Quantitative time-resolved analysis reveals intricate, differential regulation of standard- and immuno-proteasomes

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Running Title: Modeling standard- and immuno-proteasome dynamics
Abstract
Proteasomal protein degradation is a key determinant of protein half-life and hence of cellular
processes ranging from basic metabolism to a host of immunological processes. Despite its
importance the mechanisms regulating proteasome activity are only incompletely understood.
Here we use an iterative and tightly integrated experimental and modelling approach to
develop, explore and validate mechanistic models of proteasomal peptide-hydrolysis
dynamics. Different proteasome isoforms exist in mammalian cells, where standard- and
immuno-proteasomes are present as 20S proteasomes or coupled to regulatory complexes.
Here we map the highly dynamic regulation of the 20S proteasome in detail. We show how its
activity varies over time and analyse quantitatively the interactions between substrates and
products and the proteolytic and regulatory sites; the locations of these sites and the
interactions between them are predicted by the model, and experimentally supported. Our in-
depth analysis identifies that the rate-limiting step of hydrolysis is the transport of the
substrates into the proteasome. This transport efficiency varies between human standard- and
immuno-proteasomes thereby impinging upon total degradation rate and substrate cleavage-
site usage.

Introduction
Cells can be usefully thought of as communities of molecular machines that work in concert
to maintain cellular function (1). Metabolism, gene expression, protein production and
degradation, energy production, DNA replication, cell division all have their own repertoire
of associated proteins and macromolecular assemblies, and are controlled by complex and
highly dynamic signalling and regulatory networks; this picture is complemented by a slew of
structural proteins that provide cell walls, nuclear membranes, endoplasmic reticulum and
other structures of the eukaryotic cell.
But while advances in imaging and microscopy technology have allowed us to glean some insights into the structures and even dynamics at the molecular level (2), much of our knowledge about the function of, e.g., protein production at ribosomes, or degradation at proteasomes, is based on indirect observations, or on data collected at some experimentally convenient equilibrium state. Increasingly time-resolved data can also be collected, and this type of data typically provides more detailed insights into the molecular mechanisms at work. To interpret such data, mathematical modelling coupled to state-of-the-art statistical inference thus becomes a necessity (3-5) to make sense of data and design better, more discriminatory experiments.

Here we apply a modelling approach to a detailed mechanistic analysis of proteasomes, which are at the core of the ubiquitin-proteasome system (UPS) and responsible for the destruction of the majority of the cytoplasmic proteins. Proteins are usually tagged by E1-E2-E3-E4 enzyme-mediated poly-ubiquitination, carried into the proteasome proteolytic chamber where they are fragmented and, ultimately, expelled (6). Proteins, and especially oxidised proteins—i.e. those that reflect the presence of cellular stress conditions—can also be degraded by 20S proteasomes without prior poly-ubiquitination (7-10). In addition to stress response, the 20S proteasomes present in cells (11) have been shown to regulate the abundance of a host of signalling molecules involved in cell cycle progression, cellular growth control and oncogenesis (8). Additionally, 20S proteasomes form the core of the structure of 26S proteasomes and we cannot expect to understand the latter if we do not understand the mechanisms that determine the activity of the former.

Poly-ubiquitin tagging (when it occurs), transport and peptide-bond hydrolysis regulate protein half-life and thereby affect the majority of metabolic processes in the cell. Despite considerable biochemical and structural efforts, the mechanisms of proteasomal action, in particular the causes and extent of differences in substrate-specific proteolysis between different proteasome isoforms, are only incompletely understood. In this study we combine
carefully designed experimental assays with detailed modelling to shed light on these mechanisms.

There is a dearth of mechanistic analyses of proteasome functions, and the complexity of polypeptide hydrolysis has precluded detailed analysis (12). The most direct insights come from carefully chosen short fluorogenic peptides, which have a single proteasome-catalyzed cleavage site, which in turn allows us to follow the kinetics in real time. They have also been used (exclusively) to measure the proteasomal activity in cellulo (e.g. (13-16)), despite the fact that they lack the complexity of “real” proteins and they do not recapitulate proteasome proteolytic activities towards polypeptide chains (17). Nevertheless, they provide arguably the most promising class of leads of proteasomal inhibitors (18, 19). Furthermore, kinetic and structural analyses rely on their structural and biophysical characteristics (20-24); we, too, use them here for our analysis, which is based on a representative set of such peptides. As we show below the insights gained from the short fluorogenic peptides are borne out by further analysis of polypeptides.

The mechanistic analyses performed here also incorporate known structural features into a mathematical model of proteasome action. The 20S proteasome consists of four stacked seven-membered rings (denoted by α7β7β7α7). These rings form three interconnected cavities, including a pair of antechambers through which substrates are passed before reaching the central catalytic chamber. Antechamber walls are not merely structural, but interact actively with the substrates by altering their properties and keeping them accessible for hydrolysis (25). These proteasome chambers can also store two or more proteins in order to enable continuous degradation (26, 27). The central chamber contains six subunits (two β1, β2 and β5 subunits) that catalyse the peptide-bond hydrolysis and peptide splicing after binding of the polypeptide substrates nearby their active N-terminal Thr (28-30).

20S proteasomes modify their conformations and thus in turn their activity upon peptide-bond hydrolysis (20, 31), binding of regulatory complexes such as 11S or 19S to the proteasome α
rings (leading to the formation of PA28-capped proteasomes and 26S proteasomes, respectively) (31-35), and activation of non-catalytic modifier sites (22, 23, 36) whose location in the proteasome remain unknown (12).

In mammals different 20S proteasome isoforms exist, which carry different catalytic β subunits. Upon an inflammatory stimulus such as IFN-γ the catalytic standard β1, β2 and β5 subunits peculiar of the standard proteasome (s-proteasome) are replaced by the immunodominant subunits β1i, β2i and β5i in the newly synthesized immunoproteasome (i-proteasome). Proteasome isoforms degrade substrates with different rates, but generate the same peptide pool (17). And while there exist differences between substrates, the 20S i-proteasome has been shown often to have a higher polypeptide degradation rate than its standard counterpart (18). Why s- or i-proteasomes should degrade specific substrates more rapidly is a matter of ongoing debate. Different steps of the proteolysis process may be responsible for isoform specific kinetics: transport of the substrate through the gate and the antechambers; binding to substrate binding sites and ensuing peptide-bond hydrolysis (37, 38); and the release from the substrate binding sites and finally from the proteasome gate. The question is also of direct medical relevance: different degradation kinetics of specific substrates by s- and i-proteasomes, or i-proteasome carrying genetic polymorphisms/mutations, have been suggested to be involved in a variety of pathologies (18, 39). Furthermore, i-proteasome deficient mice show altered proteasome-dependent kinetics of pathogen epitope generation (39). Such different kinetics could, for example, lead to the remodelling of immunodominance of viral epitopes (40) or to the lack of CD8$^+$ T cell-mediated response towards specific viral antigens (41).

As we will show below, our framework allows us to start from simple models of proteasome function, and identify which mechanistic shortcomings they exhibit. We focus on the dynamics of the digestion of a set of exemplar peptides, and show that this process is regulated carefully and by three distinct mechanisms, that act in concert to regulate
proteasomal activity. Here considering different mechanisms, coupled to state-of-the art statistical model selection techniques (42), allows us to elucidate steps in the proteasomal dynamics that cannot be probed directly through experiments. Model selection naturally extends the conventional hypothesis testing approach, and by accounting for uncertainty fairly and correctly, can be applied to systems with many unknown kinetic parameters (which are, in any case, inferred together with their respective uncertainties) (43, 44).

Results.

Mechanistic modelling of proteasome proteolysis.

Rate of product formation changes over time.

The majority of mathematical models (45) describing enzymatic activity are Michaelis-Menten-type (MM) models, i.e. they describe the dependency of the initial reaction speed on the initial substrate concentration. Such models can capture allosteric effects and have e.g. been applied to investigate the action and dynamics of specific inhibitors. However, they assume that the initial reaction velocity is constant.

The in vitro digestion of short fluorogenic peptides by purified mouse 20S proteasomes reveals, however, substrate inhibition at high substrate concentrations (Figure 1A), which is not compatible with the classical MM model. Furthermore, we find that proteasome proteolytic activity, i.e. reaction velocity, changes over time as the product accumulates in non-linear fashion (Figure 1B). For instance, the reaction velocity for the substrate Suc-LLVY-MCA increases over time (Figure 1B); this temporal profile of proteasome activity is not due to its permanency at 37°C since Suc-LLVY-MCA degradation kinetics of the mouse proteasomes prior storing for 18 hours at 37°C or 4°C do not differ significantly (Figure 1 – figure supplement 1A).

Similarly, we detect proteasome activity inhibition at high substrate concentrations (Figure 1 – figure supplement 1B), and non-linear accumulation of product over time (Figure 1 –
figure supplement 1C), when using protein homogenate of human T2 cells, which contains 20S proteasome as well as proteasome regulatory complexes such as 19S and PA28.

In agreement with the data obtained by using the short fluorogenic substrates, we also observe that the frequency of peptide-bond hydrolysis after some cleavage sites of polypeptide substrates varies over time (Figure 1C-D). The changing substrate cleavage site usage over time might be the result of substrate depletion that leads to the re-entry and cutting of peptide products, thus acting as competitive inhibitors. This would lead to a decrease of the average length of the peptides products due to further cleavages and thus further shortening of the initial products. However, we observe only a slight reduction in the average length of the peptide products in the polypeptide degradations, only at late time points, and only when less than 50% of the substrate is still intact (Figure 1 – figure supplement 1D-E). By contrast, the changes in cleavage site usage are already becoming evident at early time points (Figure 1C-D). Therefore substrate depletion, product re-entry or further cleavage cannot explain variation in cleavage site usage over time.

To understand the proteasomal mechanisms in detail we therefore investigate product formation under carefully designed experimental conditions (43), and measure the degradation of Suc-LLVY-MCA, Bz-VGR-MCA and Z-LLE-MCA over time by mouse proteasome for different initial substrate concentrations. Based on these data sets (available on DRYAD: http://datadryad.org/) we then develop a set of increasingly detailed mathematical models of proteasomal peptide hydrolysis (Figure 2 - source data 1) and apply a Bayesian model selection (42) framework (which also ensures a level of parsimony: i.e. models that are more complicated are only preferred if they are capable of capturing the data significantly better than simpler models) to elucidate proteasome action. As a by-product we also obtain parameter estimates (including an assessment of the corresponding uncertainty).
Classical enzyme kinetic models fail to describe the time course of proteasomal peptide hydrolysis.

A useful mathematical model needs to explain the time course of product formation over time. Here, we focus especially on the inhibition of product formation at high substrate concentrations (which can be best seen in dose-response curves) and on the increase of the reaction velocity at early time points (which can be seen on the individual time series).

Substrate degradation involves: binding of substrate close to the active site; peptide bond hydrolysis; and release of the products from the active site. This scheme is often assumed for enzymatic reactions and forms the basis of the MM model (Figure 2A); but it explains neither the substrate inhibition nor the increasing reaction velocity.

In the short fluorogenic peptide assay the product has the same amino acid sequence as the N-terminal part of the substrate (the C-terminal part contains the MCA-group that is cleaved off). Because of this the product itself can bind tightly to the active site without being further processed and thereby block the cleavage of further substrates (product inhibition). We employ the two-site-modifier scheme of Schmidtke et. al. (36), who have already argued against the use of MM-types model for analysing proteasome function, and we adapt it to allow for product and substrate inhibition (Figure 2B and Figure 1 – figure supplement 2).

However, the substrate inhibition model still fails to reproduce our data, which can be clearly seen in the case of Z-LLE-MCA degradation (Figure 1 – figure supplement 3A-B, blue curves). The intermediate complexes are assumed to be in quasi-steady state (which holds for all proteases studied so far), which allows us to reduce the overall complexity of the mathematical description without loss of information (46, 47).

Because of the observed increase in reaction velocity over time until 90 – 120 min, we next investigate if a positive feedback loop could cause this. Two distinct mechanisms are possible: either (i) the product enhances binding to the active site; or (ii) the product increases the peptide-bond hydrolysis rate (Figure 1C-D). Even though both mechanisms
result in an increased reaction velocity over time, they cannot explain substrate inhibition and fail to reproduce our experimental data (Figure 1 – figure supplement 3C-D). Because of this we require and develop a more complex model that accounts for the specific steps of proteasomal peptide hydrolysis.

Substrate transport is a crucial step in modelling peptide hydrolysis

The models considered so far do not account for the proteasome structure where active sites are buried inside the proteasome’s inner cavity; substrate must enter via the proteasome gate and move along the inner cavity until it reaches the active site. This structural organisation profoundly affects proteasome dynamics.

Previous *in silico* studies (12) had assumed that substrate molecules enter and leave the proteasome by diffusion. However, the proteasome gate and the interior surface of the proteasome chambers have strong partial charges, and substrate molecules have been shown to interact at least with the latter, and a transport model based on free diffusion cannot capture the observed data (Figure 2E).

Before substrate enters the 20S core, it needs to be located close to one of the two proteasome gates (Figure 2F). Substrate affinity to the gate is described by the parameters $k_{on}$ and $k_{off}$. After entry into the central chamber through the antechamber ($v_{in}$) the substrate can bind to an active site. The number of substrate molecules that can enter the chambers is determined by the maximum capacity ($C$ – number of molecules that can be allocated in the proteasome chamber). This number depends on the volume of the proteasome chamber and the volume of the molecules. Since different substrate molecules differ in volume, the maximal capacity is expected to differ as well. The rate $v_{in}$ is dependent on how many molecules are already located inside the proteasome chamber. If the number of molecules inside the chamber reaches the maximal capacity $C$, the rate by which new molecules enter the chamber is decreased. Since the exact mechanism for this process is unknown we formulate a heuristic
expression that decreases the rate as a function of the number of molecules inside the
chamber and the maximal capacity (see Supplementary File 1—section 2).

To exit the proteasome products and unprocessed substrates need to move from the central
chamber through the antechamber, and then to the gate (τ), where they can exit the
proteasome (v_{out}). Both unprocessed substrates and products can re-enter the proteasome.

Substrates and products can enter and leave the proteasome through the same gate or through
different gates. In the model we include both gates, which are described by an outer site (G1)
and an inner site (G2). Note, in the schematics of Figure 2 we show substrate entry always
on the left hand side and substrate release always on the right hand side for simplicity. This
does not indicate two distinct gates with distinct characteristics.

This proposed transport model is the simplest realistic representation of the biophysical
transport mechanism; the possible bio-molecular interactions are summarised by rates (k_{on},
k_{off}, v_{in}, τ, v_{out}), see Figure 2—source data 2.

Peptide transport is regulated through open / closed gate conformation

The proteasomal hydrolysis rate changes over time and the kinetics vary between different
fluorogenic peptides or synthetic polypeptides (Figure 1B-D). There is already evidence for
non-catalytic regulatory sites in proteasomes (22, 23, 36). In particular, Kisselev and
colleagues (22, 23) identified two enhancers of the degradation rate of Suc-FLF-MNA and
Suc-LLVY-MNA molecules, but not of Boc-LRR-MCA. We therefore expect that Suc-
LLVY-MCA will enhance its own hydrolysis through a positive feedback loop (self-
activation). Indeed, the degradation rate of Suc-LLVY-MCA by mouse proteasome
progressively accelerates within 90 min (Figure 1B), and the degradation rate of the substrate
Bz-VGR-MCA increases in the presence of the peptide LLVY (Figure 3A) – this is indirect
evidence for the existence of feedback. The resulting time course shows an initially low
reaction velocity, which then increases over time. This phenomenon is more pronounced at
substrate concentration above 40 μM (Figure 3A). To test whether the progressive increase
of the enhancing effect of LLVY peptide over time is due to slow kinetics of the binding to
non-catalytic modifier sites we pre-incubate mouse proteasome with LLVY peptide prior to
the start of the degradation of the substrates Suc-LLVY-MCA and Bz-VGR-MCA. The
enhancing activity of LLVY is maximal for shorter pre-incubation, but the initial acceleration
remains the same (Figure 3B). Thus the progressive acceleration over time of the two
substrates’ degradations is not due to slow binding of the LLVY peptide to non-catalytic
modifier sites. Instead the data suggest that peptide-bond hydrolysis is mandatory for the
enhancing effect by LLVY peptide.
A possible explanation of our observations is that LLVY accumulates slowly inside the
proteasome where it binds to the regulatory site and then opens the gate. We can confirm this
with the help of Rpt peptides, which are short peptides deriving from subunits of the 19S
regulatory complex and known to bind the proteasome α subunit tails and open the gate (48).
When mouse 20S proteasome digests the substrates Bz-VGR-MCA in presence of Rpt
peptides no further enhancement of the degradation rate is seen when the LLVY peptide is
added (Figure 1 – figure supplement 4).
In our model we therefore have to also include regulatory sites to which substrate can bind.
Based on the in vitro results we investigate two alternatives: (i) the regulatory site is on the
outer surface of the proteasome and therefore accessible to all external peptides (Figure 2G);
or (ii) the regulatory site is located inside the proteasome chambers and thus only accessible
to peptides that are also inside the chambers (Figure 2H). In both cases binding to the
regulatory site would lead to an increase of the substrate influx and hence to an increase in
the model parameters $v_{in}$ and $v_{out}$ by a factor (parameter $X_{enh}$).
We also observe evidence for such a positive feedback loop in the degradation of Z-LLE-
MCA, where the speed of the reaction increases more slowly as the substrate concentration
increases. (Figure 1 – figure supplement 5). This substrate inhibition takes place during the
first 60 min of the gate opening process. This suggests the existence of a regulatory site, which upon binding of substrate stops the gate from opening. If such a regulatory site were inside the proteasome we should observe the effect only after enough substrate has entered the chambers, \textit{i.e.} only at later time points assuming that the chamber fills only slowly; if, however, the regulatory site were on the proteasome’s exterior surface and thus exposed to the initial substrate concentration, inhibition could take place immediately with the initiation of the reaction. Accordingly, we extend the models shown in Figure 2G and Figure 2H by adding a regulator site outside the proteasome with inhibitory effect on the gate conformation. Such inhibition could occur upon specific binding to a specific site, or upon unspecific binding of peptides to the surface of the proteasome (Figure 2I-J).

The last five models (Figure 2F-J) all contain the same description of the active site events. We employ the same substrate and product inhibition model as described for the SI model in Figure 2B and the active site events of the resulting models are described by the schematic shown in Figure 1 – figure supplement 2.

We implement all discussed models (Figure 2A-D and Figure 2F-J) and use Bayesian model selection (42) to determine which model best represents our experimental data. First we focus on data generated from mouse proteasome digestion of 80 - 480 μM Suc-LLVY-MCA. We perform model selection using our approximate Bayesian computation sequential Monte-Carlo (ABC-SMC) framework (49). We start by comparing the four non-compartmentalised models (Figure 4A). The winning model is then compared to the compartmentalised models in a pairwise manner (50). Here the “winning” model is compared to the next model, and models are compared in the order of increasing complexity. This iterative scheme (Figure 4A) provides a “best” model, which is then again tested against all other models.

Neither the model without any transport regulation, nor the model with an enhancing regulator site outside the proteasome can explain the substrate inhibition observed in our data.
(Figure 4A-B and Figure 2 – figure supplement 1A-B, D, dose-response curves). The two models with the enhancing regulatory site inside the chambers can reproduce the increased reaction velocity at early time points observed in the data, but only the model with an inhibiting regulatory site is able to fit simultaneously the substrate inhibition and is therefore able to reproduce the time course data in detail (Figure 4B and Figure 2 – figure supplement 1C, E). This is further confirmed when we use data generated by digestion of 160 - 640 μM Z-LLE-MCA using mouse proteasome and apply the model selection scheme for the last two models (Figure 2 – figure supplement 2A-C).

In summary, the integrative analysis suggest that the gate-opening regulatory site proposed previously (22, 36) should be located inside the proteasome chambers. We also find evidence for a transport inhibiting regulatory site located on the proteasome surface. Combining the description of the substrate transport and the substrate hydrolysis at the active site, the resulting model now accounts for the previously described, but never wholly explained, observations: reduction of product generation long before substrate depletion (51); and substrate inhibition at early and late time points (36, 51). Furthermore, it explains the reaction velocity increase over time, which results from the spatial organisation of the proteasome.

Proteasome regulatory experiments are predicted correctly.

After model selection we calibrate our mathematical model against the experimental data sets for the degradation of the substrates Suc-LLVY-MCA, Z-LLE-MCA and Bz-VGR-MCA by mouse proteasome. Data and model fits for the substrates are shown in Figure 3 – figure supplement 1A; we obtain posterior parameter distributions that provide us with confidence intervals for the parameters and allow us to detect potential correlations between parameters. The parameter estimates are shown in Figure 3 – figure supplement 1B and related to the model in Figure 3 – figure supplement 1C.
We first test if our kinetic model can qualitatively reproduce published results on proteasome modifier sites (which were not used in model development and calibration). Kisselev et al. (22) showed that proteasomal cleavage of the substrate Boc-LRR-MCA is enhanced by adding Suc-LLVY-MNA (or Suc-FLF-MNA), or by using the mutant Δ3αN proteasome, which has a constitutively fully open gate. Our calibrated model reproduces qualitatively the time course of Bz-VGR-MCA and Suc-LLVY-MCA hydrolysis of Kisselev et al. (22) (Figure 4C). In the same study the authors also showed dose-response curves for the substrates Suc-LLVY-MCA and Boc-LRR-MCA in dependence of the LLVY peptide concentration; our model also reproduces the results for the hydrolysis of the substrates Suc-LLVY-MCA, Z-LLE-MCA and Bz-VGR-MCA (Figure 4D). To note, in the study by Kisselev et al. (22) the authors used proteasome purified from other species rather than mouse and different short fluorogenic peptides (Suc-FLF-MNA instead of Suc-LLVY-MCA and Boc-LLR-MCA instead of Bz-VGR-MCA). These differences in the experimental setup explain why we obtain only qualitative agreement between the published data and our in silico predictions.

Modelling polypeptide degradation.

The hydrolysis of oligo- and polypeptides has previously been modelled phenomenologically (12). Our model, calibrated against degradation data of short peptides, can serve as starting point for modelling polypeptide degradation; but many possible substrate cleavage sites will make the investigation of polypeptide degradation computationally and experimentally more challenging.

As a first step we are interested in whether our fully parameterised model can explain the altered cleavage site usage over time (Figure 1C-D). We extend the model in order to describe the hydrolysis of a hypothetical peptide with two cleavage sites. A schematic of the substrate and possible resulting products is shown in Figure 3 – figure supplement 2A. To
reduce computational complexity we model the allosteric regulation of the active sites in a simplified fashion (parameters: $K_{iS}$, $K_{iP}$, $n_i$, $n_a$, $\alpha$, $\beta$); all other reaction steps and the non-catalytic regulatory mechanisms are as above. We assume that the parameters related to the peptide-bond hydrolysis at the active sites are the same for substrate and all resulting products, but the parameters related to peptide transport are substrate dependent. This allows us to test whether the cleavage site usage variation over time can be explained by transport properties of the substrate and products.

In our framework we detect all possible substrate cleavage site behaviours that we observe by digesting representative polypeptides (Figure 1C-D and Figure 3 – figure supplement 2B-E).

**Comparing human s- and i-proteasome dynamics.**

* S- and i-proteasome have different kinetic parameters.

In the last decade potentially different activities of s- and i-proteasomes have been discussed, at times controversially, although recent results (17) suggest that such differences are not of qualitative but quantitative nature. Like we have observed for the mouse proteasome, human 20S proteasome purified from T2 (s-proteasome) and LcL (i-proteasome) cell lines shows degradation dynamics of short fluorogenic peptide that vary over time (Figure 4 – figure supplement 1A). This is not due to variation of proteasome functionality as pre-incubation of the proteasome without substrate at 37°C for 18 hrs does not alter its activity (Figure 4 – figure supplement 1B), in agreement with what is observed for mouse proteasome (Figure 1 – figure supplement 1A). Similarly, the substrate cleavage preferences within the LLO$_{291-317}$ polypeptide by s- and i-proteasomes vary over time, and not because of product re-entry. We observe modification of the cleavage site usage already at early time points (Figure 4 – figure supplement 1C). However, a slight reduction in the average peptide products length, as would be expected to result from further peptide product fragmentation, is evident only at
late time points and with less than the 50% of the substrate still intact (Figure 4 – figure supplement 1D). This observation is in agreement with an analysis of the production efficiency of peptides derived from the tested polypeptide substrate: these vary already at early time points and show marked differences between s- and i-proteasomes (17).

In order to understand the origin of the kinetic differences between s- and i-proteasomes we calibrate our mechanistic model against new time course data from s- and i-proteasomes. We then compare the resulting posterior parameter distributions to determine which kinetic parameters differ between s- and i-proteasomes. Finally, we make in silico predictions about the different effects of the Rpt peptides on the substrate hydrolysis by s- and i-proteasomes, which we then validate experimentally.

We use measurements for six different initial concentrations (from 20 to 640 μM) of the substrates Suc-LLVY-MCA, Z-LLE-MCA and Bz-VGR-MCA, using 0.5 μg of the s- and i-proteasomes. Figure 5 – figure supplement 1 shows that the model is able to reproduce all experimental data sets. Due to the presence of the catalytic immuno-subunits in i-proteasomes we expected to observe differences in the parameters related to the active sites. Suc-LLVY-MCA is mainly hydrolysed by the β5 / β5i subunits (17), and we observe a higher active site affinity ($K_{aS}$) with a lower Hill coefficient ($n_a$) in i- than in s-proteasomes; similarly i-proteasomes have a lower inhibitory site affinity ($K_{iS}$) with a higher Hill coefficient ($n_i$) compared to s-proteasomes. Furthermore, the hydrolysis rate ($k_p$) is higher in i-proteasomes (Figure 5A).

The posterior parameter distribution of Z-LLE-MCA (mainly cleaved by β1 / β1i active sites) (23) also shows differences in active site parameters, and for i-proteasomes we observe lower affinity to the active site ($K_{aS}$) and a lower Hill coefficient regarding binding to the inhibitory site ($n_i$). We find no evidence for differences in parameters related to the active site for Bz-VGR-MCA (mainly cleaved by β2 / β2i active sites).
Comparing posterior parameter distributions for s- and i-proteasomes reveals differences in parameters related to peptide transport and transport regulation for all three substrates (Figure 5B). All three substrates have altered affinities to the gate ($k_{off} / k_{on}$: i-proteasome lower than s-proteasome for Suc-LLVY-MCA and Z-LLE-MCA but higher for Bz-VGR-MCA) and altered influx and efflux rates ($v_{in}$, $v_{out}$: i-proteasome higher for Suc-LLVY-MCA and Bz-VGR-MCA but lower for Z-LLE-MCA). Finally, affinity to the enhancing regulatory site inside the proteasome chambers ($R_{off} / R_{on}$) is higher in i-proteasome for all three substrates. By contrast, we find no evidence for differences in $X_{enh}$ and $\tau$. Furthermore, the marginal posterior parameter distributions for the maximal capacity $C$ do not differ between s- and i-proteasomes, which was expected because both isoforms have the same proteasomal cavity volume.

In summary, the presence of the immuno-subunits in the assembled proteasome influences not only active site parameters but also the parameters that regulate substrate transport.

Peptide transport is the main limiting step in human s- and i-proteasome.

To further understand differences between s- and i-proteasome and how substrate transport- and hydrolysis steps affect overall substrate degradation, we perform sensitivity analysis based on the posterior parameter distribution. Sensitivity coefficients inform us about the parameters that determine the overall dynamics (52, 53). Since we are interested in the rate limiting steps of the proteolysis, we need to determine the reaction that has the strongest potential to increase product formation. We test the influence of the gate affinity, peptide influx, hydrolysis, peptide translocation and peptide efflux as well as initial gate size (simultaneous change of influx and efflux) on the product formation. We compute the fold-change in product formation when increasing one of these reactions (Figure 5C), and perform this analysis in silico using 320 $\mu$M substrate (the same analysis using 80 $\mu$M can be
found in Supplementary File 1). The reaction inducing the strongest fold change in product formation is the rate-limiting step.

For Suc-LLVY-MCA we find that the initial gate size has the highest impact. Increasing the hydrolysis also increases the product formation, but to a lesser extent. This is observed for s- and i-proteasomes, and is valid independently of substrate concentration (Figure 6 – figure supplement 1A); similar results are found for Bz-VGR-MCA. The degradation of Z-LLE-MCA is limited by the efficiency of the hydrolysis, which induces the strongest increase in product formation. None of the peptide transport related reactions can increase the overall proteasome activity. When using 80 µM Z-LLE-MCA the hydrolysis is still the main rate limiting step, but the gate size has a similar impact (Figure 6 – figure supplement 1A).

Reasons for the observed differences might lie in the physical and chemical properties of the investigated substrates, which differ not only in their volumes, but also in their partial charges.

Overall peptide transport is the rate limiting step in proteasomal degradation. Our model shows how even a subtle difference in one of the transport parameters can result in strongly altered product formation kinetics. Differences in kinetic parameters between s- and i-proteasomes furthermore result in different chamber-filling kinetics (Figure 6), which are also reflected by the differences in rate limiting steps. Equivalent results are also obtained for the mouse proteasome (Box S1 and Figure 6 – figure supplement 1B-D).

Since substrate-characteristic peptide transport appears to shape the kinetics of (20S) proteasome-mediated peptide degradation, this process should naturally be the rate limiting-step of the overall reaction; furthermore it seems to vary systematically between s- and i-proteasomes.

To test the robustness of our posterior parameter distributions we alter specific steps of the proteolytic process. First, we predict in silico the kinetic effect of the Rpt peptides – which open the gate by binding the α subunit tails (48) – on the hydrolysis rate of s- and i-
proteasomes over time. According to our simulation the enhancing effect of Rpt should be strongest at early time points and decrease over time (Figure 5D). We also predict that at early time points the effect of Rpt will be larger in s-proteasome when hydrolysing Suc-LLVY-MCA than in i-proteasomes, although over time the enhancing effects are the same. A similar effect is predicted for proteasomal hydrolysis of the Bz-VGR-MCA substrate in presence of Rpt peptides, although s-proteasome is predicted to remain higher than i-proteasome also at later time points (Figure 5D). Third, we predict that both proteasome types will initially have the same enhancing effect while digesting Z-LLE-MCA substrate, but that this effect becomes stronger over time for i-proteasomes. The in vitro experiments verify these three nontrivial in silico predictions. The small quantitative deviations can be explained by the use of a simplified model for the functioning of Rpt peptides (multiplication of the parameter $v_{in}$ by a constant factor), which is expected to be more complex in reality. But this comparison validates that transport is indeed the rate limiting step (Figure 5A-C).

Discussion

Using tightly integrated experimental and computational modelling analyses, recent advances in our understanding of proteasome structure and function, and previous attempts at modelling proteasome dynamics (12), we elaborate the first comprehensive mathematical model that is able to describe the regulatory components of 20S proteasomes; and the complex interactions between substrate/product transport and substrate hydrolysis over time for representative peptides. Modelling helps us to understand how the 20S proteasome catalyzes the degradation of specific proteins in cells (7, 8, 10, 11); it also forms the basis for future studies of the main active forms of proteasome in cells, e.g. when it is bound to the regulatory complexes 19S and PA28 (11). Indeed, in 19S or PA28 single-capped proteasomes all steps considered in our model are still present, including the regulation of the gate by non-
catalytic modifier site(s) and the gate binding affinity since one of the proteasome gates is not bound to the regulatory complexes and will therefore be regulated as described here.

We observe similar proteasome dynamics over time when using purified 20S proteasome as well as protein homogenates, where 20S proteasomes are surrounded by several regulatory molecules. It suggests that with the present model we describe a core of proteasome features that could reflect proteasome dynamics also in more complex scenarios such as might exist inside the cellular environment. Our model therefore also provides an interpretative framework for general studies of proteasome action, keeping in mind that substrate-specific effects need to be accounted for (17). Simple (e.g. MM-type) models, which have been largely used in analysing proteasome functionality so far, are clearly not capable of describing proteasome dynamics; this is becoming apparent since time-course data is much less forgiving and harder to fit to than e.g. dose-response curves. The 20S proteasome is a dynamic enzyme and its catalytic activity varies over time in a way that can only be understood if the structural characteristics of the proteasome are taken into account. Kinetics change over longer time scales than the structural dynamics described by Osmulski et al. (20) and they are influenced by the interactions of substrates and products with both proteolytic and regulatory sites. Our analysis suggests that the cavity of 20S proteasomes is progressively filled by peptides that can further facilitate other molecules to enter the chambers until the effective cavity volume has been almost completely filled. In fact, the estimated numbers of substrate and product molecules (C) that are inside the proteasome cavity are close to the possible maxima (Box S2). Peptide accumulation over time leads to activation of non-catalytic modifier sites, which our analysis shows to be located inside the proteasome cavity.

Substrate transport through the gate and cavities must therefore be a key factor in the regulation of the proteolytic process, despite the fact that it had been largely neglected so far in the quantitative analysis of proteasome dynamics (12). The integration of data into our mathematical model clearly shows that peptide transport is the major rate limiting step in the
degradation of short fluorogenic substrates: all models/hypotheses that do not account for this are soundly rejected. Hydrolysis is only rate limiting for the degradation of Z-LLE-MCA. We stress that for the short fluorogenic substrates the rate limiting steps are conserved across the different proteasomes (mouse proteasome and human s- and i-proteasomes). This in turn suggests that the interspecies homology of the proteasome structure results in preserving transport characteristics, although subtle structural variations can quantitatively modify the transport, as observed by comparing s- and i-proteasomes.

Variation of the transport efficiency typically leads to quantitative changes in the degradation rates of short fluorogenic substrates. For more complex substrates, e.g. polypeptides, transport variation could change substrate cleavage-site usage and thus result in the generation of specific peptide products. If these peptides are MHC class I-restricted epitopes, substantial variations in their amount could strongly affect the cell-mediated immune response. Indeed, as we have recently shown, significant decreases of antigenic peptide amount produced by proteasome could lead to a presentation onto the MHC class I molecules that is so diminished to be not able to trigger CD8+ T cell activation (17). According to our model, modifications of the proteasome gate upon PA28 binding will for instance affect the substrate cleavage-site usage and lead to the observed alterations of MHC class I-restricted epitope repertoire (54).

Differences in gate and inner channel features are in part responsible for the different degradation rates of the short fluorogenic peptides by human s- and i-proteasomes although they diverge also in the activity of the catalytic subunits themselves. Differences in the gate between the two proteasome isoforms are supported also by Fabre et al. (11), who identified preferential binding between the α subunits of s- and i-proteasomes and specific regulatory complexes. Such differences can become dominant driving forces. For instance, the degradation of the substrate Bz-VGR-MCA is not influenced by differences in the active site-related parameters – as is correctly predicted by our model. This also agrees with comparisons of the crystallographic structures of the mouse s- and i-proteasomes, which identify only
minor variations between the $\beta_2$ and $\beta_2i$ catalytic pockets (37). By contrast, the catalytic sites of s-proteasome have stronger binding affinity for the substrate Z-LLE-MCA than the i-proteasome, which could be explained by the structural differences between $\beta_1$ and $\beta_1i$ catalytic pockets described by Huber et al. (37). For the degradation of the substrate Suc-LLVY-MCA our model predicts that the i-proteasome has higher substrate-binding affinity, higher hydrolysis rate, and lower cooperativity than the s-proteasome. This prediction is corroborated by the recent study of Arciniega et al. (38), who showed that the $\beta_5i$ pocket is more prone to bind and process substrates than the $\beta_5$ pocket; however, only s-proteasome is able to modify its conformation upon substrate binding to the $\beta_5$ subunit (38), which is also correctly captured by our model as increased cooperativity ($n_i$).

The outcomes of our integrative analysis suggest that the i-proteasome also has a higher affinity for the enhancing non-catalytic modifier site activated by all three substrates; given that our model consistently predicts this site to be located at the inner surface, it should now become possible to determine the precise location of the enhancer site using structural techniques.

Our model is a necessary and non-trivial step towards understanding protein degradation by proteasomes. A full-length protein is a much more complicated substrate and it can interact with the different regulatory and catalytic sites in a myriad of ways. In any such analysis, however, the model developed here can aid the experimental set-up (43, 55) and the interpretation of experimental data. What is already clear is that the complex interactions between a suitable complicated substrate and the proteasomal machinery will substantially shape differences in degradation rates between proteins; as the protein degradation rate is known to influence e.g. noise in signal transduction, a way to understand and rationally interfere with this process is of obvious importance.

Materials and methods.
Experimental procedures.

20S proteasome purification and protein homogenates. 20S proteasomes from LcL and T2 cells and mouse liver are purified as previously described (17). Cell protein homogenates are extracted from T2 cells as previously described (56).

In vitro digestion of synthetic polypeptides and short fluorogenic peptides. Synthetic polypeptides or short fluorogenic peptides are digested by purified 20S proteasomes or cell protein homogenates in 100 μl TEAD buffer (Tris 20 mM, EDTA 1 mM, NaN3 1 mM, DTT 1 mM, pH 7.2) over time at 37°C as previously described (17).

Peptide synthesis and quantitation. Peptides gp10035-57 (VSRQLRTKAWRQLYPEWTEAQR), LLO291-317 (AYISSVAYGRQVYLKLSTNSHS TKVKA), Rpt2 (GTPEGLYL) and Rpt5 (KKKANLQYYA) are synthesized using Fmoc solid phase chemistry as previously described (29). Quantification of produced peptides - both cleavage and spliced products - and computation of the substrate site-specific cleavage strength (SCS) are carried out by applying QME method to the LC-MS analyses (29).

Mathematical model.

The final model shown in Figure 2J contains a set of ordinary differential equations as follows:
\[ \frac{dS_{out}}{dt} = -S_{out}G_{1}k_{on} + [G_{1}S_{out}]k_{off} + [G_{2}S]_{transport_{out}} - h_{out}I_{free_{ion}} + hI_{off} \]

\[ \frac{dG_{1}}{dt} = -(S_{out} + P_{out})G_{1}k_{on} + ([G_{1}S_{out}] + [G_{1}P_{out}])k_{off} + transport_{in} \]

\[ \frac{dP_{out}}{dt} = -P_{out}G_{1}k_{on} + [G_{1}P_{out}]k_{off} + [G_{2}P]_{transport_{out}} - h_{out}I_{free_{ion}} + hI_{off} \]

\[ \frac{d[G_{1}S_{out}]}{dt} = S_{out}G_{1}k_{on} - [G_{1}S_{out}]k_{off} + transport_{in} \]

\[ \frac{dG_{1}P_{out}}{dt} = P_{out}G_{1}k_{on} - [G_{1}P_{out}]k_{off} + transport_{in} \]

\[ \frac{dS}{dt} = [G_{1}S_{out}]_{transport_{in}} - \frac{SG_{2}}{E_{0}} - v_{hydr} - R_{on} \frac{SE_{reg}}{E_{0}} + R_{off} \frac{E_{reg}S}{E_{0}} \]

\[ \frac{dP}{dt} = [G_{1}P_{out}]_{transport_{in}} - \frac{PG_{2}}{E_{0}} + v_{hydr} - R_{on} \frac{PE_{reg}}{E_{0}} + R_{off} \frac{E_{reg}P}{E_{0}} \]

\[ \frac{d[G_{2}S]}{dt} = \frac{G_{2}S}{E_{0}} - [G_{2}S]_{transport_{out}} \]

\[ \frac{d[G_{2}P]}{dt} = \frac{G_{2}(S + P)}{E_{0}} - ([G_{2}S] + [G_{2}P])_{transport_{out}} \]

\[ \frac{d[E_{reg}]}{dt} = -\frac{R_{on}E_{reg}}{E_{0}}(S + P) + R_{off}([E_{reg}S] + [E_{reg}P]) \]

\[ \frac{d[E_{reg}S]}{dt} = \frac{R_{on}E_{reg}S - R_{off}E_{reg}S}{E_{0}} \]

\[ \frac{d[E_{reg}P]}{dt} = \frac{R_{on}E_{reg}P}{E_{0}} - P - R_{off}E_{reg}P \]

\[ \frac{dI_{free}}{dt} = -(S_{out} + P_{out})I_{free_{ion}} + I_{off}([IS] + [IP]) \]

\[ \frac{d[IS]}{dt} = S_{out}I_{free_{ion}} + I_{off}([IS]) \]

\[ \frac{d[IP]}{dt} = P_{out}I_{free_{ion}} + I_{off}([IP]) \]

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\[ \frac{d[IS]}{dt} = S_{out}I_{free_{ion}} + I_{off}([IS]) \]

\[ \frac{d[IP]}{dt} = P_{out}I_{free_{ion}} + I_{off}([IP]) \]

587 with
\begin{align*}
\text{transport}_{in} &= \frac{v_{in}}{1 + X_{\text{enh}}} \frac{E_0}{1 + Y_{\text{in}}} \frac{[E_{\text{reg}}S] + [E_{\text{reg}}P]}{[IS] + [IP]} \tanh(E_0 C - S - P) \\
\text{transport}_{out} &= \frac{v_{out}}{1 + X_{\text{enh}}} \frac{E_0}{1 + Y_{\text{in}}} \frac{[E_{\text{reg}}S] + [E_{\text{reg}}P]}{[IS] + [IP]} \\
v_{\text{hydr}} &= \frac{n_{\alpha} k_p E_0 S}{xK_{aS}} \left( 1 + \frac{\beta S_{i}^{p_{i}^n}}{\alpha K_{iS}^S} + \frac{\beta P_{i}^{n_{i}^n}}{\alpha K_{iP}^P} \right) \\
 x &= 1 + \frac{S_{i}^{p_{i}^n}}{K_{aS}} + \frac{K_{iS}}{K_{aP}} P_{i}^{n_{i}^n} + \frac{S_{i}^{p_{i}^n}}{K_{aS} K_{iS}} + \frac{K_{iP}}{K_{aP}} P_{i}^{n_{i}^n} + \frac{S_{i}^{p_{i}^n}}{\alpha K_{aS} K_{iP}} + \frac{K_{iP}}{\alpha K_{aP}} P_{i}^{n_{i}^n} + \frac{S_{i}^{p_{i}^n}}{\alpha K_{iS} K_{aP}}
\end{align*}

A full list of parameters and model species is given in Figure 2 – source data 2 and 3. Details about the experimental procedures, mathematical analysis and other tested models can be found in Supplementary File 1. The model is also provided in SBML format (generated with the SBML editor from MINRES Technologies) in Supplementary File 1.

Author contribution.

JL and MM conceived the project and performed the experiments. JL and MPHS designed the computational framework. JL, HH, MPHS, MM interpreted the results. JL, HH, PMK, MPHS and MM wrote the manuscript. EB performed the experiments.

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Competing interests.

The authors declare no competing or financial interests.

References


Figure legends

Figure 1. Velocity and specific cleavage site usage by mouse proteasomes varies over time. A) The amount of products generated after 6h was measured for different initial substrate concentrations of the short fluorogenic substrates Suc-LLVY-MCA, Bz-VGR-MCA and Z-LLE-MCA by purified 20S mouse liver proteasome. B) The reaction velocity [nM/min] of the same substrates (480 μM) as in (A) by purified mouse 20S proteasome was measured over time. C-D) Cleavage rate [pmol peptide-bond hydrolysed/(min•mg proteasome)] after the residues gp10040 (Arg), gp10042 (Lys) and gp10052 (Trp) of the synthetic polypeptide gp10035-57 (C) as well as LLO298 (Tyr), LLO390 (Arg) and LLO315 (Val) of the synthetic polypeptide LLO291-317 (D) by mouse proteasome. Peptide product amount and SCS was computed by applying QME to each time point of the in vitro kinetics. Values are the mean and the SD of two independent experiments.

Figure 2. Development of the mathematical model. Schematics of the developed and tested models. Reactions involved in peptide-bond hydrolysis are indicated in red, steps involved in substrate and product transport are indicated in blue and the regulation of the transport is indicated in green. Models in A-D are without the proteasome as a separate compartment, while E-J are compartmentalised models. Note, for simplicity the schematics contain only one active site (instead of the two copies for each active site). Furthermore peptides can enter and leave the proteasome chamber through both gates.

Figure 3. Peptide-mediated enhancement of proteasome activity. A) Product formation over time from degradation of Bz-VGR-MCA by mouse proteasome in presence or absence of LLVY peptide over time. B) Product formation from degradation of Suc-LLVY-MCA and Bz-VGR-MCA (100 μM and 200 μM, respectively) after pre-incubation at 37° C of mouse proteasome with LLVY peptide over time.

Figure 4. Bayesian model selection and model validation. A) A model comparison scheme is applied to identify the best candidate among models represented in Figure 2F-J. MS stands for model selection. B) The prior model probability is 0.5 for all pairwise model comparisons, and 0.25 for the comparison of models 1-4. The model selection scheme proceeds over SMC populations (49) each of them returns an updated model probability, until the winning model has a probability of 1 in all comparisons. The colours correspond to (A). The winning model is challenged by in silico experiments. C) The posterior parameter distributions inferred from a data set using mouse proteasome (Figure 3 – figure supplement 1A-C) are used to simulate the mean behaviour of opened-gate mutant (ΔNα3) and the effect of Suc-LLVY-MCA. Simulation of the mutant (ΔNα3) is achieved by increasing the parameters $v_{in}$ and $v_{out}$ 10 fold. The model is extended to simulate the effect of the molecule Suc-LLVY-MCA, parameters are taken from the posterior parameter distribution obtained from digestions of Suc-LLVY-MCA. D) Dose response curves are simulated for the effect of the molecule Suc-LLVY-MCA on the peptide-bond hydrolysis of Suc-LLVY-MCA and Bz-VGR-MCA. In (C) and (D) the results are qualitatively comparable to the results of the experiment by Kisselev et al. (22).
**Figure 5. Human s- vs i-proteasomes.** A) Marginal posterior parameter distributions for active site related parameters that differ between s- and i-proteasomes. No evidence for differences related to Bz-VGR-MCA was detected. B) Marginal posterior parameter distributions for transport related parameters that differ between s- and i-proteasomes. Shown is the fold increase of product formation upon increase of a specific reaction. Substrate concentration is 320 μM, measurement is taken after 60 min reaction. D) In silico predictions for fold stimulation of substrate hydrolysis in presence of Rpt peptides and experimental validation. A final concentration of 40 μM Rpt peptides was added to the standard experimental setup described in experimental procedures. Dashed lines indicate 5%- and 95%-iles of the predictions.

**Figure 6. Rate limiting steps of human proteasome activity.** The mean of in silico predictions (coloured lines) is plotted over time for the degradation of the substrates Suc-LLVY-MCA (A), Z-LLE-MCA (B) and Bz-VGR-MCA (C) with varying initial substrate concentrations using human s- and i-proteasomes, respectively. The inferred posterior parameter distributions of each substrate were used to simulate the number of peptide molecules (product and substrate) and the relative amount of product vs total amount of peptides inside the chambers over time.

**Source Data legends**

**Figure 2 – source data 1. Compartment models in SBML format.** All models shown in figure 2F-J (M5-M9) are provided as SBML (Systems Biology Markup Language) model files, which can be used for model inference and numerical analysis. A description of the SBML format is provided via the BioModels Database (https://www.ebi.ac.uk/biomodels-main).

**Figure 2 – source data 2. List of mathematical model parameters.** The parameters for all models shown in figure 2 are defined in this table. All of them were unknown and had to be inferred from experimental data.

**Figure 2 – source data 3. List of mathematical model species.** The species for all models shown in figure 2 are defined. The initial conditions are known and defined in Supplementary File 1.

**Supplementary File legend**

**Supplementary File 1: Quantitative time-resolved analysis reveals intricate, differential regulation of standard and immune-proteasomes.**
A: Network diagrams with labeled nodes:
- M1: Michaelis-Menten model (Fig. 3A)
- M2: substrate inhibition model (Fig. 3B)
- M3: substrate inhibition with positive feedback on binding (Fig. 3C)
- M4: substrate inhibition with positive feedback on hydrolysis (Fig. 3D)
- M5: compartmentalised model with affinity transport, no regulation (Fig. 3F)
- M6: compartmentalised model with affinity transport and enhancing regulator site outside the chamber (Fig. 3G)
- M7: compartmentalised model with affinity transport and enhancing regulator site inside the chamber (Fig. 3H)
- M8: compartmentalised model with affinity transport and enhancing and inhibiting regulator site outside the chamber (Fig. 3I)
- M9: compartmentalised model with affinity transport and enhancing (inside the chamber) and inhibiting (outside the chamber) regulator site in the chamber (Fig. 3J)

B: Graphs showing population numbers:
- MS1: Population M1 vs. population M3
- MS2: Population M5 vs. population M4
- MS3: Population M5 vs. population M4
- MS4: Population M7 vs. population M5
- MS5: Population M8 vs. population M7
- MS6: Population M9 vs. population M7

C: In silico predictions:
- C1: Specific activity [nmol/min] vs. Suc-LLVY-MCA [μM]
- C2: Specific activity [nmol/min] vs. Bz-VGR-MCA [μM]

D: Experiments by Kisselev et al. (2002):
- D1: Fold stimulation of LLVY-MCA hydrolysis vs. LLVY [μM]
- D2: Fold stimulation of VGR-MCA hydrolysis vs. LLVY [μM]
- D3: Fold stimulation of LLVY-MCA hydrolysis vs. Suc-FLF-mna [μM]
- D4: Fold stimulation of LLR-MCA hydrolysis vs. Suc-FLF-mna [μM]