Evidence for the Existence of both Proteasomes and a Novel High Molecular Weight Peptidase in Entamoeba histolytica*

(Received for publication, September 5, 1995, and in revised form, December 27, 1995)

Henning Scholzet‡, Silja Frey⁠a, Zdenka Cejkai, and Tilly Bakker-Grunwald‡

From the Department of Biology/Chemistry, ‡Biochemistry, and ¶Microbiology, University of Osnabrück, Barbarstraße 11, D-49069 Osnabrück and ¶Max-Planck-Institut für Biochemie, D-82152 Martinsried, Germany

To screen for high molecular weight proteases in Entamoeba histolytica, we subjected a soluble amebal extract to density gradient centrifugation and tested the fractions for activity against the chymotryptic peptide substrate, Suc-leucyl-leucyl-tyrosyl-4-methylcoumaryl-7-amide. Two peaks of activity, of approximately 11 and 20 S, were clearly separated. Based on SDS-electrophoretic pattern and immunoblot analysis, we ascribe the 20 S activity to proteasomes. The 11 S protein was purified from amebal homogenates by a series of chromatographic steps. As determined by molecular sieve chromatography and nondenaturing gel electrophoresis, the native complex had an apparent Mr of 385,000 ± 10%. On SDS gels, the purified enzyme exhibited a single band of Mr 62,000 that yielded a single N-terminal sequence, indicating that the preparation was homogeneous and that the native complex consisted of six very similar or identical subunits. The enzyme preferred peptides with aromatic residues at the P1 position and had low but distinct activity toward azocasein. We conclude that the 11 S enzyme is a novel high molecular weight protease that is distinct from proteasomes.

Entamoeba histolytica is a parasitic protozoan that resides in the human gut. It frequently occurs in developing countries, causes amebic dysentery, and may lead to the formation of tumor-like abscesses in liver and spleen (1). As to its cell biology, E. histolytica is a low eukaryote that lacks mitochondria and a well-defined endoplasmatic reticulum/Golgi apparatus. Morphologically conspicuous is the enormous amount of vacuoles in the amebae. These occupy about 40% of the total cell volume and are functionally equivalent to both the lysosomes and the cytotoxic vesicles of higher eukaryotic cells (2, 3). Besides its function in cellular metabolism, protein degradation is essential for a range of regulatory processes and for antigen presentation and appears to be due both to the replacement of certain proteasomal β-subunits by others and to an enhanced response to activator protein (6, 13, 14). The three-dimensional structure of an archaeal 20 S proteasome, which should be similar to its eukaryotic counterpart, has recently been solved (7). It consists of four stacked rings with seven subunits each: two rings of 22.3-kDa α-subunits sandwiched between two rings of 25.8-kDa β-subunits. Remarkably, the bacterial ClpP possesses a similar 7-fold symmetry (15). The active site residue of the proteasome has been identified as a β-chain threonine both by site-directed mutagenesis (16) and by N-terminal modification (17).

In E. histolytica, a range of cysteine proteinases of the papain type has been extensively characterized both on the genomic and on the protein level (18, 19). These enzymes are localized in the lysosome-like vacuoles mentioned above. By contrast, nothing is known about cytoplasmic protein degradation in this organism or about the proteases involved. In this study, we present evidence indicating that E. histolytica contains both proteasomes and a novel, unrelated high molecular weight protease.

**EXPERIMENTAL PROCEDURES**

Cell Culture—E. histolytica, strain HM1-IMSS, was cultured at 37 °C in TYI-S-33 medium (20) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. After 3 days, cells were chilled on ice and centrifuged at 300 × g for 10 min at 4 °C. Cells were resuspended in phosphate-buffered saline containing the cysteine proteinase inhibitor E-64 (final concentration, 0.1 µM) and disrupted by sonication. The homogenate was centrifuged at 100,000 × g for 1 h at 4 °C, and the supernatant (soluble extract) was used for further experiments.

Peptidase and Protease Assays—Routinely, peptidolytic activity was

---

*This study was supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. 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: Universität Osnabrück, Fachbereich Biologie/Chemie, Biochemie, Postfach 4469, D-49069 Osnabrück, Germany. Tel.: 49-541-9692888; Fax: 49-541-9692870; E-mail: scholze@cipf5.biologie.Uni-Osnabrueck.de.

1 The abbreviations used are: MCP, multicatalytic protease; CAPS, 3-(cyclohexylamino)propanesulfonic acid; E-64, epoxysuccinyl-L-leucyl-lyl-tyrosyl-4-methylcoumaryl-7-amide; Suc-LLVY-MCA, succinyl-leucyl-leucyl-lyl-tyrosyl-4-methylcoumaryl-7-amide; N-But-Leu-LTR-MCA, N-butyloxycarbonyl-leucyl-erysyl-threonyl-arginyln-4-methylcoumaryl-7-amide; PAGE, polyacrylamide gel electrophoresis.

2 The human gut. It frequently occurs in developing countries, causes amebic dysentery, and may lead to the formation of tumor-like abscesses in liver and spleen (1). As to its cell biology, E. histolytica is a low eukaryote that lacks mitochondria and a well-defined endoplasmatic reticulum/Golgi apparatus. Morphologically conspicuous is the enormous amount of vacuoles in the amebae. These occupy about 40% of the total cell volume and are functionally equivalent to both the lysosomes and the cytotoxic vesicles of higher eukaryotic cells (2, 3). Besides its function in cellular metabolism, protein degradation is essential for a range of regulatory processes and for antigen presentation and appears to be due both to the replacement of certain proteasomal β-subunits by others and to an enhanced response to activator protein (6, 13, 14). The three-dimensional structure of an archaeal 20 S proteasome, which should be similar to its eukaryotic counterpart, has recently been solved (7). It consists of four stacked rings with seven subunits each: two rings of 22.3-kDa α-subunits sandwiched between two rings of 25.8-kDa β-subunits. Remarkably, the bacterial ClpP possesses a similar 7-fold symmetry (15). The active site residue of the proteasome has been identified as a β-chain threonine both by site-directed mutagenesis (16) and by N-terminal modification (17).

In E. histolytica, a range of cysteine proteinases of the papain type has been extensively characterized both on the genomic and on the protein level (18, 19). These enzymes are localized in the lysosome-like vacuoles mentioned above. By contrast, nothing is known about cytoplasmic protein degradation in this organism or about the proteases involved. In this study, we present evidence indicating that E. histolytica contains both proteasomes and a novel, unrelated high molecular weight protease.

**EXPERIMENTAL PROCEDURES**

Cell Culture—E. histolytica, strain HM1-IMSS, was cultured at 37 °C in TYI-S-33 medium (20) supplemented with 100 units/ml penicillin and 100 µg/ml streptomycin. After 3 days, cells were chilled on ice and centrifuged at 300 × g for 10 min at 4 °C. Cells were resuspended in phosphate-buffered saline containing the cysteine proteinase inhibitor E-64 (final concentration, 0.1 µM) and disrupted by sonication. The homogenate was centrifuged at 100,000 × g for 1 h at 4 °C, and the supernatant (soluble extract) was used for further experiments.

Peptidase and Protease Assays—Routinely, peptidolytic activity was
measured with Suc-LLVY-MCA as a substrate (21). In a fluorescence cuvette, 10 µl of substrate (5 mM dissolved in dimethyl sulfoxide), up to 40 µl of 1% (w/v) SDS, and 5–20 µl of sample were added to 1 ml of 10 mM Tris-HCl, pH 7.8, at 25 °C. The rate of release of 7-amino-4-methylcoumarin was determined by following the increase of fluorescence at 380 nm excitation and 460 nm emission. The cleavage of N-t-Boc-LSTR-MCA was determined under identical conditions. With benzoylcarbonyl-leucyl-leucyl-glutamyl-2-naphthylamide as substrate (end concentration, 0.1 mM), wavelengths were adjusted to 333 nm excitation and 410 nm emission, respectively (11). Proteolytic activity was determined at 37 °C with azocasein (final concentration, 10 mg/ml) as a substrate (22).

Density Gradient Centrifugation—Sucrose density gradient centrifugation was performed in 37-ml polyallomer tubes using a swing rotor (SW 28, Beckman). Soluble extract containing E-64 was loaded onto a linear gradient of 15–40% sucrose and centrifuged at 27,000 rpm for 42 h at 4 °C. Fractions of 1 ml were collected and analyzed for enzymatic activity and protein content.

Purification of the 11 S Peptidase—Soluble extract containing E-64 was applied onto a column of DEAE-cellulose (Whatman; bed volume, 30 ml) equilibrated with 10 mM Tris-HCl, pH 7.8, and eluted with a linear gradient of 0–40% sucrose and centrifuged at 27,000 rpm for 42 h at 4 °C. Fractions of 1 ml were collected and analyzed for enzymatic activity and protein content.

Purification of the 11 S Peptidase—Soluble extract containing E-64 was applied onto a column of DEAE-cellulose (Whatman; bed volume, 30 ml) equilibrated with 10 mM Tris-HCl, pH 7.8, and eluted with a linear gradient of 0–40% sucrose and centrifuged at 27,000 rpm for 42 h at 4 °C. Fractions of 1 ml were collected and analyzed for enzymatic activity and protein content.

Purification of the 11 S Peptidase—Soluble extract containing E-64 was applied onto a column of DEAE-cellulose (Whatman; bed volume, 30 ml) equilibrated with 10 mM Tris-HCl, pH 7.8, and eluted with a linear gradient of 0–40% sucrose and centrifuged at 27,000 rpm for 42 h at 4 °C. Fractions of 1 ml were collected and analyzed for enzymatic activity and protein content.

Analytical Molecular Sieve Chromatography—The apparent molecular weight of the native 11 S enzyme was determined by molecular sieve chromatography over Sephacryl S-300 after calibration of the column with the high molecular weight standards thyroglobulin (Mr 669,000), apoferritin (Mr 443,000), catalase (Mr 240,000), and aldolase (Mr 158,000).

Electron Microscopy—Drops of the purified 11 S protease were applied to carbon film-covered glow-discharged grids and negatively stained with aqueous 1% uranyl acetate (23). Electron micrographs were recorded with a Philips EM 420.

Gel Electrophoresis—SDS-PAGE was carried out in 15% polyacrylamide gels according to Ref. 24. Native gels (7.5% polyacrylamide) were run at 4 °C with a Tris/glycine system, pH 8.9, according to Ref. 25. The Suc-LLVY-MCA hydrolyzing activity was detected by overlaying the gel strip with 2 ml of 50 mM Tris-HCl buffer, pH 7.8, containing 40 µl of a 5 mM substrate solution, and visualizing the fluorescence of the released 7-amino-4-methylcoumarin on a UV fluorescent table equipped with a 354-nm lamp.

N-terminal Sequencing—The purified 11 S enzyme was subjected to SDS-PAGE and blotted on polyvinylidene difluoride membrane (Bio-Rad). The protein band was cut out and N-terminally sequenced on an Applied Biosystems 473A protein sequencer.

Immunoblotting—Gels were blotted on nitrocellulose sheets in 0.1 M CAPS-HCl, pH 11.5, and the immunoreaction was performed according to a protocol of Hoefer Scientific Instruments. Antibodies against proteasomes of Thermoplasma acidophilum, Dictyostelium discoideum, and rat proteasome were kindly provided by A. Grziwa and M. Nesper.
RESULTS

Identification of Two High Molecular Weight Proteases—To test for the presence of non-thiol proteases in *E. histolytica*, a soluble amebic extract (100,000 × g supernatant) containing the cysteine proteinase inhibitor E-64 was incubated with the fluorogenic chymotrypsin substrate, Suc-LLVY-MCA. A release of fluorophore could readily be observed. As shown in Fig. 2, this peptidolytic activity was stimulated by over 60% by SDS, with an optimum at an SDS concentration of about 0.4 mg/ml. Beyond the optimum, the curve exhibited a flat declining plateau and then decreased steeply at concentrations exceeding 0.8 mg/ml SDS.

Stimulation of peptidolytic activity by SDS has typically been found for proteasomes (27). We therefore set out to investigate whether these complexes were present in *E. histolytica*. To this end, a soluble amebic extract was subjected to sucrose density gradient centrifugation, and the fractions were tested for chymotryptic activity. As shown in Fig. 3 (closed circles), two Suc-LLVY-hydrolyzing peaks with sedimentation velocities of approximately 11 and 20 S were clearly separated. Only the second peak exhibited significant activity toward the tryptic substrate, Boc-LSTR-MCA (Fig. 3, open squares). The SDS-electrophoretic pattern of the 20 S peak (Fig. 3, inset A) was restricted to a series of bands between 25 and 30 kDa and roughly corresponded to that found for proteasomes from a number of organisms (21), whereas the 11 S fraction exhibited bands over a much wider range (data not shown) and obviously contained a crude mixture of proteins. In immunoblot experiments, we tested the reactions of a cell homogenate and of the 11 and 20 S peaks with an antibody (MCP231) against a sequence motif common to α-chains of proteasomes (26). As shown in Fig. 3, inset B, the 20 S peak (lane 3) exhibited a series of cross-reacting bands around 30 kDa. By contrast, neither the 11 S fraction (lane 2) nor the purified 11 S protease (lane 1; see below) cross-reacted with the antibody. These findings indicated that *E. histolytica* contained both 20 S proteasomes and an 11 S peptidase.

Purification and Subunit Composition of the 11 S Peptidase—At this stage, it seemed possible that the 11 S enzyme was a catalytically active proteasomal fragment that lacked α-chains; alternatively, it could be a distinct protease that had nothing to do with proteasomes. To distinguish between these possibilities, we set out to purify and characterize the 11 S enzyme. Purification was achieved by two anion exchange and two molecular sieve chromatography steps (Fig. 1; Table I). Overall, specific activity toward the chymotrypsin peptide substrate went up by a factor 320, and 730 mg of protein (from approximately 5 × 10⁸ amebae) yielded about 100 mg of peptidase (Table I). To test for the purity of the isolated enzyme, an aliquot was subjected to SDS-PAGE. Under these conditions, the protein appeared as a single band with an apparent Mr of 62,000 (Fig. 4A, right) and yielded a single N-terminal sequence; this sequence (DNYVNVKQNL) did not exhibit sig-
significant similarity to any other N-terminal sequence in the EMBL data base. Under non-denaturing conditions, the enzyme migrated as a single, peptidolytically active complex between the marker proteins catalase (Mr, 240,000) and aperfor-ritin (Mr, 443,000; Fig. 4A, left). In agreement both with this observation and with its sedimentation constant, we determined its apparent Mr of about 180,000 (Fig. 4A, middle). From these data, we infer that the native protease is probably composed of six identical or very similar subunits of 60–65 kDa. Under weakly denaturing conditions, three of these subunits remained associated as a metastable, active trimer half the size of the native complex. A cartoon illustrating these inferred relationships is given in Fig. 4B. An electron micrograph of the purified 11 S protease revealed compact particles with an average diameter of about 9 nm (Fig. 5).

Substrate Specificity and Inhibitor Profile of the 11 S Peptidase—Tables I, II, III, and IV summarize some kinetic properties of the 11 S enzyme. Table II lists its relative activity toward a range of fluorogenic substrates. It can be seen that the enzyme had relatively high chymotryptic activity (i.e. it preferred aromatic residues at the P$_1$ position), much lower trypsin-like activity (Arg at the P$_1$ position; see also Fig. 1), and did not accept any of the other peptides offered as a substrate. In particular, whereas the rough extract had considerable peptidylglutamyl peptidase activity (up to 20% of the chymotryptic activity altogether. Table III presents the kinetic parameters of Suc-LLVY-MCA hydrolyzing activity; data not shown), the purified enzyme lacked this activity; data not shown), the purified enzyme lacked this activity.
than the other inhibitors of serine and cysteine proteases that we tested. Whereas EDTA and ATP had no or little effect, enzyme activity increased by about 40% in the presence of MgCl₂ or CaCl₂ (both at 10 mM; Table V). Under all conditions tested, activity was increased up to 2-fold by the addition of 0.4 mg/ml SDS (data not shown).

**DISCUSSION**

In this study, we have identified two high molecular weight (11 and 20 S) proteases in *E. histolytica*. The heavier enzyme appears proteasome-like both from its subunit composition and from its cross-reactivity with an antibody against proteasomal α-subunits. By contrast, the lighter enzyme exhibited several novel characteristics. Clearly, its activity cannot be ascribed to a calpain inhibitor and stimulated by Ca²⁺-tolysain and amebapain, which favor arginine in P₁ and P₂ (28).

### Table V

| Compound                  | Concentration | Activity |
|---------------------------|---------------|----------|
| None                      |               | 100      |
| 3,4-Dichloroisocoumarin   | 1             | 40       |
| Phenylmethylsulfonyl fluoride | 1           | 62       |
| Leupeptin                 | 1             | 82       |
| Chymostatin               | 1             | 50       |
| Antipain                  | 1             | 44       |
| N-Ethylmaleimide          | 5             | 38       |
| p-Chloromercuribenzoate   | 1             | 27       |
| Ac-LLN-L-al (calpain inhibitor I) | 0.1     | 0        |
| EDTA                      | 5             | 100      |
| MgCl₂                     | 10            | 137      |
| CaCl₂                     | 10            | 144      |
| ATP                       | 1             | 118      |
| MgCl₂ + ATP               | 1 + 1         | 125      |

Effect of various compounds on the activity of the 11 S protease toward Suc-LLVY-MCA

Acknowledgments—We thank Drs. Hendli, Dahlmann, Grziwa, and Nesper for kind gifts of anti-proteasome antibodies, Dr. Roland Schmid for N-terminal sequencing, and Mario Pietersma for enthusiastic contribution to part of the experiments.

**REFERENCES**

1. Walsh, J. A. (1986) Rev. Infekt. Dis. *8*, 228–238
2. Löhden, U., and Bakker-Grunwald, T. (1989) Anal. Biochem. *182*, 77–83
3. Bakker-Grunwald, T. (1991) in Biochemical Protozoology (Coombs, G., and North, M., eds) pp. 367–376, Taylor & Francis, London
4. Gottesman, S., and Mauriz, M. R. (1992) Microbiol. Rev. *56*, 592–621
5. Dice, J. F. (1990) Trends Biochem. Sci. *15*, 305–309
6. Goldberg, A. L., and Rock, K. L. (1992) Nature *357*, 375–379
7. Löwe, J., Stock, D., J. B., Zwickl, P., Baumeister, W., and Huber, R. (1995) *Science* *268*, 533–539
8. Lupas, A., Zwickl, P., and Baumeister, W. (1994) Trends Biochem. Sci. *19*, 533–534
9. Ciechanover, A. (1994) *Cell* *79*, 13–21
10. McGuire, M. J., and DeMartino, G. N. (1986) Biochim. Biophys. Acta *873*, 279–289
11. Chu-Ping, M., Slaughter, C. A., and DeMartino, G. N. (1992) Biochim. Biophys. Acta *1119*, 303–311
12. Tanaka, K., Ii, K., Ichihara, A., Waxman, L., and Goldberg, A. L. (1986) *J. Biol. Chem.* *261*, 15197–15203
13. Ustrell, V., Pratt, G., and Rechtein, M. (1995) Proc. Natl. Acad. Sci. U. S. A. *92*, 584–588
14. Fresnzel, S., Psolsh-Hurt, B., Seeling, A., and Kloetzel, P. M. (1994) *J. Biol. Chem.* *269*, 975–981
15. Kessel, M., Mauriz, M. R., Kim, B., Kocis, E., Trus, B. L., Singh, S. K., and Steves, A. C. (1995) *J. Biol. Chem.* *250*, 587–594
16. Seemüller, E., Lupas, A., Stock, D., Löwe, J., Huber, R., and Baumeister, W. (1995) *Science* *268*, 579–582
17. Fentey, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) *Science* *268*, 726–731
18. Scholze, H. (1991) in Biochemical Protozoology (Coombs, G., and North, M., eds) pp. 251–256, Taylor & Francis, London
19. McKernon, J. H., Gouvier, J., Sikes, A., Reed, S., and Keene, W. E. (1991) in Biochemical Protozoology (Coombs, G., and North, M., eds) pp. 245–250, Taylor & Francis, London
20. Dondel, L. S. (1988) in Amebiasis: Human Infection by Entamoboda histolytica (Radin, J. J., ed) pp. 25–40, dahn Wiley & Sons, Inc., New York
21. Tanaka, K., Yoshimura, T., Kumatari, A., Ichihara, A., Ikai, A., Nishigai, M., Kameyama, K., and Takagi, T. (1988) *J. Biol. Chem.* *263*, 16208–16217
22. Barrett, A. J., and Kirschke, H. (1981) Methods Enzymol. *80*, 533–561
23. Peters, J.-M., Cecka, Z., Harris, J. R., Kleinschmidt, J., and Baumeister, W. (1993) in *Methods Enzymol.* *234*, 932–937
24. Douglas, M., Finkelstein, D., and Butzow, R. A. (1979) Methods Enzymol. *56*, 734–749
25. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.* *121*, 404–427
26. Hendle, K. B., Kristensen, P., and Uerkvitz, W. (1995) *Biochem. J.* *305*, 245–252
27. Tanaka, K., Yoshimura, T., and Ichihara, A. (1989) *J. Biochem. (Tokyo)* *106*, 495–500
28. Scholze, H., and Tannich, E. (1994) Methods Enzymol. *244*, 512–523
29. Croalt, D. E., and DeMartino, G. N. (1991) *Physiol. Rev.* *71*, 813–847
30. Hua, S., Li, X., Coffino, P., and Wang, C. C. (1995) *J. Biol. Chem.* *270*, 10264–10271
31. Sogin, M. L. (1989) *Am. Zool.* *29*, 487–499
Evidence for the Existence of both Proteasomes and a Novel High Molecular Weight Peptidase in *Entamoeba histolytica*

Henning Scholze, Silja Frey, Zdenka Cejka and Tilly Bakker-Grunwald

*J. Biol. Chem.* 1996, 271:6212-6216.
doi: 10.1074/jbc.271.11.6212

Access the most updated version of this article at [http://www.jbc.org/content/271/11/6212](http://www.jbc.org/content/271/11/6212)

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

[Click here](http://www.jbc.org/content/271/11/6212.full.html#ref-list-1) to choose from all of JBC's e-mail alerts

This article cites 27 references, 9 of which can be accessed free at [http://www.jbc.org/content/271/11/6212.full.html#ref-list-1](http://www.jbc.org/content/271/11/6212.full.html#ref-list-1)