The bovine lung 20S proteasome binding to reversible inhibitors: modulation by sodium ion

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Abstract The effect of sodium ion on the inhibition exerted by Cbz-Leu-Leu-Leu-CHO on the chymotrypsin-like activity of the 20S proteasome isolated from bovine lung was investigated. The experimental data were analyzed using a standard linkage formalism. The calculated equilibrium affinity constants for the sodium ion binding to the free-enzyme and the inhibitor-bound enzyme are compatible to other well-characterized ion-involving heterotropic systems. The functional interdependence between the binding events played by the inhibitor and the sodium ion conforms to a heterotropic modulatory mechanism.

Key words: 20S proteasome; Inhibition; Sodium; Linkage; Signaling

1. Introduction

The 20S proteasome or multicatalytic proteinase complex (MPC) is a widespread, unusually high molecular weight proteinase which is responsible for the non-lysosomal proteolytic processing in all eukaryotic cells [1–4]. The functional molecule is constituted by 14 non-identical subunits (M.W. 21–34 kDa), each present twice, exhibiting at least five distinct proteolytic activities [5]. Several studies show that the catalytic activities are associated to separate subunits of the complex even though some of them derive from the cooperation of two or more subunits and the structural integrity of the complex is necessary for the expression of the proteolytic activity [6]. The complex binds cooperatively some of its ligands, as appears either from the substrate saturation kinetics exhibited by the peptidyl–glutamyl peptide hydrolyzing (PGPH) activity or from the dependence of one enzymatic activity by the modulation exerted on the other proteolytic activities assembled on the complex [7,8].

Several molecules are able to affect both the catalytic properties of the complex or the mechanisms involved in the recognition of the substrate through the binding to specific sites on the catalytic subunits.

In particular it has been reported that, for the MPC proteolytic activity, these effects are played by several mono- or divalent ions such as Na^+, K^+, Mg^{2+} and Mn^{2+}.

It seems that some of these ions act as specific heterotropic effectors contemporaneously affecting, but with an opposite effect, the proteolytic activities assembled on the complex, suggesting a composite mechanism of communication among the subunits apparently mediated by the ions [8–11]. Djaballah and coworkers [12], through sedimentation velocity experiments, have shown that the effects of several compounds on the catalytic activities of the MPC complex is associated to conformational changes affecting the molecular shape. The ligand-mediated conformational changes of the proteasome experimentally confirm the allosteric nature of the modulation exerted by divalent metal ions on MPC.

Some ions have an effect only on some catalytic components, as in the case of Mg^{2+}, inducing higher PGPH activity, reducing the trypsin-like (T-L) activity and with no effect on the chymotrypsin-like (ChT-L) activity [10,13].

The ion Mn^{2+} seems to play a role in the modulation of the catalytic properties of MPC from rat liver, especially in the homotropic cooperativity exerted by the MPC toward the substrate carbobenzoxy (Cbz)-LLE-β-naphtylamide (βNA) [13].

Other compounds, such as fatty acids, heparin, RNA, ATP, polylysine, spermidine [14], are known to act as effectors of the catalytic properties of MPC.

In order to better understand the effect exerted by physiologically significant effectors on the MPC activity, here we report the results of a study performed on the MPC purified from bovine lung, focusing our attention on the interplay between the binding of a reversible inhibitor of the MPC, the peptidyl aldehyde Cbz-Leu-Leu-Leu-CHO, and the binding of sodium ion. This three-partners interaction can be seen as a model of the combined heterotropic modulation of effectors in solution on the 20S proteasome catalytic properties.

2. Materials and methods

2.1. Materials

Bovine lung was obtained from the local slaughter-house. The inhibitor Cbz-Leu-Leu-Leu-CHO (Mg132) and the synthetic substrate Cbz-Gly-Gly-Leu-*p*-nitroanilide (pNa) were obtained from Sigma-Aldrich. (USA).

All other chemicals were of analytical grade.

2.2. Enzyme purification

MPC was isolated from bovine lung using a purification strategy as previously described [15] and essentially based on a fractionation from 40 to 60% in ammonium sulfate, an ionic exchange chromatography

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Abbreviations: MPC, multicatalytic proteinase complex; ChT-L, chymotrypsin-like; T-L, trypsin-like; PGPH, peptidyl–glutamyl peptide hydrolyzing; Cbz, carbobenzoxy; βNA, β-naphtylamide; pNa, *p*-nitroanilide

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and two gel filtration columns which favor the removal of lower molecular weight contaminants. A higher degree of purification was obtained adding a hydrophobic interaction chromatography step, which seems to improve the separation of MPC from the co-purifying chaperonin Hsp90. In detail, the first chromatographic step was a DEAE–Sephacel column (5 cm×20 cm) in 0.01 M Tris–EDTA, pH 7.4; elution was carried out with a linear gradient established between 1 l of 0.01 M Tris–EDTA, pH 7.4, and 1 l of 0.5 M Tris–EDTA buffer at the same pH, with a flow rate of 2.75 ml/min. Fractions of 10 ml were collected, assayed for the ChT-L activity and the active ones (from 1580 to 1770 ml) were pooled, concentrated by ultrafiltration using YM10 membranes (Amicon) and loaded onto a Sephacryl S-300 (HiPrep 16/60, Pharmacia) in Tris–EDTA 200 mM, pH 8.3, with a flow rate of 0.5 ml/min. Active fractions (from 47 to 52 min) were collected, concentrated and rechromatographed on a Superose 6 (10/30, Pharmacia) equilibrated with 150 mM Tris–EDTA, pH 7.5, with a flow rate of 0.5 ml/min (the active fractions came out from 13 to 14 min). The last step of the purification was a hydrophobic interaction chromatography on a TSK Phenyl-5PW column (8×75 mm, LKB), equilibrated in Tris–EDTA 50 mM containing 2 M $(NH_4)_2SO_4$, pH 7.5, and eluted with a linear gradient, in 60 min, between the equilibrium buffer and 50 mM Tris–EDTA, pH 7.5, using a flow rate of 0.5 ml/min. Active fractions (from 3 to 4 min) were concentrated and the buffer was exchanged with 0.01 M Tris–EDTA, pH 7.4.

The active fractions from the last purification step were pooled and the resulting enzyme was homogeneous to a native PAGE (4.6%) prepared using the standard method of Ornstein and Davis [16,17].

2.3. Determination of enzyme activity

The activity of MPC was determined measuring the ChT-L activity of the complex. The assay mixture contained 40 μl of a 10 mM solution in dimethyl sulfoxide of the chromogenic substrate Cbz-Gly-Gly-Leu-pNa (0.4 mM, final concentration), a variable volume of the enzyme solution, dialyzed in the assay buffer and containing about 40 μg of protein, and 50 mM Tris–HCl, pH 8 (reaction buffer) up to a final volume of 1 ml. All the added salts were dissolved in the reaction buffer up to the desired concentration. The complications arising from non-specific ionic strength effects were eliminated using an inert chloride salt at constant ionic strength. All experiments were conducted at a constant ionic strength of 32 mM by diluting the buffer containing NaCl with choline chloride [18]. The reaction was started by the addition of the substrate, following at 405 nm and 37°C the formation of the hydrolysis product for at least 20 min.

When the effect of the inhibitors was assayed the dialyzed enzyme was previously incubated for 10 min with the inhibitor.

One enzymatic unit is defined as the amount of enzyme that produces 1 μmol of product per hour.

2.4. Protein content

Protein content was determined according to Bradford [19] using bovine serum albumin as standard.

3. Results

3.1. Effect of sodium ion on the binding of the reversible inhibitor Cbz-LLL-CHO

The modulation of the binding of the Cbz-LLL-CHO by

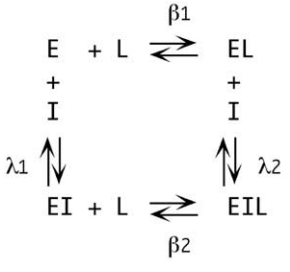

Fig. 1. General binding scheme for a coupled phenomenon such as the binding of a reversible inhibitor and a release/uptake of a modulating ion.

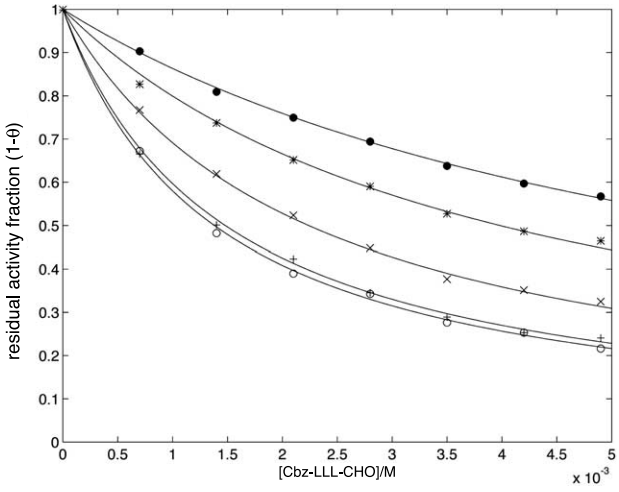

Fig. 2. The residual enzyme activity $(1-\theta)$ of MPC inhibited by Cbz-LLL-CHO at different ion concentrations: 1 μM (○), 31.6 μM (+), 316 μM (x) 1.78 mM (*) and 31.6 mM (●) NaCl. For details see Section 2.

Na^+ can be seen as a general linkage scheme [20], where the enzyme presents binding sites either for the ligand L (in our case the inhibitor Cbz-LLL-CHO) or for the ion I, as shown in Fig. 1, where the parameters β_1 and β_2 are the equilibrium association constants for the enzyme–inhibitor complex in the absence and presence of the modulating ion, respectively. The other two parameters refer to the enzyme–ion complex equilibrium association constants in the absence (λ_1) and presence (λ_2) of the reversible inhibitor.

If the two phenomena (the binding of the reversible inhibitor and the release/uptake of the modulating ion) are linked, the equilibrium association constant between the enzyme and the inhibitor, measured at different ion concentrations β_{app}, will depend on the ion concentration. The apparent association constant β_{app} will change its value from β_1 (no ion present) to β_2 (saturating ion concentration).

If the ion negatively modulates the enzyme–inhibitor complex stability, then $\beta_1 > \beta_2$, and, by symmetry, the condition $\lambda_1 > \lambda_2$ holds.

The modulating activity of the sodium ion toward the enzyme–inhibitor complex stability has been studied as shown in Fig. 2.

The fraction of free enzyme or residual activity fraction $(1-\theta)$ decreases upon the addition of increasing amounts of reversible inhibitor Cbz-LLL-CHO. The inhibitory effect is reduced at increasing concentrations of sodium ion. The experimental data of the residual enzyme activity fraction $(1-\theta)$ at increasing Cbz-LLL-CHO concentrations shown in Fig. 2 can be analyzed using a standard binding isotherm:

$$(1-\theta) = \frac{1}{1+\beta_{app}[\text{Cbz-LLL-CHO}]} \quad (1)$$

where the apparent equilibrium association constant β_{app} depends on the ion concentration following a general linkage relationship:

$$\beta_{app} = \beta_1 \frac{1+\lambda_2[Na^+]}{1+\lambda_1[Na^+]} \quad (2)$$

At a given Na^+ concentration, the free enzyme fraction $(1-\theta)$ was measured at n increasing inhibitor concentrations

Table 1
Parameter values obtained by global non-linear regression analysis of the experimental data presented in Fig. 1 using Eqs. 1 and 2 as fitting functions

Parameter name	Parameter value (M^{-1}) ± S.D.
λ_1	3020 ± 150
λ_2	631 ± 92
β_1	724 ± 60

The standard deviations for the parameters were obtained by a standard Monte Carlo procedure [21].

(at least $n = 7$). The determinations were repeated at m increasing Na^+ concentrations ($m = 12$). The resulting $n \times 2 \times m$ matrix constituted the experimental dataset, which was globally analyzed by the Marquardt–Levemberg non-linear least-squares fitting procedure [21] using Eqs. 1 and 2 as fitting functions.

Data analysis results are shown in Table 1, where the best-fitted values for the parameters together with the standard deviations are reported.

Fig. 3 shows the apparent equilibrium constant as a function of the ion concentration. The negative modulation of the ion is depicted as a decrease of the equilibrium association constant for the enzyme–inhibitor complex.

The modulation of the EL complex stability is not the same at all the L concentrations. We should refer to the $\gamma = \partial \overline{X}/\partial \mu_y$ function (where μ_y is, in this case, the Na^+ chemical potential) as defined by Wyman and Gill [20] to have a deeper understanding of the modulating interplay between the inhibitor L and the ion I as a function of their concentrations.

Using the best-fitted values obtained from the data shown in Fig. 2, it can be noticed that at low concentrations of the ion and the inhibitor, the heterotropic function γ does not change much (see Fig. 4). The same situation is valid at high concentrations.

The maximum value of the intermodulating heterotropic function γ is reached at intermediate concentrations. The value of γ is always non-positive, confirming the negative modulation qualitatively shown in Figs. 2 and 3.

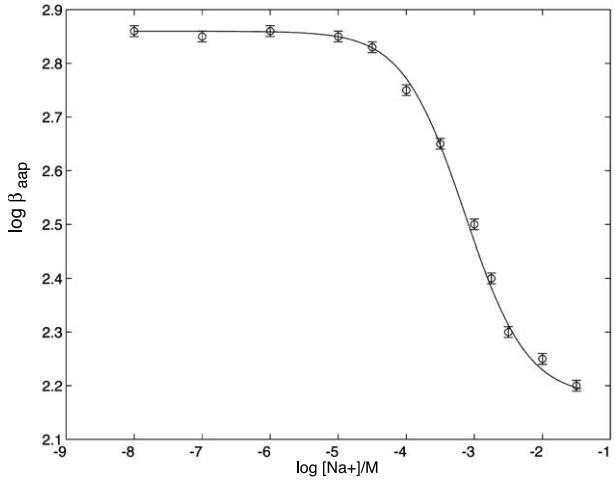

Fig. 3. Apparent equilibrium constant as a function of the ion concentration. The calculated apparent equilibrium constants calculated for each binding curve shown in Fig. 2 are reported vs. log NaCl (M). The sigmoid curve is the theoretical curve obtained using Eq. 2 and the best-fitted values for the λ_1 and λ_2 parameters reported in Table 1.

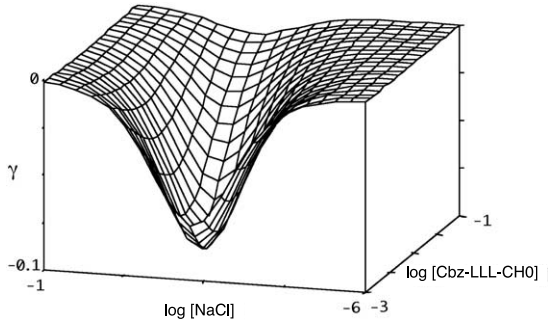

Fig. 4. Heterotropic second derivative γ for the interaction of MPC with Na^+ and Cbz-LLL-CHO. The three-dimensional plot has been obtained from the $\gamma = \partial \overline{X}/\partial \mu_y$ function, using the parameters reported in Table 1.

The modulating effect exerted by sodium ion can be seen as a signaling system, as illustrated by Wyman and Gill [20]. Upon the addition of one mole of inhibitor, the enzyme releases ($Na^+_L - Na^+_U$) moles of sodium ion, where Na^+_U are the moles of ion bound to the inhibitor-free enzyme, and Na^+_L are the moles of ion bound to the inhibitor–enzyme complex. This effect depends on the sodium ion concentration following the signaling equation:

$$(Na^+_L - Na^+_U) = \frac{\lambda_2[Na^+]}{1 + \lambda_2[Na^+]} - \frac{\lambda_1[Na^+]}{1 + \lambda_1[Na^+]} \quad (3)$$

Fig. 5 shows the release of ($Na^+_L - Na^+_U$) moles of sodium ion upon the binding of one mole of inhibitor to the enzyme, as described by Eq. 3.

4. Discussion

Several mono- or divalent ions such as Na^+, K^+, Mg^{2+} and Mn^{2+} affect the MPC proteolytic activity [8–11].

Some of these ions act as specific allosteric modulators contemporaneously affecting, but with an opposite effect, the assembled proteolytic activities on the complex, suggesting a

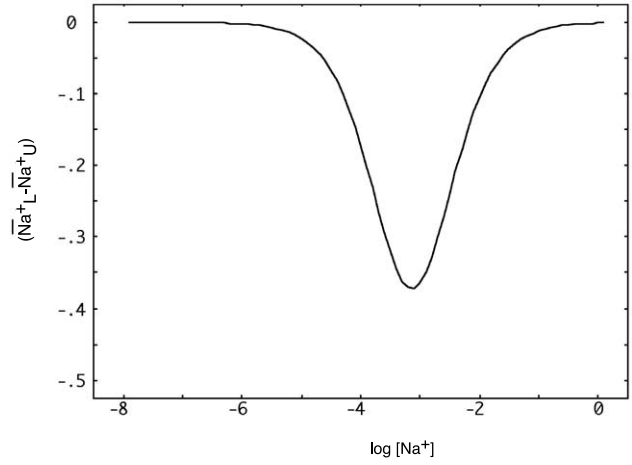

Fig. 5. Release of sodium ion upon inhibitor binding to the proteasome as a function of increasing sodium ion concentration as obtained by Eq. 3, where the best-fitted values for the λ_1 and λ_2 parameters reported in Table 1 were used.

composite mechanism of communication among the subunits apparently mediated by the ions.

The focus of the present work is the quantitative analysis of the functional interplay between the binding of the peptide Cbz-Leu-Leu-Leu-CHO, a reversible inhibitor of the MPC, and the binding of sodium ions.

The analysis of the experimental data clearly indicates a negative heterotropic effect mutually exerted by the peptidyl–aldehyde inhibitor and the sodium ion. Considering the different nature of the two ligands (a small ion and a mainly hydrophobic small peptide), it is reasonable to suppose that Na^+ plays its role as modulatory effector, as seen for other proteases [18]. In addition, the inhibitor participates in the heterotropic mechanism despite its size. This result is relatively unexpected because similar effects on proteolytic systems have been previously described only for the binding of macromolecular inhibitors [22]. This effect confirms the proteasome's peculiar conformational sensitivity in comparison with other proteases [5].

The experimental data can be analyzed through a model based on a 1:1 stoichiometry between the two ligands, even if the linkage functional analysis used is able to handle only overall events. In fact, structural studies would be necessary to dissect the overall negative heterotropic process into distinct events which could even be locally positive [23,24].

The equilibrium stability constants for the enzyme–sodium complex and for the ternary enzyme–inhibitor–sodium complex, namely λ_1 and λ_2, albeit not high, are comparable with the affinity values of ions for other proteins, such as chloride binding to human hemoglobin [20,25] or aluminum binding to chymotrypsin [22].

The negative heterotropic effect can be seen as an intracellular signaling system, at least in the thermodynamical framework defined by Wyman [20]. The maximum signaling effect is around the λ_1 value (3 mM) (as shown in Fig. 4), in the physiological range of intracellular sodium concentration, suggesting a physiological relevance for this mechanism. In fact, while the Cbz inhibitor is small in size in comparison to other effectors in solution, it is noteworthy that the proteasome recognition can be modulated even in the case of small reversible inhibitors by sodium ion.

There is direct and indirect evidence that the 20S proteasome exists in distinct functional conformations [8,26]. The existence of at least two distinct but interconverting conformations has been demonstrated for the 20S proteasome: the macromolecular architecture can be switched by allosteric effectors, including small peptides and bivalent metal ions [26]. The addition of the reversible inhibitor Cbz-LLL-CHO resulted mainly in a closed-barrel distribution, similar to those obtained upon addition of weak ligands and irreversible inhibitors. The effect of sodium ion as heterotropic ligand presented in this paper suggests that the simple two-state model could be an approximation of the complex modulation mechanism of the 20S proteasome functionality.

In conclusion, while the interplay between sodium and the reversible inhibitor Cbz-LLL-CHO binding to MPC is an excellent model for modulatory mechanisms in large macromolecules, the effect of sodium ion is relevant. In fact Na^+ could play a significant role in the MPC modulation 'in vivo', where its intracellular concentration is close to the one observed having the maximum heterotropic effect.

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