 15, 30, 60, 120 and 240 nM) of rDer p 1 N52Q in HBS buffer were injected at a flow rate of 70 µl/min. Kinetic rate constants (k_a and k_d) were fitted using: A the two-state reaction model including a conformational change, and B the heterogeneous ligand model. Lower panel correspond to the residual value of the fitting for each rDer p 1 concentration.
Such a model is often observed when the ligand has multiple attachment sites to the sensor chip. Two of the seven lysine residues, which mediate the covalent immobilization of the propeptide, are located in the C-terminal part of the propeptide. Thus, the lower affinity constant value (76 nM) could correspond to the propeptide molecule immobilized through these two residues, in a less accessible orientation for Der p 1. Even if these two models reflect two different phenomena, the calculated constants for the two models are in the same order of magnitude and are in good agreement with those obtained in solution.

The validity of the two states model was also analysed by fitting the different rate constants determined by SPR to the different experimental product formation curves obtained at different propeptide concentrations. Primary fitting and optimization analysis of each time curves permitted to determine different sets of optimal rate constants for each initial propeptide concentration higher than 50 nM. The different rate constants obtained from the primary analysis agreed fairly well with the values determined by SPR. Based on these data, the different sets of rate constants were averaged and recomputed for all product formation curves. The resulting optimized rate constants are listed in Table II. Computed values permitted to follow the evolution of the concentration of the different enzyme species during the reaction time (Figure 6A).

Free enzyme concentration (E) decreases rapidly as a well shaped exponential curve whereas the initial complex concentration (EI) increases in the first quarter of the reaction time, then decreases to reach about 55 % of the [E₀] at the end of the reaction time. Final complex (EI*) appears rapidly after EI, reflecting the rapid conversion of the initial complex in the more stable EI* complex. Final complex (EI*) concentration then increases to reach the remaining 45 % of the initial enzyme concentration.

FIGURE 6: Simulation of the evolution of the different complexes species during the reaction time. A: Left ordinate: Evolution of the computed free enzyme [E] and complexes species ([EI] and [EI*]) concentrations versus time. Right ordinate: Experimental product formation curve measured in presence of 70 nM propeptide and the corresponding simulated fitting curve. Data were computed from the experimental points recorded with an initial propeptide concentration of 70 nM using the optimized rate constants presented in Table II. B: Left ordinate: Evolution of the initial rates versus initial propeptide concentrations. Right ordinate: Evolution of the ratio of the maximal velocity over the calculated initial velocities as a function of the propeptide concentrations. Linear regression was performed using Grafit software (Eriphacus Software Ltd.).
3.4.5 pH dependent conformational changes of the propeptide

The shift in the propeptide inhibition profile from pH 5.5 to 4 (Figure 4B) could be explained by conformational changes of the propeptide. The near UV CD spectrum of the propeptide at neutral pH shows two maxima of ellipticity at 278 nm and 286 nm, suggesting that at least one of the three tyrosine residues present in the N-terminal globular domain is in an asymmetric environment (Figure 7A). Comparison of the near UV CD spectra recorded from pH 7 to pH 2 reveals minor changes in the tertiary structure organization when pH shifts from 7 to 4, followed by a total loss of the near UV CD signal between pH 4 and 2. On the other hand, far UV CD measurements (Figure 7B) indicate that the free propeptide is characterized by a high α-helical content, which is in good agreement with the analysis of the zymogen structure (Figure 1).

**FIGURE 7:** Near and far UV CD spectra of full length Der p 1 propeptide (100 µM) at different pH values. Spectra were recorded at 25°C in 10 mM polybuffer in presence of 100 µM or 20 µM of full length propeptide for near and far UV CD spectra respectively. The inset shows the pH-induced unfolding transition of the tertiary and secondary structure content followed respectively at 278 and 222 nm. The far UV CD spectra recorded at different pH values display no major change in secondary structure at pH ≥ 4, whereas at pH 3 and 2 the spectra reveal a significant loss of the secondary structure content. Changes in the near UV CD spectra between pH 7 and 4 can be related to an increase of the intrinsic fluorescence of the tyrosine located in the N-terminal part of the propeptide. Furthermore, the pH dependence of the tyrosine fluorescence emission was recorded at 305 nm (Figure 8A). A significant increase in intensity is observed between pH 7 and 4, which coincides with the gentle decrease in CD signal observed at 278 nm. These changes in the optical properties of the propeptide can be explained by an increase in solvent accessibility of at least one tyrosine residue, with maximum exposure at pH 4 and a midpoint at pH 5.5. Taken together, these data suggest that acidification of the solution up to pH 4 leads to conformational changes in the globular N-terminal domain of the propeptide, which are characterized by an increase in solvent accessibility of at least one tyrosine residue and the conservation of a large part of the secondary and tertiary structures. At pH values lower than pH 4, the propeptide loses both its tertiary and secondary structures.

These results were confirmed by the study of the propeptide flexibility at different pH values (Figure 8B). The minor increase of the value of the Stern-Volmer constant (K_{SV}) observed between pH 5 to 4 could be related to the conformational changes observed in circular dichroism and intrinsic fluorescence measurements, whereas the large increase of the K_{SV} value observed between pH 4 and 2 can be ascribed with the loss of tertiary and secondary structures observed in near and far UV CD
experiments, respectively (Figures 8, 9). All these data indicate that, upon acidification to pH 4, the propeptide is transformed into a molten globule-like intermediate.

Further evidence for the existence of this intermediate was obtained by monitoring the fluorescence of ANS in the presence of propeptide at different pH values. ANS fluorescence showed a maximum of intensity at pH values comprised between 3 and 4, confirming the presence of a molten globule state under these pH conditions (Figure 8C).

Finally, we could show that, under acidic conditions (i.e. pH ~4), rDer p 1 rapidly degrades its propeptide into low molecular weight fragments (Figure 9). This proteolysis could be associated with the loss of the propeptide inhibition ability during incubation at pH 4 because no hydrolysis could be detected at pH values higher than pH 4.5. Similar experiments performed with rDer p 1 treated with E-64, a specific irreversible inhibitor of cysteine proteases, did not lead to propeptide hydrolysis even at pH 4, showing that the degradation of the propeptide was due to the enzymatic activity of Der p 1.
3.4.6 Molecular Modelling
Two average conformations of the propeptide are determined considering the time interval from 61.5 to 100 ps for the first one, and 601 to 1100 ps for the second one. They are soaked in a 5 Å layer of water molecules and the geometries of the whole systems are optimized. The two final geometries of the propeptide are compared with the starting one and superpositions, performed by taking into account all the heavy atoms of the propeptide (Figure 10). It is clear that the major conformational changes from the propeptide geometry in the complex to its geometry as a free entity are occurring before 100 ps in the simulation since in the final geometry the two skeletons change much less than in the intermediate geometry. The geometry variation is the largest at the hinge between $\alpha_2$ and $\alpha_3$ helices, which mainly translates in a large change in two dihedral angles ($p_i$) defined by the C$_\alpha$ of four residues: residues 54-53-52-51 for $p_1$, 55-54-53-52 for $p_2$; $p_1$ varies from $-119^\circ$ to $-45^\circ$ during the first 100 ps while $p_2$ changes from $-110^\circ$ to $-155^\circ$. The conformational changes result in the formation of a hydrophobic cluster at the ($\alpha_2$-$\alpha_3$) hinge as shown in Figure 10. Furthermore, a significant change at the hinge between $\alpha_3$ and $\alpha_4$ helices also occurs after 100 ps (Figure 10C).

3.5 Discussion
Previous studies highlighted difficulties to correctly mature recombinant proDer p 1 expressed in different hosts and suggested that the glycosylation level of the zymogen proDer p 1 could interfere with the activation process (van Oort et al. 2002; Takai et al. 2006). In this study, we have investigated both the influence of the different potential N-glycosylation sites in the maturation process and the pH influence on the propeptide association.

3.5.1 Identification and localization of the cleavage sites in the activation of proDer p 1
Elimination of the propeptide N-glycosylation motif present in the proDer p 1 led to partial maturation of the corresponding zymogen during the fermentation process. This maturation was not observed for the WT proDer p 1 expressed in the same conditions. Moreover, ProDer f 1, a homologous zymogen from D. farinae, which only possesses one N-glycosylation motif in its protease (N53) domain is easily activated in vitro (Takahashi et al. 2001). N-terminal sequencing of the bands obtained at the end of the fermentation for the N16pQ/N52Q proDer p 1 mutant revealed the presence of a multistep activation mechanism initiated by the cleavage of the Tyr19p-Ala20p bond. The sequence of the first intermediate (ATFE-) showed that the cleavage site is located in the solvent exposed coil which...
connects the first two α-helices of the propeptide (Figure 1). On the other hand, the far UV CD spectra of the full length propeptide monitored at pH 4 showed that the propeptide retained a large part of its secondary structures at this pH value. This observation together with the location of the cleavage site indicated that the first cleavage occurred in a solvent exposed part of the propeptide structure, which does not possess secondary structure at pH 4. This hypothesis is strengthened by the fact that the N-truncated propeptide was unable to inhibit rDer p 1 even if present in large concentration. However, we cannot exclude the possibility that this propeptide partially inhibits the allergen activity when still anchored to the enzyme. The fact that the formation of the ATFE- intermediate, which is only three residues away from the propeptide N-glycosylation site, is the limiting step could explain the absence of partial activation observed for WT proDer p 1. This observation is in good agreement with the previous studies of the influence of the glycosylation on the activation process. This hypothesis could also explain the different results reported by Jacquet et al. and VanOort et al. during their experiments. Van Oort et al. did not succeed in obtaining maturation of recombinant proDer p 1 WT with the correct N-terminus, while Jacquet et al. reported that the proDer p 1 WT lacking the first 19 residues was easily converted into mature allergen after acidic treatment (Massaer et al. 2001; van Oort et al. 2002). However, the fact that the proDer p 1 N52Q mutant also presents the ATFE-intermediate at the end of the production may be attributed to the heterogeneous glycosylation of the propeptide as previously proposed (Takai et al. 2006). Therefore, its presence could be attributed either to the partial activation of the minor glycosylated form or to the decrease of the steric hindrance corresponding to the lack of the glycosylation of site 52 in the hypothesis of an intermolecular activation of the zymogen.

For the proDer p 1 N52Q mutant, the maturation led to the appearance of a new band with a molecular mass intermediate between those of the ATFE- form and mature allergen. This intermediate is characterized by the SNGG- N-terminal sequence. This motif is located at the end of the second alpha helix of the propeptide in a solvent accessible area. Furthermore, this intermediate could correspond to the intermediate observed during the activation of the ATFE- intermediate obtained with proDer p 1 WT (Jacquet et al. 2002). These two cleavages occurred in regions which correspond to sequences specifically recognized by Der p 1. Indeed, recent studies demonstrated that natural Der p 1 displays a predominant preference for hydrophobic residues in position P2 and a slight preference for basic or polar amino acids in positions P1 and P3 respectively (Schulz et al. 1998; Harris et al. 2004). Considering this substrate specificity, the two cleavage sites identified in the propeptide contained adequate residues at P3-P2-P1 positions, i.e. Lys17p-Ser18p-Tyr19p and Tyr38p-Val39p-Gln40p, consistent with the Der p 1 specificity. N-terminal sequencing of the mature allergen forms indicated that cleavages also occurred at the end of the propeptide and generated the mature enzyme with a two residues extension or with the correct N-terminus. This phenomenon was also reported for other cysteine proteases and has been previously observed for proDer p 1 maturation (Menard et al. 1998; Jacquet et al. 2002; Takai et al. 2002). These two cleavages occurred at sequences Asp76p-Leu77p-Asn78p and Asn78p-Ala79p-Glu80p and were in agreement with the Der p 1 substrate specificity. In other papain-like protease precursors, additional cleavages occurred in a region located just after the second β strand (McQueney et al. 1997; Menard et al. 1998). The lack of such cleavages in proDer p 1 activation could be explained by the shorter length of the Der p 1 propeptide, or by the presence of the fourth alpha helix which could decrease the proteolysis accessibility of the corresponding area.
3.5.2 Propeptide inhibition

In this study, we investigated for the first time the interaction between soluble unglycosylated Der p 1 propeptide with its recombinant parent enzyme in terms of activity inhibition and SPR interaction analysis. The propeptide inhibits unglycosylated and glycosylated Der p 1 with K\text{D} of 7 nM and 9 nM, respectively. These dissociation constants values were confirmed using the surface plasmon resonance technology. Surface plasmon resonance permits to study the association and the dissociation as two independent events which is complementary to the enzymatic approach. Fitting the SPR curves to different models allowed to show that two models could describe the interaction between rDer p 1 and its propeptide. From the comparison of the kinetics rate constants obtained from these two models and kinetics rate constants obtained by enzymatic analysis (Table II), we could not exclude one of these two models. The dissociation constants determined by these two approaches were lower than the dissociation constants observed for the other papain-like propeptides, but are similar to the results obtained for cathepsins K and L (K\text{D} \approx 2.6 nM) for which the propeptide shows a high similarity with the Der p 1 propeptide (Billington et al. 2000; Guo et al. 2000). The presence of a two-step reaction mechanism had been previously proposed to explain the association of propeptide with cathepsin L (Guo et al. 2000). This association could be initiated by weak contacts between enzyme and propeptide inducing conformational changes in one or both partners leading to a more stable complex. Evidence of these conformational changes in the case of rDer p 1 could be demonstrated by the comparison of the initial and steady state rates of the enzyme in presence of increasing concentrations of propeptide (Figure 3A and 7B). Analysis of the first part of the product formation curves showed an instantaneous decrease of the initial velocity when propeptide concentration increased which indicated immediate inhibition and allowed the determination of the dissociation constant of 36.7 nM for the first equilibrium (75 nM for SPR). This transition complex was readily converted into a more stable complex with a global K\text{D} of 7 nM and 12.9 nM for enzymatic and SPR analyses respectively.

The presence of a two states interaction model was also analysed and confirmed by fitting the experimental inhibition curves. Computer analysis permitted to determine the different rate constants describing the experimental curves by a two-equilibrium reaction model. Simulated values were in good agreement with those determined by SPR except for k_{a1} and k_{d1} which are slightly different. This observation underlines the difficulty to compare data recorded for the interaction between free partners or with one of them immobilized at the surface of a sensor-chip. Analysis of the evolution of the two different complexes concentration during the reaction time permitted to show the presence of both initial and final complexes in the early reaction time, which could explain the difficulty to point out a two states model in our kinetic inhibition analysis. Moreover, detection of a two state model by inhibition analysis could be complicated by the presence of both inhibited complexes related by a rapid conversion rate (K_{DFinal} = 0.2).

As an illustration of the conformational changes between the initial (EI) and final (EI*) complexes, molecular modelling and dynamics showed that in water, the free propeptide undergoes fast conformational changes from its geometry in the complex which are mainly characterized by a rotation of the third and fourth alpha helices in comparison with the N-terminal globular domain (Figure 10). This conformational change could explain the observation of a two-equilibrium interaction model. Indeed, interactions could be initiated by loose contacts between the hydrated free
propeptide forming the initial complex (EI) followed by a redocking of the fourth alpha helix in the active site cleft driven by dehydratation and the presence of a stereocomplementary charged area and hydrophobic patches leading to the formation of a more stable complex (EI*). In this state, the propeptide could adopt a conformation similar to that prevalent in the zymogen.

3.5.3 Free propeptide structure and pH unfolding

Previous studies of the interactions between protease and propeptide did not intensively analyse the evolution of the structure of the free propeptide during acidification. Studies of the pH unfolding of the cathepsin L propeptide revealed that it exhibited a high content of secondary structures and a tertiary structure at neutral pH. On the other hand, at pH 4, it lost a large part of its tertiary structure but retained its secondary structure content (Jerala et al. 1998). In these conditions, the obtained propeptide intermediate was able to bind ANS. Our data showed that, as for the cathepsin L propeptide, the Der p 1 propeptide and its C-terminal truncated form were folded as compact globular domains at neutral pH. The ability of the free propeptide to adopt a correct fold independently of the protease domain, demonstrates that this propeptide is a good candidate for acting as an intramolecular chaperone, as previously reported for other cysteine protease propeptides (Wiederanders 2003; Muntener et al. 2005). No tertiary structure was detected by intrinsic fluorescence emission or by near UV CD spectra for the N-truncated propeptide. This absence of a tertiary structure was due to the deletion of the first alpha helix, which seems to play a key role in the folding of the propeptide.

Activation of the proDer p 1 zymogen in the mature allergen must likely occur when the interactions between propeptide and protease become weaker. For cysteine protease zymogens, this condition is reached by lowering the pH to 4. Proteolysis of the zymogen propeptide generates free propeptide or non-inhibitory propeptide fragments. Cleavages can result from intramolecular or intermolecular processes, depending on the zymogen (McQueney et al. 1997; Menard et al. 1998). In the present study, we showed that the free propeptide of Der p 1 undergoes conformational changes under acidic conditions. At pH 4, this conformational transition led to a minor decrease of the tertiary structure but a large increase of the solvent accessibility and flexibility of the residues located in the N-terminal globular domain. In this state, the propeptide retains a high amount of secondary structures and binds ANS. At lower pH, we have shown that the propeptide loses its residual tertiary and secondary structures and therefore its ability to bind ANS. No ANS binding was detected for the C-truncated propeptide whereas increase of the ANS fluorescence was detected with the N-truncated form suggesting that the C-terminal part of the propeptide plays a major role in the formation of the molten globule state. This may be due to the presence of the long fourth amphiphatic alpha helix. The conformational changes recorded during acidification can be correlated to the shift of the inhibitory properties of the propeptide at acidic pH. Our results clearly show a relationship between the conformational changes recorded for the propeptide when pH decreases from pH 7 to pH 4 with a decrease of the interaction between the propeptide and the protease monitored by enzymatic inhibition analysis. From these results, we can conclude that zymogen activation occurs at pH values inducing changes in the propeptide structure, and permitting a sufficient activity of the mature protease to enhance the activation process.
In conclusion, according to our results, the maturation process of proDer p 1 under acidic conditions is a multi-step process. In these conditions, the solvent accessibility of the propeptide residues increases and exposes the sequence specifically recognized by Der p 1. A first cleavage between Tyr19p and Ala20p of the propeptide liberates the first alpha helix and thus, further decreases the interaction between the two partners. This decrease results in rendering other parts of the propeptide such as the end of the second alpha helix or the C-terminal extremity of the propeptide more sensitive to proteolysis.

3.6 Acknowledgements

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3.7 References


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pH dependence of propeptide inhibition


pH dependence of propeptide inhibition