Rapid Communication J. Biochem. 127, 941–943 (2000)

New Crystal Forms and Low Resolution Structure Analysis of 20S Proteasomes from Bovine Liver[1]

Yoshikazu Tomisugi,* Masaki Unno,† Tsunehiro Mizushima,† Yukio Morimoto,*,[2] Nobuyuki Tanahashi,† Keiji Tanaka,† Tomitake Tsukihara,‡ and Noritake Yasuoka*

Faculty of Science, Himeji Institute of Technology, 3-2-1, Kohto, Kamigori, Hyogo 678-1297; †The Tokyo Metropolitan Institute of Medical Science, 3-18-22, Hon-Komagome, Bunkyo, Tokyo 113; and ‡Institute for Protein Research, Osaka University, 3-2, Yamadaoka, Suita, Osaka 565

Received March 9, 2000; accepted April 11, 2000

20S proteasomes from higher eukaryotes have immunological functions rather than those from archibacteria or yeast. To clarify the mechanism of the sorting and production of antigen-presenting peptides, it is important and worthwhile to determine the structure of mammalian proteasomes using a third generation synchrotron radiation source. Here we report new crystal forms of 20S proteasomes from bovine liver and preliminary structure analysis of them. The crystals belong to the same space group but have different cell dimensions. One crystal (form I) belongs to space group $P2_12_12_1$ with unit cell dimensions of a = 124.8, b = 197.4, c = 323.8 Å, and diffracts to 3.0 Å resolution. The other crystal (form II) belongs to the same space group with a = 115.1, b = 205.6, c = 316.0 Å, and diffracts to 4.0 Å resolution. The diffraction data for the form I crystal provided an interpretable electron density map for presenting the structural differences from yeast proteasomes.

Key words: crystal, immunoproteasomes, synchrotron radiation, X-ray analysis.

Proteasomes (or multicatalytic protease complexes) are widely distributed in eukaryotes, ranging from man to yeasts (*1*, *2*). They are involved not only in the selective destruction of short-lived regulatory proteins but also in the removal of abnormal, misfolded or improperly assembled proteins generated in cells (*3*, *4*). There are growing lines of evidence of the importance of proteolysis mediated by proteasomes and their partner, ubiquitin, which is responsible for many biological processes, including the cell cycle, apoptosis, signal transduction, metabolic regulation and the stress response (*3*, *5*). In addition, in higher eukaryotes, proteasomes are known to act as antigen-processing enzymes responsible for the generation of peptide ligands presented by major histocompatibility complex (MHC) class I molecules (*6*).

Proteasomes have molecular masses of approximately 750 kDa and sedimentation coefficients of approximately 20S (*3*). They are barrel-like particles formed through the axial stacking of four rings comprising two outer α-rings and two inner β-rings, associated in the order of αββα (*7*). The catalytic β-type subunits are located in a chamber formed by the centers of the abutting β rings and the α subunits form a physical barrier that prevents substrates reaching the active sites (see below). The outer α-rings are almost completely closed, indicating that 20S proteasomes exist as latent forms in cells.

The cellular functions of proteasomes are achieved through the formation of two kinds of active complexes with proteasome activators (PA), which are likely to be involved in opening of the α-ring gate for the entry of a protein substrate (*8–10*). One is the 26S proteasome, a complex with a PA700 activator with a molecular mass of 700 kDa, which degrades ubiquitin-tagged proteins in the presence of ATP. The other is a complex with a PA28 activator that participates in a cellular immune response, because PA28 is greatly induced by γ-interferon, which is known as a major immunomodulatory cytokine. This cytokine is also known to produce "immunoproteasomes" that play a key role in the generation of antigenic peptides in the cytoplasm of cells (*6*).

In 1995, we reported that bovine 20S proteasomes have a highly ordered structure and a relative low temperature factor in the crystal, which indicates the possibility of high-resolution X-ray analysis (*11*). A little later, Lowe *et al.* (*12*) reported 20S proteasomes from the archaebacterium *Thermoplasma acidophilum* at 3.4 Å resolution, and then Groll *et al.* (*13*) presented ones from yeast at 2.4 Å resolution. These reports generally state that eukaryotic proteasomes are composed of two copies of a hetero-heptamer of α-type subunits and one of β-type subunits, $(\alpha_{1\text{-}7}\beta_{1\text{-}7})_2$, whereas prokaryotic enzymes are homo-heptamers of identical α- and β-type subunits, $(\alpha_7\beta_7)_2$, and that they have three major cavities in the complex body (*7*). The active sites of 20S proteasomes are scattered in the wall of the central cavity made of 14 β-subunits. Two cavities at the end of the complex body seem likely to be involved in the selection and/or control of various proteins to be degraded.

To date, there is no information on the tertiary structure

[1] This research was partly supported by Grants-in-Aid for Scientific Research (Nos. 10680640, 09267233, and 11169234 to Y.M.) from Monbusho and The Inamori Foundation (1998 to Y.M.), for which the authors are very grateful.

[2] To whom correspondence should be addressed. Tel: +81-791-58-0178, Fax: +81-791-58-0177, E-mail: morimoto@sci.himeji-tech.ac.jp

Abbreviations: MCA, 4-methylcoumaryl-7-amide; Suc, succinyl.

of mammalian proteasomes, and thus we still intend to study bovine proteasomes, which may help to clarify their immunological role and the mechanism underlying the interaction with PA28, which is not present in yeast. In the present paper, we report new crystal forms of 20S proteasomes that are different from those we previously reported, and preliminary crystallographic studies to determine the tertiary structure with an interpretative electron density map. A synchrotron radiation source is necessary for the acquisition of diffraction data for such a supra-molecular complex, and the third-generation one showed especially good quality of diffraction, a low background level, sharp spots and a good S/N ratio. We then reached a high quality level of X-ray data by means of multiple iterative data collection. The electron density map calculated from these X-ray data clearly shows the molecular packing of 20S proteasomes without any collision in the orthorhombic crystal lattice.

20S proteasomes were isolated from bovine liver by the method described previously (*11*). In order to obtain a better yield of proteasomes and to produce X-ray grade crystals, purification was carried out using two large columns of hydroxyapatite (22 × 250 mm; Bio-Rad Industries) and a heparin-affinity gel (22 × 250 mm; Pharmacia LKB Biotechnology). The final enzyme solution was concentrated to 35 mg/ml by ultracentrifugation (160,000 ×*g*, Hitachi RP55T rotor) and membrane filtration (Amicon YM100). The crystallized enzyme was dissolved in a small amount of water for measurement of the peptidase activity using Suc-Leu-Leu-Val-Tyr-MCA (Peptide Institute) as a substrate (*14*). Crystals were grown by the hanging drop vapor diffusion method at 25°C (Fig. 1). The drops contained 3 μl of protein and 3 μl of the reservoir solution, with 0.15 M magnesium acetate, 0.1 M Na-cacodylate (pH 6.5), and 35% 2,4-methylpentanediol. These crystals were quickly frozen in a stream of cold nitrogen gas (Oxford Cryosystem) and then kept in liquid nitrogen to prevent degradation prior to the synchrotron experiments.

The acquisition of native X-ray data for crystals was carried out with a synchrotron source at the BL6A of the Photon Factory (PF, Tsukuba), and further high resolution experiments were carried out with a third-generation undulater at the ID2 of the European Synchrotron Radiation Facility (ESRF, Grenoble) and the BL41XU of the SPring-8 (Harima) under the conditions summarized in Table I. All of the X-ray experiments were carried out under 100 K in a stream of nitrogen gas.

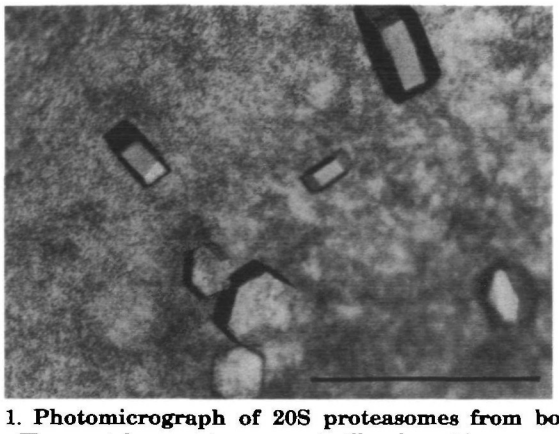

Fig. 1. **Photomicrograph of 20S proteasomes from bovine liver.** The crystals were transparent pillar-shaped hexagons. The bar indicates 0.5 mm.

A total of 1,288 diffraction images for 20S proteasome crystals were processed and evaluated with the DENZO (*15*), SCALEPACK (*15*), MOSFLM (*16*), and CCP4 suite (*17*) programs. All computing was performed on a personal IRIS workstation (O2, Octane and Impact) in our laboratory. We found two crystal forms during the processing of diffraction images, which have the same space group but exhibit slight differences in their unit cell dimensions. Although the preparation, purification and crystallization processes were not changed, the two crystal forms (forms I and II) appeared in the same crystallization drop.

A total of 339,924 measurements were recorded and merged with an R factor of 11.8% to give 78,752 unique reflections and 200–3.5 Å resolution. The orthorhombic crystal belongs to space group $P2_12_12_1$, with cell parameters of a = 124.8, b = 197.4, c = 323.8. On the other hand, form II native data derived from a total of 537,013 measurements were reduced to 64,560 unique reflections with an R factor of 11.9% and 200–4.0 Å resolution. The space group was the same as that of form I, but the cell parameters were slightly different, *i.e.* a = 115.1, b = 205.6, c = 316.0 Å. Assuming a molecular mass of 750 kDa, the respective V_m values are 2.65 and 2.49 Å^3/Da. We have obtained another crystal form of the 20S proteasomes from bovine liver, in which the 20S proteasomes could be packed into a hexagonal $P6_122$ or $P6_522$ cell with $a = b$ = 121.83 (2) Å, c = 930.68 (6) Å, and the V_m value was 2.7 Å^3/Da. A crystallographic twofold axis goes through the center of the 20S complex and is available for the stacking of a half complex ($\alpha_{1\text{-}7}\beta_{1\text{-}7}$) (*11*). On the other hand, 20S proteasomes from yeast have a local twofold axis in the 20S complex, and this local symmetry is used to form a 20S complex in an asymmetric unit (*13*). The 20S proteasomes from an archaebacterium also have a local twofold axis in the 20S complex packed in the same space group ($P2_12_12_1$) with similar cell dimensions (a = 311.9, b = 209.0, c = 117.2 Å) (*12*) to as in our latest research. Such good important information might lead to the easy structure analysis of bovine 20S proteasomes by means of a molecular replacement method, and good quality electron-density maps calculated from observed structure factors and phases derived with the electron density modification method are now being interpreted (Fig. 2). Further model building of bovine proteasomes will be well achieved when the amino acid sequences of all subunits can be determined.

The slight differences in the cell dimensions and V_m values may have been caused by an irregular combination of the components which form the 20S proteasomes. A gap in the crystallographic isomorphism between forms I and II may occur generally for some reasons, such as molecular

TABLE I. **Experimental conditions at various synchrotron facilities.**

	ID2/ESRF	BL6A/PF	BL41XU/ SPring-8
Source	Undulator	Bending magnet	Undulator
Wavelength (Å)	0.996	1.00	0.708
Camera length (mm)	400	430	500
Oscillation angle (°)	0.5/frame	0.5	0.5
Exposure time (s)	90/frame	120	60

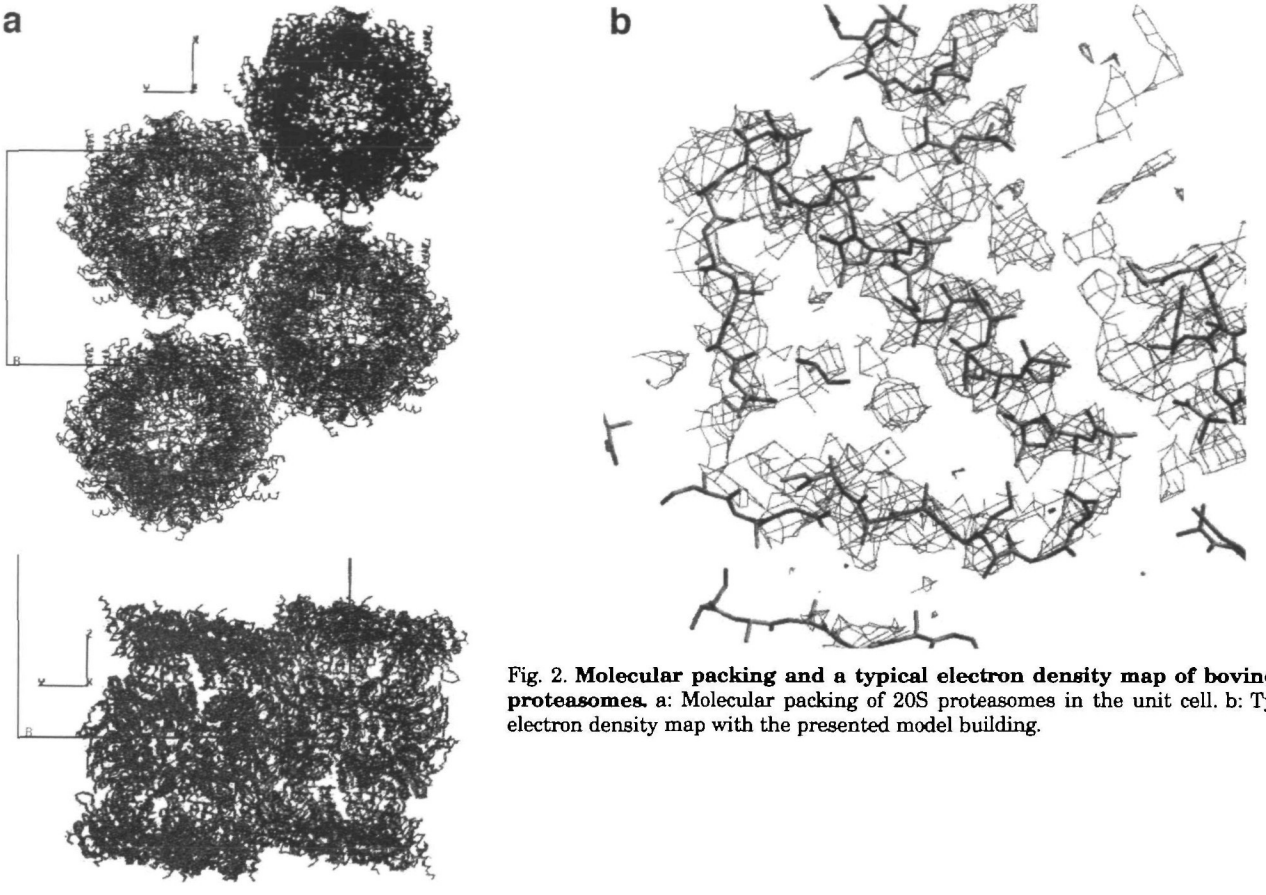

Fig. 2. **Molecular packing and a typical electron density map of bovine 20S proteasomes.** a: Molecular packing of 20S proteasomes in the unit cell. b: Typical electron density map with the presented model building.

packing and structural variation involving the fluctuation of a flexible loop. Immunoproteasomes have three exchangeable and γ-interferon–inducible subunits (LMP7, LMP2, and MECL1) in the central part of the 20S complex body, and three constitutive subunits (X, Y and Z, respectively) could be substituted by these γ-interferon subunits when the cells were treated with γ-interferon (*5*). The amino acid homology between a pair of two respective subunits is about 60–70%, therefore, the differences in their tertiary structures caused by such nonhomologous amino acid sequences are expected to be a minimum alteration. The two crystal forms grew simultaneously in the same crystallization drop but were not complementary to each other. In our case, substitution of the exchangeable subunits occurred in bovine proteasomes and brought about slight differences in the cell dimensions.

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