Mechanistic Studies on the Inactivation of the Proteasome by Lactacystin

A CENTRAL ROLE FOR δAsto-LACTACYSTIN β-LACTONE*

(Received for publication, January 11, 1996)

Lawrence R. Dick†, Amy A. Cruikshank, Louis Grenier, Francesco D. Melandri, Sandra L. Nunes, and Ross L. Stein
From ProScript, Inc., Cambridge, Massachusetts 02139

Lactacystin is a Streptomyces metabolite that inhibits cell cycle progression and induces differentiation in a murine neuroblastoma cell line. The cellular target of lactacystin is the 20 S proteasome, also known as the multicatalytic proteinase complex, an essential component of the ubiquitin-proteasome pathway for intracellular protein degradation. In aqueous solution at pH 8, lactacystin undergoes spontaneous hydrolysis to yield N-acetyl-L-cysteine and the inactive lactacystin analog, clasto-lactacystin dihydroxy acid. We have studied the mechanism of lactacystin hydrolysis under these conditions and found that it proceeds exclusively through the mechanism of lactacystin hydrolysis under these conditions and that the latter is the sole species that interacts with the proteasome.

In 1991, Omura and his colleagues (1) reported the discovery of lactacystin, a natural product that could induce differentiation in cultured neuronal cells. Lactacystin attracted the attention of chemists who sought and found routes to its total synthesis (2, 3) and biochemists who sought to understand its mechanism of action as an inhibitor of the 20 S proteasome (5). The 20 S proteasome is a large (700 kDa) multiprotein complex that constitutes the catalytic core of the 26 S proteasome, a ubiquitous intracellular protein-degrading machine of eukaryotic organisms (for reviews see Refs. 6–12). The latter endeavor culminated in a recent thesis (2, 3) and biochemists who sought to understand its mechanism of proteasome inactivation by lactacystin. In this report, we show that lactacystin hydrolyzes in aqueous solution through a mechanism that involves transient formation of the intermediate clasto-lactacystin β-lactone and that lactacystin, per se, is not a proteasome inhibitor. Rather, the intermediate β-lactone is the sole inhibitory species.

EXPERIMENTAL PROCEDURES

Synthesis—Lactacystin, clasto-lactacystin β-lactone, and clasto-lactacystin dihydroxy acid (DHA)1 were prepared by the method of Corey et al. (2, 3). The structure of the synthetic materials was confirmed by proton magnetic resonance spectrometry and fast atom bombardment mass spectrometry. The purity of the materials was assessed at >95% by HPLC.

Spectrophotometric Analysis of Lactacystin Hydrolysis—Disappearance of the lactacystin thioester as a function of time was monitored continuously by measuring the decrease in absorbance at 238 nm by HPLC. For measurements of N-acetyl-L-cysteine (NAC) dependence, the starting NAC concentration was varied. Lactacystin concentration was varied. The decrease in absorbance of the sample at 238 nm versus an appropriate buffer reference was monitored for 1 h. For measurements of N-acetyl-L-cysteine (NAC) dependence, the starting NAC concentration was varied. The decrease in absorbance at steady state (i.e. [NAC] >> [β-lactone] and [NAC][dt = 0]). Values for kobs at steady state were calculated from a linear regression of log A238 vs. time over the range from 40–60 min of incubation. NAC stock solutions (0.2 mM) were titrated with 1 eq of NaOH so that NAC addition would not perturb the pH of the assay buffer.

HPLC Analysis of Lactacystin Hydrolysis—0.5 mM lactacystin was incubated at 37 °C in 20 mM HEPES, 0.5 mM EDTA, pH 8. At various times, 100 μl aliquots of the sample were removed and acidified with 10 μl of 4% (v/v) TFA to stop the reaction. 100 μl of each sample was then subjected to reverse-phase HPLC on a C18 column (Vydac, 218TP54). The column was equilibrated with water containing 0.06% (v/v) TFA at a flow rate of 0.75 ml/min. The material was eluted with a linear gradient of methanol containing 0.05% TFA starting at 5 min after injection. The absorbance of the column eluate at 210 nm was monitored with an in-line detector. The UV chromatograms were digitized and integrated using Millenium (Waters) software. Quantitation of the chromatographic peaks was based on peak area relative to external standards run under the same conditions as the experimental samples.

Purification of 20 S Proteasome and Proteasome Activator PA28—20 S proteasome was purified by a modification of procedures by Hough et al. (13) and Ganoth et al. (14). PA28 activator was purified by a modification of procedures of Chu-Ping et al. (15). Rabbit reticulocytes were obtained from Green Hectares (Oregon, WI). The reticulocytes were washed three times by suspending them in ice-cold phosphate-buffered saline and centrifuging at 2000 × g for 15 min. After the final wash, the cells were lysed in 1.5 volumes of cold distilled water containing 1 mM EDTA. The mixture was passed through a Glasc-O Bio-Nebulizer at 250 p.s.i. to ensure complete cell lysis. The lysate was then centrifuged at 2000 × g for 1 h to remove debris. The supernatant was brought to 20 μM HEPES, pH 7.6, 1 mM DTT (Buffer A), filtered through a 0.2-μm filter, and applied to a DE52 anion exchange column equilibrated in Buffer A. The column was washed with 5 volumes of Buffer A, and absorbed proteins were eluted with 25 volumes of Buffer A containing 500 mM NaCl. The eluate (Fraction II) was brought to 38% saturation with ammonium sulfate and centrifuged at 10,000 × g for 20 min. The supernatant was brought to 85% saturation with ammonium sulfate and centrifuged 10,000 × g for 20 min. The resulting pellets were resuspended in a minimal volume of Buffer A and dialyzed versus 4 liters of Buffer A overnight. The dialysate (Fraction IIB), which

1 The abbreviations used are: DHA, clasto-lactacystin dihydroxy acid; HPLC, high performance liquid chromatography; NAC, N-acetyl-L-cysteine; TFA, trifluoroacetic acid; AMC, 7-amino-4-methylcoumarin; MES, 2-(N-morpholino)ethanesulfonic acid.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: ProScript, Inc. 38 Sidney St., Cambridge, MA 02139. Tel.: 617-374-1470; Fax: 617-374-1488.
contains both 20 S proteasome and PA28 activator, was applied to a Mono Q anion exchange column. Elution was performed by a 40-column volume gradient from 0–500 mM NaCl.

20 S Proteasome—Column fractions were assayed for SDS-stimulated Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity. The active fractions were pooled, diluted to 50 mM NaCl, and applied to a 3-ml Superdex 200 size exclusion column. Elution was performed with a 20-column volume gradient from 50–500 mM NaCl. Active fractions were pooled and concentrated using a 30,000 MWCO Centricon centrifugal concentrator. The concentrate was applied to a Superose 6 size exclusion chromatography column and eluted with Buffer A containing 100 mM NaCl. The active fractions that were judged to be pure by SDS-polyacrylamide gel electrophoresis analysis were pooled and stored at −80 °C for subsequent use.

PA28—Column fractions (see above) were assayed for their ability to stimulate the Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity of exogenous 20 S proteasome. The active fractions were pooled and diluted to 10 mM NaCl. This material was then further purified and concentrated on a 1-ml Resource Q anion exchange column with a 40-column volume gradient from 10–500 mM NaCl. Active fractions were pooled and concentrated using a 10,000 MWCO Centricon centrifugal concentrator. The concentrate was applied to a Superdex 200 size exclusion chromatography column and eluted with Buffer A containing 100 mM NaCl. The active fractions that were judged to be pure by SDS-polyacrylamide gel electrophoresis analysis were pooled and stored at −80 °C for subsequent use.

Lactacystin Inactivation of Proteasome Activity—2 ml of assay buffer (20 mM HEPES, 0.5 mM EDTA, pH 8.0) and Suc-Leu-Leu-Val-Tyr-AMC in methanol were added to a 3-ml fluorescent cuvette, and the cuvette was placed in the jacketed cell holder of a Hitachi F-2000 fluorescence spectrophotometer. The temperature was maintained at 37 °C by a circulating water bath. 0.34 μg of proteasome and 3.5 μg of PA28 were added, and the reaction progress was monitored by the increase in fluorescence at 440 nm (λex = 380 nm) that accompanies production of free AMC. The progress curves exhibited a lag phase lasting 1–2 min resulting from the slow formation of the 20 S-PA28 complex. After reaching a steady state of substrate hydrolysis, lactacystin was added to a final concentration of 1 μM, and the reaction was monitored for 1 h. The fluorescence (F) versus time (t) data were collected on a microcomputer using LAB CALC (Galactic) software. k_{inact} values were estimated by a nonlinear least-squares fit of the data to the first order equation:

$$F = A(1 - e^{-kt}) + C \quad \text{(Eq. 1)}$$

where C = F_{t = 0} and A = F_{t → ∞} − F_{t = 0}. Owing to the complex kinetics of lactacystin hydrolysis and its mechanism of inactivation of the proteasome (see below), the kinetics of proteasome inactivation are also complex, and this fact is reflected in the curve fitting. There was a small systematic deviation of the measured progress curves from the "best-fit" curves generated by fitting the data to Equation 1. Nevertheless, we judged that estimates of k_{inact} obtained using this simple kinetic model were sufficiently accurate for the present work.

RESULTS AND DISCUSSION

Hydrolysis of Lactacystin—In the course of our studies on the mechanism of inactivation of the 20 S proteasome by the natural product lactacystin, we examined the stability of lactacystin in aqueous buffer and found that it is hydrolyzed to the dihydroxy acid with complex kinetics. Progress curves for the disappearance of the thioester are biphasic and characterized by an initial burst of thioester disappearance followed by a slower first order decay (Fig. 1). This result is consistent with the mechanism of lactacystin hydrolysis shown in Scheme I. According to this mechanism, lactacystin is hydrolyzed to the dihydroxy acid (DHA) and N-acetyl-L-cysteine (NAC) through the intermediary of clasto-lactacystin β-lactone.

This mechanism predicts: 1) formation of the β-lactone as an observable intermediate and 2) that the addition of NAC to solutions of lactacystin will slow the latter’s hydrolysis. The first prediction was realized with HPLC experiments as shown in Fig. 2. Panel A shows a chromatogram of the lactacystin hydrolysis products after 10 min in the assay buffer. At this time, approximately 20% of the starting material has disappeared and there is a corresponding accumulation of NAC. We see in B that, as NAC accumulates, the rate of lactacystin disappearance slows down, so that the observed half-life is 60 min (i.e. the intersection of the progress curves for lactacystin and NAC). Over the first 30-min interval, there is a time-dependent formation and decay of β-lactone (C). At 10 min, the concentration of β-lactone reaches a maximum that corresponds to 10% of the lactacystin concentration at that time. Over the interval from 60–120 min, the reactionapproximates a steady state (d[β-lactone]/dt ≈ d[NAC]/dt ≈ d[β-lactone]/dt ≈ 0), and here the concentration of β-lactone corresponds to approximately 2% of the lactacystin concentration. The second prediction is based on the rate law of Equation 2 which describes the NAC dependence of the steady-state rate constant for hydrolysis of lactacystin by the mechanism of Scheme I. According to Equation 2, k_{obs} should approach 0 at high concentrations of NAC. This is born out experimentally in Fig. 3 where we see a NAC-dependent decrease in k_{obs}. That k_{obs} does indeed go to 0 at high concentrations of NAC also indicates that direct attack of water on lactacystin to produce the dihydroxy acid contributes insignificantly to the overall hydrolysis of lactacystin.

Inactivation of the 20 S Proteasome by Lactacystin—These experiments raised the possibility that at least a portion of the inactivation of the proteasome by lactacystin could be accounted for by the β-lactone that accumulates spontaneously when lactacystin is in the assay buffer. To test this, we deter-
Mechanism of Proteasome Inactivation by Lactacystin

Fig. 2. HPLC analysis of lactacystin hydrolysis and demonstration of formation of the β-lactone as an intermediate. A, reverse-phase chromatogram of lactacystin and its hydrolysis products after 10 min of incubation at pH 8, 37°C. The reaction conditions and sample analysis are described under “Experimental Procedures.” B, progress curves for the hydrolysis of lactacystin and accumulation of N-acetyl-l-cysteine (NAC), dihydroxy add (DHA), and β-lactone. The data are derived from chromatographic analysis as illustrated in A. C, progress curve for the transient accumulation of β-lactone during lactacystin hydrolysis. The data shown are the same as in B with an expanded scale on the ordinate.

Fig. 3. The NAC dependence of the rate constants for lactacystin hydrolysis (kobs, open circles) and proteasome inactivation by lactacystin (k_{inact}/[I], closed circles). Lactacystin hydrolysis was assayed spectrophotometrically as described under “Experimental Procedures.” The hydrolysis of the β-lactone to the dihydroxy acid was assayed spectrophotometrically (data not shown), and we obtained an estimate for k_b (Equation 2) of 8.99 ± 0.12 × 10^{-4} s^{-1}. Using this value, the k_{obs} data of this figure were fit to Equation 1 and we obtained k_b = 2.61 ± 0.08 × 10^{-4} s^{-1} and k_p = 8.89 ± 0.53 × 10^{-4} s^{-1}. Inactivation of the chymotrypsin-like activity of the 20 S proteasome-PA28 activator complex was assayed fluorometrically as described under “Experimental Procedures.”

Table I

Conditions that stabilize lactacystin decrease its efficacy as a proteasome inactivator

| pH, temperature | k_b/K_m | k_{obs} | k_{inact}/[I] |
|-----------------|---------|---------|---------------|
| 8.0, 37°C       | 132,000 | 2       | 20,000        |
| 6.3, 25°C       | 140,000 | 0.02    | 8,000         |

Mechanism of Proteasome Inactivation by Lactacystin

Hydrolysis of the substrate Suc-Leu-Leu-Val-Tyr-AMC by the 20 S proteasome-PA28 activator complex was assayed at 37°C in 20 mM HEPES, 0.5 mM EDTA, pH 8.0, or at 25°C in 20 mM MES, pH 6.3. k_b/K_m values were calculated from the slopes of the steady-state velocity versus [S] curves in the limit of low substrate concentration (S = 5 μM). Values for the steady state rate of lactacystin hydrolysis, k_{obs}, were measured spectrophotometrically as described under “Experimental Procedures.” The k_{inact} values were measured as described under “Experimental Procedures” using β-lactone = 0.1 μM, [lactacystin] = 1 μM, and [S] = 5 μM.

To further test this mechanism, we conducted an experiment in which we studied the inactivation of the proteasome by lactacystin at pH 6.3 instead of the usual pH 8.0. At pH 6.3, lactacystin is stable (i.e. k_{obs} < 0.02 s^{-1}) and does not undergo cyclization to the β-lactone (Table I). When tested as a proteasome inactivator at this pH, we observed that lactacystin is inactive while its β-lactone is still a potent inactivator (see Table I). This result is consistent with the β-lactone being the sole species that interacts with the proteasome.

Prior to the discovery that the proteasome is the molecular target for lactacystin, Fenteany et al. (4) studied the biological activities of several lactacystin analogs and concluded that the hydroxysobutyl group and substituents of the γ-lactam ring were important for activity but that the NAC moiety was not required. Also, they observed that the analogs that retain activity all have the potential of forming a β-lactone, and they hypothesized that this species might act as an intermediate. Our data support their hypothesis and suggest that the β-lactone is the sole active species. Furthermore, we predict that lactacystin analogs that differ in the NAC moiety will have intrinsic potencies in direct proportion to their ability to form the β-lactone. Subsequently, when these investigators found that the proteasome was the molecular target (5), they observed that lactacystin and the β-lactone inhibited 3 different activities of this multiproteinase complex. The different rates of inactivation for the different activities suggested that the mechanism involved modification of ≥ 3 different sites on the enzyme. Nevertheless, the potencies of the two compounds for inactivating the three peptidase activities were in roughly the same proportion. Thus, it appeared that although the intrinsic potency of lactacystin was approximately 1/10 that of the β-lactone, the two did not discriminate for binding to different sites on the proteasome. We have had occasion to examine two novel lactacystin analogs that contain substitutions for the NAC moiety and have observed the same pattern for inactivation of the different proteasome peptidase activities. In light of the mechanism elucidated here, this result is predicted.

References

1. Omura, S., Matsuzaki, K., Fujimoto, T., Kosuge, K., Furuya, T., Fujita, S., and Nakagawa, A. (1991). J. Antibiot. 44, 113–118

2. L. R. Dick, A. A. Crulshank, L. Grenier, F. D. Melandri, S. L. Nunes, and R. L. Stein, unpublished data.
2. Corey, E. J., and Reichard, G. A. (1992) J. Am. Chem. Soc. 114, 10677–10678
3. Corey, E. J., Reichard, G. A., and Kania, R. (1993) Tetrahedron Lett. 34, 6977–6980
4. Fenteany, G., Standaert, R. F., Reichard, G. A., Corey, E. J., and Schreiber, S. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3358–3362
5. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) Science 268, 726–731
6. Goldberg, A. L., Stein, R. L., and Adams, J. (1995) Chem. Biol. 2, 503–508
7. Rubin, D., and Finley, D. M. (1995) Curr. Biol. 3, 854–858
8. Tanaka, K. (1995) Mol. Biol. Rep. 21, 21–26
9. Ciechanover, A. (1994) Cell 79, 13–21
10. Peters, J. M. (1994) Trends Biochem. Sci. 19, 377–382
11. Wilk, S. (1993) Enzyme & Protein 47, 187–188
12. Rivett, A. J. (1993) Biochem. J. 291, 1–10
13. Hough, R., Pratt, G., and Rechsteiner, M. (1987) J. Biol. Chem. 262, 8303–8313
14. Ganoth, D., Leshinski, E., Eytan, E., and Hershko, A. (1988) J. Biol. Chem. 263, 12412–12419
15. Chu-Ping, M., Slaughter, C. A., and DeMartino, G. N. (1992) J. Biol. Chem. 267, 10515–10523
Mechanistic Studies on the Inactivation of the Proteasome by Lactacystin: A CENTRAL ROLE FOR clasto-LACTACYSTIN β-LACTONE
Lawrence R. Dick, Amy A. Cruikshank, Louis Grenier, Francesco D. Melandri, Sandra L. Nunes and Ross L. Stein

J. Biol. Chem. 1996, 271:7273-7276.
doi: 10.1074/jbc.271.13.7273

Access the most updated version of this article at http://www.jbc.org/content/271/13/7273

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/271/13/7273.full.html#ref-list-1