Kinetics, inhibition and oligomerization of Epstein-Barr virus protease

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Abstract Epstein-Barr virus (EBV) is an omnipresent human virus causing infectious mononucleosis and EBV associated cancers. Its protease is a possible target for antiviral therapy. We studied its dimerization and enzyme kinetics with two enzyme assays based either on the release of paranitroaniline or 7-amino-4-methylcoumarin from labeled pentapeptide (Ac-KLVQA) substrates. The protease is in a monomer–dimer equilibrium where only dimers are active. In absence of citrate the Kd is 20 μM and drops to 0.2 μM in presence of 0.5 M citrate. Citrate increases additionally the activity of the catalytic sites. The inhibitory constants of different substrate derived peptides and α-keto-amide based inhibitors, which have at best a KI of 4 μM, have also been evaluated.

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Keywords: Epstein-Barr virus; Serine protease; Dimerization; Alpha-keto-amide; Enzymology; Protease inhibitor

1. Introduction

Human herpesviruses are divided into three sub-families, the α-herpesviruses: herpes simplex viruses (HSV) 1 and 2, and Varicella zoster virus (VZV), the β-herpesviruses: cytomegalovirus (CMV) and human herpesvirus (HHV) 6 and 7, and the γ-herpesviruses: Kaposi’s sarcoma-associated herpesvirus (KSHV) or HHV8 and Epstein-Barr virus (EBV). The latter infects the vast majority of the world’s population. After primary infection, which has no characteristic symptoms and often goes unrecognized if it occurs early in childhood, EBV establishes and maintains a lifelong persistence in the infected host. If the primary infection occurs during adolescence or adulthood it often appears as infectious mononucleosis. The majority of its cases recover uneventfully but serious complications (hemophagocytic syndrome, neurologic complications, splenic rupture or severe airway obstruction, long lasting fatigue) may occur and fatal outcomes of infectious mononucleosis have been described in immunocompromised and immunocompetent hosts. Due to higher hygiene in developed countries, this delayed primary infection is globally gaining importance. Furthermore EBV can lead to EBV-induced malignant B-cell lymphoma in immunosuppressed patients, and is implicated to different degrees in the pathogenesis of several cancers in the immunocompetent population such as Burkitt’s lymphoma, nasopharyngeal carcinoma and gastric carcinoma (see [1] for a recent review). EBV shares this oncogenic potential with KSHV.

During latency, only a limited set of reading frames is expressed, whereas the lytic cycle is characterized by the expression of the full set of viral proteins needed for viral replication, such as the DNA polymerase and the late structural proteins including the protease. The lytic cycle of EBV infections is active in infective mononucleosis where shedding of infectious virus lasts much longer than the actual disease [2]. The currently licensed antiviral drugs against human herpesviruses inhibit the viral DNA polymerases. These are either acyclovir and related compounds, or foscarnet or cidofovir. These drugs are thus directed against the lytic cycle of the viral infection, principally of herpes simplex virus and CMV, but some may still have an effect in a number of cases of EBV related immunoproliferative disease [3], despite a controversial role of the contribution of the lytic cycle to this syndrome.

Herpesvirus proteases are essential for the production of infectious virions. They disrupt the scaffold formed by the assembly protein after assembly of the capsid in order to make space for the viral DNA. They are synthesized as part of a polyprotein comprising the protease domain and the assembly protein domain, whereas individual assembly protein molecules are also translated from a second overlapping reading frame [4]. The protease mainly recognizes two types of sequence, the maturation (M) site close to the C-terminus of the assembly protein domain and the release (R) site between the assembly protein domain and the release (R) site between...
the protease and assembly protein domains. Herpesvirus proteases form a class of serine proteases with a catalytic triad composed of Ser-His-His. They exist in a monomer–dimer equilibrium in vitro where only the dimer is active [5–7]. X-ray crystallographic studies on the protease from CMV, HSV1, HSV2, VZV, KSHV and EBV have shown the presence of a conserved dimer structure [8–14].

Despite extensive studies on the herpesvirus proteases of HSV, CMV and VZV [15], no active-site based inhibitors have entered clinical trials yet. Some sub-micromolar peptide-based inhibitors against CMV protease have been reported [16,17]. Drug development has certainly been hampered by the shallow active site of the herpesvirus proteases, leading to a search for new approaches for antiviral action on these molecules, such as dimer disruption. A peptide derived from the interface helices of KSHV showed a limited efficiency as its Ki is in the range of 300 μM [18].

The effect of kosmotropic (anti-chaotropic) salts, glycerol or sucrose on the activity of herpesvirus protease has been studied [6,19,20] and they are commonly used in order to enhance the protease activity in assays. Examples are the use of 25% glycerol for HSV1 protease [21], 0.5 M Na2SO4 and 10% glycerol for CMV [17], 30% sucrose for CMV [22]. 0.8 M potassium phosphate and 25% glycerol for KSHV protease [23]. As most of the enzymological studies have been done in the presence of such agents little kinetic data has been determined in their absence. The mechanism of this activation goes along with a promotion of dimerization but has not been studied in detail.

So far, the EBV protease has been the least studied of this group of enzymes. The monomer–dimer equilibrium of EBV protease has been studied by Buisson and coworkers [24], who also described an optimal heptapeptide substrate. The structure of EBV protease was solved by X-ray crystallography [14].

In the prospect of designing inhibitors directed against the EBV protease, our test for enzymatic activity based on a chromogenic substrate [14] has been adapted for use within a 96-well plate reader and the EBV protease activity has been quantified in the absence or presence of citrate. We found that citrate promotes dimerization and stimulates activity. These finding have been confirmed using a fluorogenic substrate based on the release of 7-amino-4-methylcoumarin (AMC). The inhibitory constants of different peptides derived from the recognition site and peptide-based inhibitors containing an α-keto-amide moiety have been determined.

2. Materials and methods
tert-Butyloxycarbonyl (Boc)- and Fmoc-aminoacids, N-Fmoc-
triazol-1-yl-oxy) (dimethylamino)methylene]-N-methylmethyleneamni-
unium hexafluorophosphate (HBTU) and resins were purchased from Senn Chemicals International (Gentilly, France) or Advanced ChemTech (Louisville, USA). The reagents and solvents for the solid phase syn-
thesis were obtained from Acros (Noisy-le-Grand, France) or Sigma-
Aldrich Fine Chemicals (Saint Quentin Fallavier, France) and were used without additional purification. All other chemicals were of the purest grade available. The peptides 1–3 (Table 1) were synthesized as described [24].

2.1. Synthesis of the α-keto-amide inhibitors, 4–8

Compounds 4–8 (Table 1) are derived from the sequence P5–P1 (nomenclature of Schechter and Berger [25]; sequence Lys-Leu-Val-
Gln-Ala) of the M site, with or without added P1’ and/or P2’ residues. The P5 lysine residue was replaced by 6-aminoacaproic acid or desa-
mino-lysine (Aca), which confers resistance to aminopeptidases. P1’ Ala was replaced by its α-keto-acyl derivative (“Aka-CO”). The P1’ resi-
due is either the wild-type Ser residue (compounds 7 and 8) or an hydrophobic phenylalanine residue (compounds 5 and 6). The activated carbonyl component was obtained from the corresponding α-hydroxy acid (norstatine derivative of alanine). The Fmoc protected α-hydroxy acid derivative of alanine was synthesized in five steps from Boc-Ala-OH. The first two steps (i.e. coupling of Boc-Ala-OH to O,N-dimethylhydroxylamine and reduction of the formed Weinreb amide using LiAlH4) led to the aldehyde Boc-Ala-H and were performed as described [26]. The aldehyde was then reacted with KCN (4 equiv.) in ethylacetate:water:methanol (5:2.5:2.5) for four days at room tem-
perature. The obtained cyanohydrine was hydrolysed (removing at the same time the Boc protecting group) by treatment with conc.
HCl (10 ml per g) for three days at room temperature, giving the deprotected norstatine derivative. N-(Fmoc-oxy)-succinimide was used for protection. Once the sequence was complete, oxidation of the hydroxy group to ketone was performed on the resin using Dess–Martin periodinane (2 equiv. in dimethylsulfoxide (DMSO), overnight). Then, deprotec-
tion and cleavage from the resins were carried out by treatment with trifluoroacetic acid:tris-isopropylsilane:H2O (9.5:0.25:0.25, 10 ml/g re-
sin, 2 h). The crude products were purified by reverse-phase HPLC on a C18 column (Deltapack Waters 40 x 100 mm) by means of a lin-
er gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid over 25 min (flow rate 28 ml/min). Their purity and identity were assessed by reverse-phase HPLC and electrospray mass spectrometry (4, exper-
imental mass, 569.3 ± 0.2 u; calculated mass, 569.3 u; 5, experi-
tal mass, 716.4 ± 0.2 u; calculated mass, 716.4 u; 6, exper-
tal mass, 716.3 ± 0.2 u; calculated mass, 716.4 u; 7, exper-
tal mass, 656.4 ± 0.2 u; calculated mass, 656.4 u; 8, exper-
tal mass, 727.4 ± 0.2 u; calculated mass, 727.4 u). For each compound, a sec-
tral mass of 727.4 ± 0.2 u was observed and corresponds to the hydrated form of the activated keto group.

Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Definition</th>
<th>Ki (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Ac-KLVQASA-NH2</td>
<td>M-site, positions P5–P2’</td>
<td>24b</td>
</tr>
<tr>
<td>2 Aca-LVQA-NH2</td>
<td>M-site, positions P5–P1</td>
<td>4100b</td>
</tr>
<tr>
<td>3 Ac-SYLLKA-OH</td>
<td>R-site, positions P5–P1</td>
<td>1200b</td>
</tr>
<tr>
<td>4 Aca-LVQA-CONH2</td>
<td>α-Keto-amide inhibitor based on M-site, positions P5–P1</td>
<td>3,7b, 4b</td>
</tr>
<tr>
<td>5 Aca-LVQA-CO-o-Phe-NH2</td>
<td>α-Keto-amide inhibitor based on M-site, positions P5–P1’, with an hydrophobic side chain at P1’ position</td>
<td>20b</td>
</tr>
<tr>
<td>6 Aca-LVQA-CO-Phe-NH2</td>
<td>α-Keto-amide inhibitor based on M-site, positions P5–P1’, with an hydrophobic side chain at P1’ position</td>
<td>5b</td>
</tr>
<tr>
<td>7 Aca-LVQA-CO-Ser-NH3</td>
<td>α-Keto-amide inhibitor based on M-site, positions P5–P1’</td>
<td>200b</td>
</tr>
<tr>
<td>8 Aca-LVQA-CO-Ser-Ala-NH2, ZnSO4</td>
<td>α-Keto-amide inhibitor based on M-site, positions P5–P2’, Cysteine-histidine ligand</td>
<td>65b, 1700b</td>
</tr>
</tbody>
</table>

*aObtained with the chromogenic assay.
*bObtained with the fluorogenic assay.
2.2. Chromogenic assays

The purification of the EBV protease domain, Pr-wt, was performed as described [14,24]. The EBV protease activity was determined by monitoring the cleavage of Ac-KLVQA-pNA, a chromogenic substrate derived from the M site peptide of EBV assembly coupled with paranitroaniline as described previously [14]. The assay was performed at 25 °C and pH 7.5 in a buffer composed of 20 mM Tris and 80 mM NaCl. Whenever indicated, 0.5 M sodium citrate was added. The absorption of free paranitroaniline (pNA), produced by the hydrolysis of the paranitroanilide substrate, was followed spectrophotometrically at 450 nm using a 96-well microplate reader (IEMS, ThermoLabSystem). The amount of hydrolyzed substrate was calculated using a calibration with free pNA (Sigma-Aldrich) in assay buffer. For 200 μl/well the conversion factor was 5.9 AU μM⁻¹ independently of the presence of citrate.

2.3. Kinetic studies using the chromogenic substrate

The enzyme was pre-incubated for 15 min at 25 °C in the assay buffer before addition of the substrate. The specificity constant $k_{cat}/K_m$ and the individual parameters $K_m$ and $k_{cat}$ for the chromogenic substrate were determined from initial rate kinetics by a fit of the Michaelis–Menten equation using XPLOR [27]. Only the initial linear part of the plot of absorption versus time was used. In the absence of citrate, the assay included 0.5 mg/ml bovine serum albuminue (Roche Diagnostics) and the time course lasted for 7 h, whereas in the presence of citrate, the time course lasted for 3 h 30 min at maximum. Experiments for the determination of the initial rate as a function of the protein concentration were carried out in duplicate. Series (3–6) of experiments were used for the calculation of the kinetic constants.

2.4. Fluorogenic assays

The substrate Ac-KLVQA-AMC has been synthesized in the following manner: the protected fragment Ac-Lys(Boc)-Leu-Val-Glu(Tri)-OH was assembled on a chlorotrityl resin following Fmoc strategy and using HBTU as the coupling agent. The protected peptide was released from the solid support with the help of a dichloromethane-trifluoroethanol:AcOH (7:2:1) mixture (1 h, then 10 min) and used without purification for the following step. The second fragment, Ala-AMC (TFA salt), was prepared by coupling Boc-Ala-OH to AMC (1.2 equiv.) in the presence of diisopropylethylamine (3 equiv.) for 30 min. The fluorogenic substrate was finally treated with a mixture of TFA:triisopropylsilane:water (95:2.5:2.5) for 1 h to yield the expected protected fluorogenic substrate was finally treated with a mixture of TFA:triisopropylsilane:water (95:2.5:2.5) for 1 h to yield the expected two fragments (1 equiv. each) were then condensed in DMF in presence of diisopropylethylamine (3 equiv.) and butyldicarbonate (1.2 equiv.) in the presence of diisopropylethylamine (3 equiv.). The final product was purified by reverse-phase HPLC. Its purity and identity were assessed by reverse-phase HPLC and electrospray mass spectrometry (exp. mass, 756.3 ± 0.2 u; calculated mass, 756.3 u).

The release of AMC has been monitored in a 96-well fluorescence plate reader (Wallac 1420) with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The linearity of the response plate reader (Wallac 1420) with an excitation wavelength of 355 nm. The linearity of the response was verified and the assay calibrated using different dilutions of AMC (Sigma-Aldrich). The substrate concentration was verified after the linearity of the response was verified and the assay calibrated using different dilutions of AMC (Sigma-Aldrich).

2.5. Modelling of the monomer–dimer equilibrium

The concentration of the protease dimer is given by:

$$[P_{2}] = \frac{K_d}{K} \left[ \left[ 1 + \frac{4[E]}{K_d} \right] \sqrt{1 + \frac{8[E]}{K_d}} - 1 \right]$$  \hspace{1cm} (1)

where $[E]$ is the total protease concentration (expressed as monomers) and $K_d$ is the dissociation constant of the monomer–dimer equilibrium. The specific activity

$$v_i/[E] = \frac{d_{0i}}{d_0} \cdot \frac{2[P_{2}]}{[E]}$$  \hspace{1cm} (2)

is proportional to the fraction $f$ of functional active sites, where $d_0$ corresponds to the maximal value of $v_i/[E]$. Eq. (2) was fitted to the experimental points using XPLOR [27], where $K_d$ and $d_0$ have been adjusted.

2.6. Dynamic light scattering

Samples were centrifuged for 10 min at 13000 rpm in an Eppendorf centrifuge in order to eliminate coarse aggregates and dust. Dynamic light scattering experiments used a Dynapro apparatus (ProteinSolutions) with a temperature regulated 15 μl cuvette. Measurements were taken at 25 °C and data were analyzed using the Dynamics v5.26.41 software. Poorly determined initial measurements were rejected based on the “baseline limit” parameter. Theoretical values for Stokes’ radii of monomers and dimers have been calculated from the 3-dimensional structure of the protease (pdb entry 106E, [14]) using the software HYDROPRO [28].

2.7. Size exclusion chromatography and multi-angle laser light scattering

Size exclusion chromatography was performed with a Shodex Protein KW-804 HPLC column (300 mm x 8.0 mm). The column was equilibrated in 20 mM Tris–HCl, 80 mM NaCl buffer at pH 7.5 in the absence or presence of 500 mM sodium citrate. Separations were performed at 20 °C with a flow rate of 0.8 ml min⁻¹. Stokes’ radii were determined by calibrating the column with globular proteins of known Stokes’ radius. On-line detection was performed by multi-angle laser light scattering (MALLS) using a DAWN-EOS detector (Wyatt Technology Corp., Santa Barbara, CA) equipped with a laser emitting at 690 nm and by refractive index measurement using a R21000 detector (Schambeck SFD). Light scattering intensities were measured at different angles relative to the incident beam, and analysis of the data was performed with the ASTRA software (Wyatt Technology Corp., Santa Barbara, CA). The excess light scattering intensity at angle $\theta$ ($R_\theta$) is related to molecular mass of solute particle according to Zimm’s formalism of the Rayleigh–Debye–Gans model for a diluted polymer solution [29]. For small (gyration radius smaller than 100 nm), non-interacting particles $R_\theta$ is given by

$$R_\theta = \frac{N_s}{k_B T} = \frac{1}{k_B T} \left[ \frac{2\pi n_0}{\lambda} \right] \left( \frac{2\pi n_0}{\lambda} \right)^2$$  \hspace{1cm} (4)

where $N_s$ is Avogadro’s number, $n_0$ is the refractive index of the solvent at the incident radiation wavelength (1.33 for a diluted aqueous buffer), $\lambda$ is the specific refractive index increment of the solute (0.185 ml g⁻¹), and $\lambda$ is the wavelength of the incident light in void (690 nm). Within the elution peak, the chromatogram is divided in slices, and for each slice, MALLS and refractive index measurements are used to calculate the molecular weight. Number-averaged ($M_n$) and weight-averaged ($M_w$) molecular weights are obtained from the molecular weight distribution across the elution peak.

2.8. Competition of inhibitors with the chromogenic substrate

Experiments were carried out in the presence of 0.5 M citrate using a protease concentration of 0.2 μM and a preincubation time of 30 min with the inhibitor (except for compound 1, which is a substrate). Concentrations were as follows: compound 1 (Ac-KLVQA-NH₂), 0.001–0.1 mM, substrate between 0 and 0.4 mM; compound 2 (Ac-LVQA-NH₂), 0.5, 0.75 and 1 mM, substrate between 0 and 0.1 mM; compound 3 (Ac-SYLKA-OH) 0.3–0.6 mM, substrate between 0 and 0.15 mM; compound 4 (Ac-LVQA-COH₂), 0.001–0.2 mM, substrate between 0 and 0.15 mM; ZnSO₄, 0.01, 0.1 and 1 mM, substrate between 0.05 and 0.4 mM. Cornish–Bowden plots were used to verify competitive inhibition and $K_s$ were derived from Dixon plots.
2.9. Competition of inhibitors 4–8 with the fluorogenic substrate
Inhibitors and substrate were solubilized in DMSO to stock concentrations of 10 mM. Different final inhibitor concentrations ranging from 0.2 μM to 500 μM were used in the assay. Inhibitors in 10 μl DMSO were added to 95 μl of buffer (0.5 M sodium citrate, 80 mM NaCl and 20 mM Tris–HCl at a pH of 7.5) containing the enzyme. 95 μl of substrate solution using the same buffer have been added to start the reaction (12.5–37.5 μM final) leading to a final concentration of the enzyme of 0.2 or 0.3 μM. The substrate concentration was limited by its solubility. The fluorescence change was measured as described above. The initial linear part of the time-course was used for rate determinations. Inhibition constants were determined from Dixon plots.

3. Results

3.1. Determination of the dissociation constant of the protease dimer in the absence and presence of citrate from the enzymatic activity
First the chromogenic substrate (Ac-KLVQA-pNA) was used and the liberation of pNA was followed spectrophotometrically using a 96-well plate reader. As herpesvirus protease monomers are inactive [5–7], determining the specific activity v/[E] as a function of the protease concentration should allow to determine the dissociation constant K_d of the protease dimer. When v/[E] was plotted as a function of the total concentration of protease monomers [E] (Fig. 1A), the data points could be fitted by a theoretical curve based on a simple monomer–dimer equilibrium where monomers were inactive as described in the Section 2. This yielded a dissociation constant of 18 ± 3 μM for the protease dimer.

Using the fluorogenic substrate (Ac-KLVQA-AMC) a dissociation constant of 20 ± 2 μM was obtained under the same conditions (Fig. 2A).

The chromogenic assay in the presence of 0.5 M sodium citrate showed a shift of the dissociation constant to 0.2 μM (Fig. 1B). But data points scatter considerably at low activity due to a lack of sensitivity whereas values for enzyme concentrations above 0.5 μM have a tendency to level off more rapidly than the theoretical curve limiting the precision of the obtained dissociation constant.

As the enzyme concentration is imposed, the large dynamic range of the enzymatic activity in function of protease and citrate concentrations is an experimental challenge. In order to overcome the sensitivity limitations of the assay, the same experiment was carried out using the fluorogenic substrate. A K_d of around 0.4 μM was obtained (Fig. 2A) although the increase of the assay sensitivity was not as large as expected due to drifts of the baseline fluorescence signal. Again, at high protease concentrations, the specific activity has a tendency to be lower than expected. This strongly affects the fit of the theoretical curve and creates an uncertainty of the value for K_d, which is comprised between 0.1 and 0.8 μM.

In order to further characterize the effect of citrate, we determined the specific activity of the enzyme at different enzyme and citrate concentrations (Fig. 2A). In general, specific activities seemed to level off for high enzyme concentrations at citrate concentrations above 250 mM, where the theoretical curve no longer fits well. At low citrate concentrations, the fit of the experimental points to the theoretical curve is excellent over a large range of protease concentrations. The experiments at variable citrate concentrations show that the dissociation constant dropped already at citrate concentration as low as 30 mM and reaches a plateau value for 250 mM citrate (Fig. 2B). In parallel there is an effect on the maximal specific activity a_0 obtained from the fit of Eq. (2) as shown in Fig. 2C which rises continuously, in particular at high citrate concentrations (Fig. 2C).

3.2. Oligomerization state of the protease in the absence or presence of citrate
When we used dynamic light scattering (DLS), the scattered signal for equivalent concentrations of the protease was much higher in the presence of 0.5 M citrate than at in its absence (Fig. 3A). The detection of the protease was still possible at concentrations where it does not yield any measurable signal in the absence of citrate. This indicates the presence of an aggregated form of the protease in the presence of citrate even at very low enzyme concentrations. Determination of the size of these aggregates by DLS was impossible, suggesting rather an aggregation than an oligomerisation with a defined particle size. When a sample containing 0.5 M citrate was diluted stepwise, the aggregates disappeared between concentrations of 0.25 M and 0.125 M and a species with a size between those
of a monomer and a dimer was observed again (data not shown).

In the absence of citrate, a concentration dependent average Stokes' radius of the molecule between 2.6 and 3.4 nm was observed (not shown). For comparison, the theoretical values for monomers and dimers derived from the 3-dimensional structure of the protease, pdb entry 1O6E, [14], without taking into account its hydration, are 2.6 and 3.3 nm, respectively. In addition, in the absence of citrate the amplitude of the scattered signal is in agreement with the one expected for a protein of the size of the protease (Fig. 3A). On a HPLC SEC sizing column, in the absence of citrate, the protease elutes in a major peak with Stokes' radius of 3.2 nm and a shoulder with a radius of approximately 4.0 nm (Fig. 3B). Detection by multi-angle laser light scattering and refractive index indicated that, in the absence of citrate, the different components have in majority (85%) an average molecular weight of 45 kDa (expected 52 kDa for a dimer), with a small proportion of trimer (11%) with a mass of 83 kDa (expected 77 kDa) and an even smaller proportion (4%) of higher molecular weight species. In the presence of 0.5 M citrate, the protease is re-
tarded on the SEC column and elutes in the same volume as small molecules such as citrate, and the refractive index measurement is contaminated by the change in the refractive index caused by the mismatch of the citrate concentration between protein sample and buffer. Only in the first part of the elution peak the refractive index can be measured accurately. Despite of the above mentioned aggregates, most of the enzyme was still present in solution (>30% of the protein input are found in the half peak). The average molecular weight $M_w$ varies from 70 kDa to 200 kDa in the left half of the peak, suggesting different oligomeric species (Fig. 3B) in addition to dimers and monomers which could not been measured due to the refractive index mismatch between protein solution and buffer. As judged from the refractive index measurement, large aggregates correspond to only a small fraction of the total mass of the protein.

3.3. Determination of the kinetic constants using the chromogenic assay

The kinetic constants of the protease using Ac-KLVQA-pNA in the absence and in the presence of 0.5 M citrate were derived from Michaelis–Menten plots. Because of the poor solubility of the substrate, it was impossible to measure initial rates at substrate concentrations higher than 0.4 mM, a value well below the $K_m$ of the enzyme and the accuracy of the values for $K_m$ and $k_{cat}$ is rather low, whereas a more accurate value can be obtained for $k_{cat}/K_m$ derived from the initial slope of the Michaelis–Menten plots (Fig. 4).

In the case of an enzyme submitted to a monomer–dimer equilibrium, we obtained an apparent value for $k_{cat}$ as only a fraction of the enzyme is dimeric and thus active. Using the calculated concentration of the dimer and its $K_d$ determined previously, a $k_{true}$ value can be calculated, which we define as the catalytic rate of one active site of a protease dimer. In the absence of citrate, we obtained $k_{true} = 2.5 \pm 0.6 \text{ min}^{-1}$ and $K_m = 2.6 \pm 0.4 \text{ mM}$. For $k_{true}/K_m$ we obtained $0.95 \pm 0.08 \text{ min}^{-1} \text{mM}^1$ from Michaelis–Menten plots (Fig. 4A).

In the presence of 0.5 M citrate we obtained $k_{cat}$ and $K_m$ values of $14 \pm 2 \text{ min}^{-1}$ and $0.9 \pm 0.2 \text{ mM}$, respectively. The determination of $K_m$ is rather inaccurate, but the increased $K_m$ compared to the one in presence of citrate becomes obvious from the lower curvature of the plots in Fig. 4A compared to 4B. For $k_{cat}/K_m$ we obtained $16.4 \pm 0.7 \text{ min}^{-1} \text{mM}^{-1}$ from the initial slope. Although four different enzyme concentrations between 0.3 and 10 \text{ mM} have been used in this experiment, the experimental points for $v/[E]$ superpose (Fig. 4B). This shows rather an independence of the kinetic parameters from the protease concentration as observed as well in Figs. 1B and 2A and is unexpected for an enzyme subject to a monomer–dimer equilibrium. As the fraction of active catalytic sites could not be predicted accurately, we resided to calculate the $k_{true}$ value as for the equilibrium in the absence of citrate.

3.4. Inhibition of EBV protease

In order to reduce the consumption of enzyme, further experiments were carried out in the presence of citrate. First, as Zn$^{2+}$ ions have been described to be efficient inhibitors of CMV protease [22] we tested ZnSO$_4$, which appeared to be only a poor inhibitor with a $K_i$ of 1.7 mM (Table 1). In a study of substrate derived peptides we determined the inhibition constant of a heptapeptide containing the M cleavage site as Zn$^{2+}$ ions have been described to be efficient inhibitors of CMV protease [22] we tested ZnSO$_4$, which appeared to be only a poor inhibitor with a $K_i$ of 1.7 mM (Table 1). In a study of substrate derived peptides we determined the inhibition constant of a heptapeptide containing the M cleavage site...
Dixon plot gave an inhibition constant $K_i$ of 24 $\mu$M, while the the Cornish–Bowden plot showed parallel lines consistent with pure competitive inhibition (not shown). As this peptide is a substrate of the protease, the measured $K_i$ value corresponds to its $K_m$ [30]. In order to evaluate the importance of the N-terminal part of the cleavage site, we tested pentapeptides corresponding to positions P5–P1 derived from the M (compound 2) and R (compound 3) sites and found inhibition constants of $4.1 \text{ mM}$ and $1.2 \text{ mM}$, respectively. An $\alpha$-keto-amide inhibitor based on the M-site substrate of EBV protease (compound 4) showed an inhibitory constant $K_i = 3.7 \text{ M}$ using the fluorogenic (Fig. 5) and $4 \text{ M}$ using the fluorogenic assay. The inhibition by compounds 5–8 has only been analysed in the fluorogenic assay yielding inhibition constants of 5–200 $\mu$M (Table 1).

4. Discussion

In the absence of citrate, the protease activity in function of its total monomer concentration can be modelled by an equilibrium between active dimers and inactive monomers as described for other herpesvirus proteases [5–7]. The value of the dissociation constant of 20 $\mu$M obtained from enzymatic assays for the EBV protease dimer compares to a dissociation constant of 57 $\mu$M obtained by analytical ultracentrifugation [24]. Although the results probably agree within the experimental errors of the ultracentrifugation experiment, it is possible that the difference may be due to a protease construct which carried two additional residues at the C-terminus (Arg-Ser) which was used at the time of the ultracentrifugation [24]. We showed later the crucial role for a correct C-terminus as constructs carrying additional residues have a strongly reduced catalytic activity and tend to aggregate [14]. In the absence of citrate, the measurements of the apparent size of the protease in function of its concentration by DLS are in rough agreement with a monomer–dimer equilibrium (not shown).

Sodium citrate, a kosmotropic agent, for example commonly used as precipitant for protein crystallisation, has been shown to strongly promote the activity of the EBV protease [24] and lowered considerably the dissociation constant (in the range of 0.1–0.8 $\mu$M) as reported for other herpesvirus proteases (see below). But whereas the concentration dependence of the specific activity $v/[E]$ of the enzyme could be very well described by the theoretical curve in the absence of citrate or at low citrate concentrations, fits between model and data are rather poor at citrate concentrations above 250 $\mu$M. Above an enzyme concentration of 0.5 $\mu$M, the value of $v/[E]$ rather reaches a plateau value, which is difficult to explain. The two different activity assays, a chromogenic and the more sensitive fluorogenic one led to very similar results.

It appeared that a competing effect might inactivate the enzyme at high citrate concentrations leading to the apparent plateau value, due to an auto-inhibition or the formation of inactive oligomers as suggested from light scattering results (Fig. 3). The majority of the enzyme appears to form dimers or small oligomers as shown by the results from static light scattering experiments (Fig. 3B). These experiments excluded the presence of large amounts of large aggregates. The uncertainty about the fraction of active enzyme did not allow to define a $k_{true}$ value for the rate of an active site as we did in the absence of citrate.

The dissociation constant obtained in the presence of citrate is comparable to the $K_i = 0.23 \mu$M [7] reported for the monomer–dimer equilibrium of HSV1 protease at a citrate concentration of 0.5 M. For CMV, the results of Margosiak et al. [6] showed an activation of the enzyme in the presence of citrate and suggested the existence of an additional aggregated state, furthermore, the authors observed a similar effect of citrate and glycerol, where the increase of the catalytic efficiency was more pronounced for citrate leading to a 26-fold increase of $k_{cat}/K_m$ at 800 $\mu$M. Margosiak et al. interpreted the relationship between activity and enzyme concentration in terms of a monomer dimer equilibrium which is dramatically shifted in presence of 25% glycerol ($K_d = 8 \mu$M in the absence of any agent to $1.9 \text{ M}$ in the presence of 25% glycerol) accompanied by a 3–6-fold increase of $k_{cat}/K_m$.

A common feature between EBV and CMV proteases is the presence of a long disordered loop next to the active site (loop L9, residues 121–135 in EBV [14], or 136–153 in CMV) which is likely to be involved in substrate binding. This loop is poorly ordered in the crystal structures and changes its conformation upon substrate binding for CMV protease [31]. In the EBV protease structure the loop is involved in the crystal contact between the dimers. Conformational changes of this loop or its interaction with other protease molecules in the presence of citrate are likely to have an effect on enzyme activity or may promote further oligomerization.

For EBV protease, the affinity for the substrate ($K_m = 0.9 \text{ mM}$) and the $k_{cat} = 14 \text{ min}^{-1}$ are, respectively, decreased and increased by factors of almost 3 and 6 in the presence of 0.5 M citrate, compared to the values in the absence of citrate ($K_m = 2.6 \text{ mM}$, $k_{true} = 2.5 \text{ min}^{-1}$) resulting in an about 17-fold increase of the specificity constant $k_{cat}/K_m$ (from 0.95 to 16.4 $\text{ M}^{-1} \text{ min}^{-1}$). This is comparable to the results from Margosiak et al. for CMV protease [6].

The $K_m$ value of EBV protease in the absence of citrate is similar to the ones obtained using pNA based substrates with HCMV protease (substrate succinyl-AGVVNA-pNA, $k_{cat} = 11 \text{ min}^{-1}$, $K_m$ about 1.9 $\text{ mM}$ measured in the presence of 20% glycerol and 10% DMSO, [32]) and HSV-1 protease (LVLA-pNA, $k_{cat} = 20 \text{ min}^{-1}$, $K_m = 2.0 \text{ M}$ in the presence of 20 % glycerol [33]). Still the rate constant for EBV protease in the absence of citrate is much lower than the ones described but these assays contained glycerol which has also a stimulatory effect [20]. When our results are compared to published [19,20], at first glimpse the activation by citrate appears to be smaller, which is due to the fact that we compared the $k_{true}$ rate constant of the active dimer. In the absence of citrate, the fraction of active dimers at low enzyme concentrations is very small and the apparent activation in the presence of 0.5 M citrate can be much higher due to the dimerization of the enzyme.

In order to characterize further the substrate specificity of the protease, we tested several peptides and peptidomimetics derived from the natural cleavage sequences. Pentapeptides corresponding to positions P5–P1 derived from the M (compound 2, Table 1) and R sites (compound 3) yielded inhibition constants of 4.1 $\text{ mM}$ and 1.2 $\text{ mM}$, respectively, 170 or respectively 50 times worse than the $K_i$ of 24 $\mu$M for the substrate heptapeptide obtained in competition experiments.
showed that residues from the P’ side contribute to the affinity of the substrate. This agrees with the relatively ill-defined and open character of the binding pockets S5–S2 [14] whereas the sites P1 and P1’ are located in a more pronounced cleft and it is likely that in particular the serine residue at position P1’ contributes to the affinity. The presence of a hydrophobic group (pNA) C-terminal to the cleavage site as present in the pNA-based chromogenic substrate (which has been derived from the M site) confirmed the importance of a P1’ residue for the affinity because the $K_m$ (0.64 mM) is smaller than the $K_i$ of the peptidic part alone (4.1 mM), although not as good as for the heptapeptidic substrate (24 μM).

The introduction of hydrophobic groups on the C-terminal side of the cleavage site has also been used to increase the affinity of peptide-based inhibitors containing an α-keto-amide moiety between residues P1 and P1’ which were reported for the CMV protease [16,17,31]. Such inhibitors form a reversible covalent complex through the serine residue of the catalytic triad. The covalent complexes of two of them (IC₅₀ of <0.05 μM and 0.3 μM) with the CMV protease have been studied crystallographically [17,31]. Like the EBV enzyme, the hepatitis C protease NS3-4A has a relatively open catalytic site which sparked a great interest in the development of α-keto-amide based inhibitors [34,35]. Recently, the efficiency of the α-keto-amide based anti-hepatitis-C-virus drug VX-950 [36] was shown in phase 1b clinical trials where the compound was administered orally.

We first designed an inhibitor with a C-terminal α-keto-amide group (compound 4, Table 1) based on the M-site substrate of EBV protease, which showed an inhibition constant of 4 μM. As pointed out above and as reported for the HCMV protease [16], addition of interacting residues on the P’ side should increase the inhibitory potency of this class of compounds toward the EBV protease. Although optimization of α-keto-amide containing peptides may be difficult to achieve, they may be a promising class of inhibitors and we designed first inhibitors with an internal α-keto-amide group (compounds 5–8) and either a hydrophobic phenylalanine residue or the natural substrate sequence at the C-terminus. Unexpectedly they did not show any potency increase compared to compound 4 (Table 1). The presence of a serine residue in position P1’ is detrimental to the inhibition constant and part of the affinity is recovered by the introduction of an additional alanine residue (compound 8) as present in the natural substrate peptide (1). But despite of the covalently binding α-keto-amide, the affinity of inhibitor 8 is not increased over the natural substrate peptide (1). Probably the increased length of the α-keto-amide compared to a peptide leads to an inadequate positioning of the P’ part of the peptide. The addition of hydrophobic groups at the P1’ position, which was suggested by the better affinity of the chromogenic substrate compared to the peptide 2 led to compounds 6 (L-Phe in P1’) and 5 (D-Phe in P1’) with inhibitory constants of 5 and 20 μM, respectively, but did not improve the inhibitory potency compared to the initial compound 4.

Due to the presence of the conserved cysteine 143 in the active site [14] as well as the presence of the two histidine residues of the catalytic triad, we postulated that the enzymatic activity may be susceptible to zinc ions which could be chelated by these residues as described for the CMV protease [22]. However, an elevated inhibition constant (1.7 mM, Table 1) was obtained for ZnSO₄ showing that zinc interfered only poorly with enzyme activity.

In conclusion, the EBV protease activity assays in a 96-well plate format combined with our characterisation of its monomer–dimer equilibrium allows the accurate measurements of the kinetic constants at a defined oligomeric state. It provides the basis for further screening of compounds with an inhibitory action on EBV protease activity. Such compounds could either target the active site such as optimized α-keto-amides or other features of the protease such as the dimer interface. The effect of the kosmotropic agent sodium citrate, a representative of a series of stimulating agents commonly used in herpesvirus protease assays, on oligomerisation state and enzyme activity has been characterized in detail. We showed that there is a multifaceted effect of citrate leading on one hand to an almost 100-fold reduction of the dimer dissociation constant and on the other hand a stimulation of the enzymatic activity. For the search for dimer-disrupting inhibitors of herpesvirus proteases it may be better to carry out enzyme assays in the absence of citrate.

Co-crystallization trials of the protease with α-keto-amide based inhibitors with a C-terminal extension will be needed in order to establish their mode of binding and to understand their relatively poor inhibition. Still, the inhibition constant of compounds with an α-keto-amide at the position of the scissile bond can be in the micromolar range and further optimisation of these compounds may lead to promising inhibitors.

Acknowledgements: We thank Patrice Morand and Jean-Marie Seigneurin for support of the project and Lucy Freeman for corrections of the article. We are grateful to Bérangère Reynaud and Judith W. Seeber for chemical synthesis.

References


