Peptides displayed on the cell surface by major histocompatibility class I molecules (MHC class I) are generated by proteolytic processing of protein-antigens in the cytoplasm. Initially, antigens are degraded by the 26 S proteasome, most probably following ubiquitination. However, it is unclear whether this proteolysis results in the generation of MHC class I ligands or if further processing is required. To investigate the role of the 26 S proteasome in antigen presentation, we analyzed the processing of an intact antigen by purified 26 S proteasome. A recombinant ornithine decarboxylase was produced harboring the H-2Kb-restricted peptide epitope, derived from ovalbumin SIINFEKL (termed ODC-ova). Utilizing recombinant antizyme to target the antigen to the 26 S proteasome, we found that proteolysis of ODC-ova by the 26 S proteasome resulted in the generation of the Kb-ligand. Mass spectrometry analysis indicated that in addition to SIINFEKL, the N-terminally extended ligand, HSIINFEKL, was also generated. Production of SIINFEKL was linear with time and directly proportional to the rate of ODC-ova degradation. The overall yield of SIINFEKL was approximately 5% of the amount of ODC-ova degraded. The addition of PA28, the 20 S, or the 20 S-PA28 complex to the 26 S proteasome did not significantly affect the yield of the antigenic peptide. These findings demonstrate that the 26 S proteasome can efficiently digest an intact physiological substrate and generate an authentic MHC class I-restricted epitope.

Cells display foreign and altered intracellular antigens to cytotoxic T lymphocytes (CTL) through MHC class I molecules. Antigenic peptides presented through class I molecules are generated in the cytoplasm by proteolytic degradation of endogenously synthesized antigens. Suitable peptides are then translocated through specialized peptide transporters (termed TAP) to the lumen of the endoplasmic reticulum, where they bind and subsequently stabilize newly synthesized MHC class I molecules. Assembled class I molecules then migrate to the cell surface for recognition by T cells.

There is now substantial evidence implicating the proteasome in antigen processing. When membrane-permeable inhibitors of proteasomes were added to cells, they severely inhibited proteasome activity in vitro, the cellular turnover of short and long lived proteins, and assembly of class I molecules as well as presentation of ovalbumin (OVA) introduced into the cytoplasm.

Proteasomes are multicatalytic complexes that constitute the major proteolytic activity in the cytosol and nucleus of all eukaryotes. Proteasomes are found in the cytoplasm as 20 and 26 S particles. The 20 S proteasome is a barrel-shaped complex consisting of four stacked rings, each composed of seven related subunits. The outer rings are formed by noncatalytic α subunits, whereas catalytic β subunits occupy the inner two rings. The 20 S proteasome is an ATP-independent protease that in vitro cleaves only peptides. It can also digest several unfolded proteins, but only when activated by treatment with SDS (4, 5).

The physiological function of the 20 S proteasome is therefore obscure. The 20 S complex forms the catalytic core of the 26 S proteasome. The 26 S proteasome is formed by an ATP-dependent association of the 20 S core particle with one or two ATPase regulatory complexes termed PA700 (or 19 S particle) at one or both ends of the 26 S proteasome barrel, respectively. The 26 S proteasome is an ATP-dependent protease that degrades mainly ubiquitinated proteins (4, 6).

In vertebrates, Interferon-γ induces the replacement of three constitutive catalytic β subunits of the 20 S proteasome, (X, Y, and Z) with three homologues (LMP7, LMP2, and MECL-1) in newly synthesized proteasomes (termed “immunoproteasomes”). Through use of precursor peptides, it has been demonstrated that incorporation of LMP2 and LMP7 may alter the cleavage specificity of the 20 S proteasome in a manner that favors the generation of antigenic peptides (7, 8). In vivo, it has been shown that LMP2 and LMP7 are not obligatory for antigen presentation (9, 10). However, LMP2 and LMP7 can restore defects in surface presentation of certain viral antigens in LMP-deficient cell lines (11).

The 20 S proteasome can also associate with the PA28 activator complex (11 S regulator) that enhances in vitro cleavage of short peptides but not of proteins (12, 13). Interferon-γ induces the synthesis of the two homologous subunits of PA28 (α and β) (14) and the formation of 20 S-PA28 complexes in vivo (15). PA28α stably expressed in a mouse fibroblast line signific-
ically enhanced class I-mediated presentation of two viral epitopes (16). The reason for the augmenting effect seems to be favorable modulation of proteasome activity (17, 18). To explain the effect of PA28 on antigen presentation in vivo, it was proposed that the 26 S proteasome initially degrades protein-antigens into long peptides, which are then processed and presented to the MHC ligands by either the 20 S or the 20 S-PA28 complex (19). Another possibility is that a hybrid PA700-20 S-PA28 complex can generate the MHC ligand in one step (19, 20).

It has been shown that increased susceptibility to ubiquitination can facilitate class I-restricted presentation of antigens in vivo (21–23). We have directly demonstrated that ubiquitination of modified OVA is obligatory for the generation of a specific MHC class I-restricted epitope in an in vitro system from lymphocyte lysate (24). Therefore, it is most likely that degradation by the 26 S proteasome is the initial step in the processing of antigens. However, it is not known whether the breakdown of the antigen by the 26 S proteasome generates the MHC ligand or only longer intermediates that require additional trimming.

Previously, extensive research has focused on the mode of action and regulation of the 20 S proteasome in antigen processing. These studies indicated that isolated 20 S proteasomes and 20 S-PA28 complexes can generate MHC class I-restricted epitopes from long peptides and chemically denatured proteins (3). However, the physiological relevance of these artificial model experiments is uncertain, since antigens are most likely globular proteins and as such are probably degraded by the ATP-dependent 26 S proteasome (25). Indeed, Yellen-Shaw et al. (26) recently demonstrated that processing of peptides might be different from that of proteins. The researchers demonstrated that a point mutation in the flanking sequence of an influenza nucleoprotein-derived epitope that inhibited class I presentation from full-length nucleoprotein had no effect when the same epitope was expressed as a minigene (26).

The major impediment to an investigation of the function of the 26 S proteasome in antigen processing was lack of physiological protein-antigens that could serve as substrates for this protease. Most proteins are targets to proteolysis by the 26 S proteasome through prior conjugation to ubiquitin. Unfortunately, ubiquitin-antigen conjugates are extremely difficult to prepare. These studies indicated that isolated 20 S proteasomes and 20 S-PA28 complexes can generate MHC class I-restricted epitopes from long peptides and chemically denatured proteins (3). However, the physiological relevance of these artificial model experiments is uncertain, since antigens are most likely globular proteins and as such are probably degraded by the ATP-dependent 26 S proteasome (25). Indeed, Yellen-Shaw et al. (26) recently demonstrated that processing of peptides might be different from that of proteins. The researchers demonstrated that a point mutation in the flanking sequence of an influenza nucleoprotein-derived epitope that inhibited class I presentation from full-length nucleoprotein had no effect when the same epitope was expressed as a minigene (26).

The major impediment to an investigation of the function of the 26 S proteasome in antigen processing was lack of physiological protein-antigens that could serve as substrates for this protease. Most proteins are targets to proteolysis by the 26 S proteasome through prior conjugation to ubiquitin. Unfortunately, ubiquitin-antigen conjugates are extremely difficult to produce and purify in quantities required for in vitro processing experiments.

We circumvented the requirement for antigen-ubiquitin conjugates by utilizing the unique mechanism by which ornithine decarboxylase (ODC) is targeted to degradation. Whereas ubiquitination is required for the degradation of most proteins by the 26 S proteasome, ODC becomes susceptible to enhanced ATP-dependent degradation (without ubiquitination) through prior association with a chaperone-like protein termed antizyme (AZ) (27). ODC is a homodimer but becomes a heterodimer upon binding to AZ. This induces a conformational change that targets the protein for degradation by the 26 S proteasome (28).

We expressed a recombinant ODC harboring the OVA-derived K<sub>b</sub>-restricted epitope SIINFEKL. We then utilized an in vitro degradation system previously described by Togunaga and co-workers (29) to test the proteolytic processing of the antigen (termed ODC-ova). In a system that contains purified AZ and 26 S proteasome, we show that in the presence of ATP, the recombinant antigen is degraded by the 26 S proteasome and that proteolysis results in the generation of the K<sub>b</sub>-epitope.

**EXPERIMENTAL PROCEDURES**

**Materials**

Pyridoxamine 5'-phosphate was synthesized by Al-Coat (Ness Ziona, Israel). Amylose was from Amersham Pharmacia Biotech. Synthetic SIINFEKL was synthesized by Anspec (San Jose, CA). Succinyl-Leu-Leu-Leu-Tyr-AMC was purchased from Sigma. The multiple antigen peptide SIINFEKL was synthesized by Peptide Technologies Corp. (Gaithersburg, MD). Amylose affinity resin was from New England Biolabs Inc. Bovine was from Calbiochem. Complete<sup>TM</sup> protease inhibitors (referred to as protease inhibitors) were from Roche Molecular Biochemicals. All standard reagents and reagents for cell culture were from Sigma. Fluorescein isothiocyanate-conjugated F(ab')<sub>2</sub> fragment goat anti-mouse IgG was from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Dr. Angel Foggard (National Institutes of Health, Bethesda, MD) kindly provided monoclonal antibody 25-D1.6.

**Preparation of SIINFEKL-specific Antiserum**

Anti-SIINFEKL antiserum was raised in rabbits immunized with the multiple antigen peptide SIINFEKL. Two-month-old New Zealand White rabbits were subcutaneously injected with 1 mg each of multiple antigen peptide SIINFEKL in incomplete Freund’s adjuvant. The animals then received two boosts of 0.5 mg each of multiple antigen peptide SIINFEKL in incomplete Freund’s adjuvant at intervals of 3 weeks. Ten days after the second boost, the rabbits were bled, and serum was prepared. The serum was tested by immunoblot analysis using OVA as antigen and preimmune serum as control.

**Plasmid Constructions**

The mouse full-length ODC cDNA in pBluescript with a Ncol site spanning the initiator ATG was a kind gift from Dr. Chaim Kahana (The Weizmann Institute, Rehovot, Israel). The full-length ODC cDNA was subcloned in pUC18 at the KpnI (5') and BamHI (3') sites, resulting in the plasmid pUC-ODC. The ODC cDNA was then isolated from pUC-ODC as an Ncol/BamHI fragment and cloned in the Ncol (5') and BamHI (3’) sites of pET19b (Novagen), resulting in the expression plasmid pET-ODC. To generate the ODC-ova expression vector, an adapter encoding the peptide SIINFEKL with BetXI-compatible ends was generated by annealing the two synthetic oligonucleotides, 5'-ATAGATTAAACTCAACCTGAAAACGTAGCCT-3’ and 5’-TCAGTTTT-TCGACCACTGGTATTTACTAGGCG-3’. The adapter was then inserted in frame at the unique BetXI site in the ODC sequence in pUC-ODC to generate the plasmid pUC-ODCova. The ODC-ova DNA was then isolated from pUC-ODCova as an Ncol/BamHI fragment and cloned in the Ncol (5') and BamHI (3’) sites of pET14b (Novagen), resulting in the expression plasmid pET-ODCova. The insertion of the adapter in the correct orientation was confirmed by DNA sequencing. The plasmids pET-ODC and pET-ODCova were used to express ODC and ODC-ova (respectively) both in Escherichia coli and in reticulocyte lysate.

The plasmid encoding the fusion protein maltose-binding protein-antizyme (MBP-AZ) was constructed as follows. The rat full-length AZ cDNA in pET8 (kindly provided by Dr. Chaim Kahana) was isolated as an Ncol fragment and then treated with DNA polymerase Klenow fragment to produce blunt ends. The blunted, Ncol fragment was then ligated in frame in pMALTM (New England Biolabs Inc.) that had been digested with Xbal after end filling of the 5’-overhangs. A plasmid clone with the correct cDNA orientation was then selected. The resulting plasmid pMAL-AZ was used for expression of AZ in bacteria.

**Preparation of Pyridoxamine 5’-Phosphate Affinity Matrix**

Pyridoxamine 5’-phosphate was coupled to Affi-Gel 10-agarose (Bio-Rad) exactly as described previously (30).

**Expression and Purification of ODC and ODC-ova**

**Expression**

For expression of ODC and ODC-ova, pET-ODC and pET-ODCova (respectively) were transformed into E. coli strain BL21 (DE3). A culture (300 ml) was grown until absorbance at 600 nm reached 0.6–0.8. Expression was then induced with 1-thio-D-ribulose (300 ml) and lysed in French pressure press cell (Amisco Slim Instruments, Inc., Urbana, IL). After lysis, the extract was supplemented with 5 mM DTT, and the insoluble material was removed by centrifugation (10,000 × g for 15 min).

**Purification**

**Step 1: Ion Exchange Chromatography**—The bacterial lysate (270 mg of protein) was loaded on a 4.5 × 1.6-cm Q-Sepharose column (Ameri...
sham Pharmacia Biotech) equilibrated in buffer L. The column was washed with 20 ml of buffer L and then developed with a linear gradient of 0–1 M NaCl (in buffer L). Both ODC and ODC-ova eluted from the column between 0.35 and 0.4 M NaCl. The peak fractions were combined and subjected to affinity chromatography.

Step 2: Affinity Chromatography—The combined protein fraction from the Q-Sepharose column was directly loaded on pyrodoxamine 5'-phosphate-agarose column (1 × 7 cm) equilibrated in buffer L containing 0.1 mM EDTA and 0.1 mM L-ornithine. The sample was applied to the column at a flow rate of 35 μl/min (17 h) at 4 °C. The column was then washed with 80 ml of buffer L containing 15 mM NaCl. The protein was then eluted from the column by successive additions of 7-ml portions of buffer L containing 10 μM pyridoxal 5'-phosphate. All of the bound protein that eluted in the first five fractions was combined and then concentrated to 0.5–2 μg/ml in Centricon 30 concentrator (Millipore Corp., Bedford, MA) and stored in aliquots at −80 °C.

Preparation of 35S-labeled ODC-ova in Bacteria

For production of 35S-labeled ODC-ova in bacteria, pET-ODCova was transformed into the methionine auxotroph E. coli strain B834 (DE3) (Novagen Inc., Madison, WI). A 50-ml culture was grown at 37 °C in M9 minimal medium supplemented with thiamine (20 μg/ml) and all 20 amino acids at 0.2 mM until absorbance at A600 reached 0.6–0.7. 1-Thio-γ-n-galactopyranoside and Pro-mix L-[35S] (Amersham Pharmacia Biotech) (0.5 μCi) were then added for a further incubation at 22 °C for 16 h. Purification of 35S-labeled ODC-ova was then carried out exactly as described above.

Expression and Purification of MBP-AZ

For expression of MBP-AZ, pMAL-AZ was transformed into E. coli strain DH10B. A bacterial culture (300 ml) was induced with 0.3 mM 1-thio-γ-n-galactopyranoside. After 2 h of induction at 37 °C, the cells were harvested and then washed with ice-cold buffer P (10 mM sodium phosphate (pH 7.0), 30 mM NaCl, 1 mM DTT, 1 mM EDTA) supplemented with a 1:25 (v/v) solution of protease inhibitors. The cells were then resuspended in 20 ml of buffer P adjusted to 0.5 mM NaCl and lysed in a French pressure press cell. The insoluble material was removed by centrifugation (10,000 × g, 15 min), and the bacterial lysate was stored at −80 °C for further purification.

Purification

Step 1: Affinity Chromatography—A sample of the bacterial lysate (25 mg of protein) was applied to a 1-ml amylase resin. The column was then washed with buffer P, and MBP-AZ was eluted from the column by the sequential addition of 1-ml portions of buffer P containing 10 mM maltose. The first 2 ml that contained the bulk of the recombinant protein (approximately 1 mg) were combined and further purified by ion exchange chromatography.

Step 2: Ion Exchange Chromatography—The affinity-purified protein from step 1 was diluted in 10 ml of buffer L (25 mM Tris-HCl, pH 7.5, 2.5 mM DTT) and loaded on a Mono-Q 5/5 column (Amersham Pharmacia Biotech) equilibrated in buffer L. The column was washed with buffer L containing 0.1 mM NaCl and then developed with a linear gradient of 0.1–1.0 mM NaCl in buffer L. MBP-AZ eluted from the column as a sharp protein peak at 0.4 mM NaCl. The protein was concentrated to 2 mg/ml in Centricon 30 and stored in aliquots at −80 °C. The Mono-Q-purified MBP-AZ was used in all of the experiments described in this study.

Preparation of 35S-labeled ODC-ova in Reticulocyte Lysate

Radiolabeled ODC-ova was produced from pET-ODC-ova that was incubated in a T7 polymerase-driven transcription-translation (TNT)-coupled system from reticulocyte lysate (Promega Corp., Madison, WI) in the presence of [35S]methionine. Following the translation reaction, unincorporated [35S]methionine was removed by ion exchange chromatography.

Degradation Assays

The activity of the 26 S proteasome was determined by its ability to degrade radiolabeled ODC-ova produced in reticulocyte lysate. Degradation reaction mixtures contained the following components in a final volume of 25 μl: 40 mM Tris-HCl (pH 7.5), 2 mM DTT, 5 mM MgCl2, 1 mM ATP, 10 mM creatine phosphate, 1.25 unit of creatine phosphokinase, 35S-labeled ODC-ova (approximately 20,000 cpm), 2 μg of MBP-AZ, and purified 26 S proteasome as indicated. Incubation was for 10 min at 37 °C. Reactions were then stopped by the addition of trichloroacetic acid. Degradation was determined by measuring the amount of soluble radioactivity after the addition of trichloroacetic acid.

One unit of 26 S proteasome was defined as the amount of enzyme that degraded 1% of 35S-labeled ODC-ova in 1 min under the conditions specified above.

Purification of 26 S Proteasome Complex

All purification procedures were performed at 4 °C.

Step 1: Preparation of Liver Lysate—The 26 S proteasome complex was prepared from livers of C57Bl mice (10–13 weeks old). A typical preparation was from 10 livers. The livers were thoroughly washed with phosphate-buffered saline and then homogenized using a motor-driven Potter-Elvehjem Teflon tissue grinder. Homogenization was in 5 ml/liver of buffer A containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 1.5 mM ATP, and 0.25 mM sucrose. The crude extract was then subjected to fractional centrifugation at 10000 × g and then at 10,000 × g. The 10,000 × g supernatant was subjected to ultracentrifugation for 1 h at 70,000 × g. The resulting supernatant (lysate) was then subjected to ammonium sulfate precipitation.

Step 2: Ammonium Sulfate Precipitation—This procedure separates 20 from 26 S proteasomes. The lysate containing approximately 250 mg of protein was supplemented with 5 mM MgCl2, 10 mM phosphocreatine, 10 μM g/ml creatine phosphokinase and was incubated for 1 h at 37 °C. The proteasome complex was then precipitated with ammonium sulfate at 38% (w/v) saturation as described previously (4).

Step 3: Gel Filtration Chromatography—The 38% ammonium sulfate sediment was dissolved in buffer B containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM ATP, and 20% (v/v) glycerol. The sample was loaded onto a Sephacrose 6B fast flow column (2.5 × 40 cm) (Amersham Pharmacia Biotech) equilibrated in buffer B. Fractions (2 ml) were collected, and 26 S proteasome activity was assayed in 2-μl samples of column fractions.

Step 4: Ion Exchange Chromatography—The proteasome peak from step 3 was combined and loaded onto a 1 × 4-cm Resource-Q column (Amersham Pharmacia Biotech) equilibrated in buffer B. The column was then washed with 10 ml of buffer B and developed by a linear gradient from 0 to 0.8 M NaCl in buffer B over 50 ml. The 26 S proteasome eluted from the column between 0.35 and 0.4 M salt.

Step 5: Glycerol Density Gradient—The 26 S proteasome complex from the ion exchange column was concentrated to 250 μl by ultracentrifugation in a Centricon 30 (Amicon). The sample was loaded onto a Sephacrose 6B fast flow column (2.5 × 40 cm) at 38% (w/v) saturation. After centrifugation at 28,000 rpm for 18 h at 4 °C, fractions of 0.4 ml were collected, and 26 S proteasome activity was assayed in 1-μl samples.

Purification of 20 S Proteasome and PA700

The 20 S proteasome and PA700 complex were purified from bovine erythrocytes as described previously (31, 32).

Expression of PA28a

PA28a in the plasmid pET16b was expressed in E. coli strain BL21 (DE3) and then purified as described previously (33).

Fluorogenic Peptide Assays

Peptidase activity of 20 and 26 S proteasome was assayed using the fluorogenic peptide sLLVY-MCA as described previously (34).

Substrate Overlay Assays

To analyze proteasome complexes, these complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to nitrocellulose. The proteasome complexes were detected using monoclonal antibodies J4D5 and J4D6 against the Thr-Pro and Lys-Pro tetrapeptide sequence, respectively.

Antigen Processing by Purified 26 S Proteasome
**Step 2: Acid Extraction and Isolation of Low Molecular Weight Material**—Following incubation at 37 °C for various time periods as indicated, the reaction mixture was adjusted to pH 2 by the addition of trifluoroacetic acid and then sonicated for 30 s at full power in a bath sonicator. The acid extract was microcentrifuged in an Amigon Microcon 10 microconcentrator (Millipore Corp., Bedford, MA). The filtrate was collected and lyophilized.

**Step 3: Isolation of Peptides**—The lyophilized low molecular weight material from step 2 was then separated on a 2.1 × 150-mm C18 column (Vydac, Hesperia, CA) (eluants A, 0.1% trifluoroacetic acid, 4% acetonitrile; eluant B, 0.085 trifluoroacetic acid, 90% acetonitrile; gradient 4–50% B in 45 min; flow rate of 0.2 ml/min). Based on the position of elution of the synthetic SIINFEKL that eluted reproducibly between 33 and 34% B, the material eluting between 31 and 36% B was routinely pooled and tested for biological activity.

**Step 4: Detection of SIINFEKL**—The combined peptide fraction from step 2 was incubated with RMA/S cells (45). The cells were then incubated with mAb 25-D1.16 followed by a second incubation with fluorescein isothiocyanate-labeled F(ab)\(^2\) goat anti-mouse IgG as described previously (36). The stained cells were then analyzed by flow cytometry using a Beckton Dickinson FACSort flow cytometer (Mountain View, CA). For cytotoxicity assays, the peptide fraction from step 2 was incubated with \(^{35}\)S-labeled RMA/S cells. The cells were then tested for recognition by SIINFEKL-specific CTL in a standard cytotoxicity assay as described previously (24).

Mass Spectrometry Analysis—Peptides extracted from an ODC-ova processing reaction (step 2) were resolved by reverse phase HPLC on a 150-mm C18 column (Vydac, Hesperia, CA) (eluants A, 0.1% trifluoroacetic acid, 4–65% acetonitrile; eluant B, 0.025% trifluoroacetic acid in 61 min at a flow rate of 40 μl/min). The sample was electrosprayed directly from the HPLC column into an electrospray ion trap mass spectrometer (LCQ, Finnigan, San Jose, CA). The mass spectrometry analysis was performed in the positive ion mode using alternating full MS scan and an MS/MS scan (collision-induced fragmentation) on the most abundant ions. The MS and MS/MS spectra collected during the run were compared with the simulated fragmentation pattern of the peptides using the program MS-Product (MS-Prospect; University of California, San Francisco).

**RESULTS**

**Components of the Cell-free System for the Degradation of ODC-ova**—The OVA-derived K\(^+\)-restricted epitope SIINFEKL (amino acids 257–264 of the OVA sequence) was juxtaposed directly at the N terminus of the PEST II region of ODC (Fig. 1A). The recombinant antigen was purified by chromatography on a Mono-Q column following affinity chromatography on immobilized pyridoxamine phosphate (30) (Fig. 1B). Immunoblot analysis with SIINFEKL-specific antiserum shows that the antibody recognized ODC-ova and native OVA but not ODC (Fig. 1C).

When ODC-ova was incubated with purified 26 S proteasome, it was degraded only in the presence of ATP and AZ (Table I), indicating that the recombinant protein retained the degradation mechanism of native ODC.

To characterize the primary antigenic end product generated by the 26 S proteasome, it was necessary to eliminate the possibility of additional degradation by contaminating proteases or peptidases. To minimize this possibility, we used only highly purified preparations of ODC-ova, AZ, and 26 S proteasome (Fig. 1B, lane 3, Fig. 2, and Fig. 3A, respectively). In addition, all processing experiments were performed in the presence of nonspecific protease inhibitors and bestatin, a potent aminopeptidase inhibitor (37). Another source of secondary peptidase activity might have been free 20 S particles that dissociated from the 26 S proteasome and co-purified with it. To confirm that the 26 S does not contain free 20 S, highly purified 26 and 20 S proteasome samples were separated by nondenaturing gel electrophoresis and then visualized following in situ peptidase activity assay (35). The 26 S proteasome samples (Fig. 3B, lanes 1 and 2) appear as two closely migrating active protein bands at the top of the gel. These bands correspond to proteasome capped with two (26 S\(^+\)) or one (26 S\(^0\)) regulatory PA700 complexes, respectively (38). The 20 S proteasomes show, as expected, a faster migrating peptidase activity (Fig. 3, lane 3). No trace of peptidase activity was detected in the lanes of the 26 S samples in the position of the free 20 S proteasome, including lane 2, which was overloaded with 26 S.

**Degradation of ODC-ova by the 26 S proteasome**

\(^{35}\)S-labeled ODC-ova, expressed in reticulocyte lysate (20,000 cpm) was incubated for 10 min at 37°C with the indicated components in the presence of ATP as described under “Experimental Procedures.” The reaction was stopped by the addition of trichloroacetic acid. Degradation was determined by measuring the amount of soluble radioactivity after the addition of trichloroacetic acid and was computed as the percentage of trichloroacetic acid-soluble material out of the total radioactivity input. Each value presented in the table is an average of duplicate incubations.

| Additions                      | Degradation % of total |
|-------------------------------|------------------------|
| MBP-AZ                        | 1.6                    |
| 26 S proteasome               | 3.0                    |
| 26 S proteasome, MBP-AZ       | 23.6                   |

**Proteolytic Processing of ODC-ova by the 26 S Proteasome Yields the H-2K\(^+\)-restricted, OVA-derived Peptide**—We tested whether degradation of ODC-ova by purified 26 S proteasome liberates the inserted SIINFEKL sequence. To this end, we incubated ODC-ova with AZ and 26 S proteasome in the pres-
ence of ATP for various time periods. Peptides were then extracted and purified by reverse phase chromatography on HPLC. The HPLC-purified peptides were then incubated with RMA/S cells. The cells were then further incubated with 25-D1.16, a monoclonal antibody that specifically recognizes cell-bound K^b–SIINFEKL complexes. The amount of SIINFEKL that was produced by proteolysis of ODC-ova was much less than that necessary for saturating the peptide binding capacity of empty K^b molecules on the surface of RMA/S cells (24). Therefore, the binding of 25-D1.16 was directly proportional to the amount of processed SIINFEKL.

Production of the antigenic peptide was detected with antibody 25-D1.16 only in the presence of the 26 S proteasome and AZ (Fig. 4, b and c). SIINFEKL-specific CTL also reacted with RMA/S cells loaded with degradation products of ODC-ova (Fig. 4e). Due to their higher sensitivity, the CTL detected antigenic peptide that was generated in the absence of AZ. This small amount of biologically reactive peptide is most likely generated by slow, basal (AZ-independent) proteolysis of ODC by the 26 S proteasome (28). As expected, no reactive peptide was produced when ODC was processed instead of ODC-ova (Fig. 4d).

Mass Spectrometry Analysis of ODC-ova-specific Degradation Products—To learn more about the processing of ODC-ova, it was necessary to determine whether, in addition to the antigenic peptide, other SIINFEKL-derived peptides were also generated. To test whether such peptides had been produced, the ODC-ova digestion products were resolved by HPLC followed by on-line electrospray mass spectrometry analysis. To detect SIINFEKL-derived peptides, we searched for masses corresponding to the antigenic peptide or any possible portion of it, either alone or with flanking ODC-derived sequences (with up to four and two residues at the N and C terminus, respectively). The spectrogram of the masses revealed only two specific masses, one between 963.0 and 964.0 that peaked at 26 min and one between 1100.0 and 1101.0 that peaked at 24 min, suggesting that only SIINFEKL and HSIINFEKL had been generated (Fig. 5, a and b). These masses were not observed when ODC (instead of ODC-ova) was processed (data not shown). To confirm the identity of the peptides, ODC-ova degradation products were once again subjected to mass spectrometry analysis. However, this time, the masses eluting at 26 and 24 min were further characterized. Mass spectrometry of these fractions revealed a mass of 963.6 corresponding to SIINFEKL (at 26 min) and a mass of 1100.7 corresponding to HSIINFEKL (at 24 min) (Fig. 6, a and c). Fragmentation of the two masses by collision-induced dissociation produced the characteristic internal fragment ions, further confirming the identity of the peptides (Fig. 6, b and d).

Samples from the HPLC fractions eluting between 23 and 29 min were also analyzed for biological activity. The individual peptide fractions were incubated with RMA/S cells, and the cells were then tested for recognition by mAb 25-D1.16. Analysis of the biological activity indicated that the SIINFEKL-containing fractions were highly active, whereas the HSIIN-
FEKL-containing fractions were only weakly active (Fig. 5c). When synthetic HSIINFEKL was incubated with RMA/S cells, the cells were stained approximately 10-fold less efficiently compared with SIINFEKL. However, at high peptide concentrations (>0.8 μM) both peptides were equally active (not shown). Consequently the low activity of HSIINFEKL compared with that of SIINFEKL reflects reduced affinity of the extended peptide to K\(^{\text{b}}\) (rather than the inability of 25-D1.16 to recognize K\(^{\text{b}}\)-HSIINFEKL complexes). Based on the activity of the synthetic peptides and that observed in Fig. 5c, we estimated that, upon processing of ODC-ova by the 26 S proteasome, SIINFEKL and HSIINFEKL are produced at a molar ratio of approximately 5:3, respectively. Nevertheless, most (if not all) of the measured biological activity is derived from SIINFEKL. Thus, these results clearly indicated that SIINFEKL is the major antigenic peptide produced by processing of ODC-ova by the 26 S proteasome.

We next tested the proportion of processed SIINFEKL relative to the amount of degraded ODC-ova. To this end, we incubated bacterially expressed \(^{35}\)S-labeled ODC-ova with the 26 S proteasome and MBP-AZ for various time periods. After each incubation period, the formation of SIINFEKL and the degradation of ODC-ova were quantified. As shown in Fig. 7, bacterially expressed ODC-ova was degraded much more slowly than ODC-ova expressed in reticulocyte lysate (Table I). However, degradation of the antigen was linear at a rate of 2 pmol/h. Generation of the antigenic peptide was also linear at a constant rate of approximately 0.1 pmol/h. The yield of SIINFEKL is therefore approximately 5% of the maximal theoretical yield expected if every degraded ODC-ova molecule would result in the formation of one molecule of SIINFEKL. This yield is relatively high considering the recent report that only 10–15% of the 26 S proteasome digestion products are peptides of 8 or 9 residues in length (39).

Effect of 20 S, PA28, and the 20 S-PA28 complex on ODC-ova Processing by the 26 S Proteasome—It has been proposed that 20 S and 20 S-PA28 may stimulate antigen processing by editing 26 S degradation products. Since processing of ODC-ova also produced HSIINFEKL and possibly smaller amounts of longer peptides that were not detected by mass spectrometry, it was important to test if the 20 S or the 20 S-PA28 complex could increase the yield of SIINFEKL. We also tested whether PA28 could directly enhance 26 S processing activity. We preincubated the 20 S and PA28\(^{a}\) to allow the formation of the 20 S-PA28 complex. When the 20 S proteasome was incubated with PA28\(^{a}\) its peptidase activity was stimulated 15-fold in the presence of PA28\(^{a}\) (Table II), indicating that the 20 S-PA28 complex had been formed (33). The 20 S, the PA28 particle, and the preformed 20 S-PA28\(^{a}\) complex were then further incubated with 26 S, ODC-ova, and AZ, and the amount of SIINFEKL was measured. As shown in Table II, except for the 20 S-PA28 complex, which slightly enhanced the production of the antigenic peptide, none of the complexes had a significant stimulatory effect on processing of ODC-ova by the 26 S proteasome.

**DISCUSSION**

In this study, we analyzed the processing of a physiological protein-antigen by the 26 S proteasome in vitro. The problem that we wanted to test was whether processing of antigens by this protease yields MHC ligands or only longer peptides that must be further processed. The function of the 26 S proteasome
in the processing pathway can be evaluated only if the products processed by this protease are amenable to analysis. To this end, we developed a novel recombinant antigen, ODC-ova, that is targeted directly to the 26 S proteasome by a known physiological route (Table I and Fig. 4). Thus, the experimental system presented in this study is likely to reflect rather faithfully the in vivo process of peptide generation by the proteasome.

Structural analysis of the antigenic epitope produced by degradation of ODC-ova by the 26 S proteasome showed that two major products are produced: the minimal and biologically active Kb ligand SIINFEKL and the ligand extended by one amino acid (derived from the ODC sequence) at the N terminus, HSIINFEKL, that is only weakly active (Figs. 5 and 6). We did not find any peptides with an extension at the C terminus, suggesting that the C-terminal leucine residue is a dominant cleavage site. The amino acid sequence of the regions flanking SIINFEKL in the ODC-ova context differs from the natural flanking sequences, and yet the epitope is accurately produced. We have previously shown that ubiquitin-mediated degradation of ovalbumin also yields SIINFEKL (24). This suggests that processing of this peptide is, to a large extent, directed by intrinsic sequence information. This conclusion is also supported by previous findings that production of SIINFEKL, from an extended peptide by the 20 S proteasome in vitro, was unaffected by LMP2 and LMP7 (8). We were also unable to detect any peptides containing portions of the epitope, implying that dominance of SIINFEKL is also determined by the absence of significant internal cleavage sites.

Processing experiments in this study were carried under linear time conditions in the presence of saturating amounts of substrate. Kinetic and quantitative analysis of processing of ODC-ova indicated direct correlation between the rate of degradation and the rate of SIINFEKL production (Fig. 7). The frequency of SIINFEKL production was relatively high (approximately 5% of the maximal expected yield). Thus, the efficiency of processing of ODC-ova is similar to that previously reported for an unrelated antigen by Villanueva et al. (40). They estimated that, in vivo, approximately 35 molecules of murein hydrolase, a Listeria monocytogenes-derived antigen, are required to yield one antigenic peptide (40). Hence, our results clearly demonstrate that the 26 S proteasome may

Fig. 5. Separation of processed peptides by reverse phase-HPLC. The degradation products of ODC-ova were separated by reverse phase-HPLC chromatography. The eluant was split postcolumn; 30% of the sample was electrosprayed directly from the HPLC column into an electrospray ion trap mass spectrometer, and 70% was collected for biological assay. Elution profiles are shown of the masses between 963 and 964 (a) and between 1100 and 1101 (b). c, biological activity. The individual peptide fractions eluting between 23 and 29 min were incubated with RMA/S cells, which were subsequently assayed for recognition by mAb 25-D1.16 by flow cytometry as described under “Experimental Procedures.” Results are expressed as the value of the log of fluorescence intensity measured for each peptide fraction.
effectively produce antigenic peptides during the initial breakdown of the antigen.

The high yield of SIINFEKL can be attributed to several factors. Intrinsic properties of the antigenic peptide including a dominant C-terminal cleavage site and weak internal sites presumably ensure high frequency of excision of the intact epitope. However, it has been demonstrated that flanking regions might adversely affect antigen processing. For example, processing of SIINFEKL from a longer precursor peptide was strongly inhibited by the introduction of a flanking proline residue at the C terminus or a glycine-rich flanking sequence at the N terminus (41). Presentation of both the Kb-restricted OVA-derived and the Db-restricted nucleoprotein-derived epitopes was also markedly inhibited by alteration of C-terminal flanking residue (42). When the minimal Ld-binding epitope produced out of the cytomegalovirus immediate early antigen was expressed in two different positions within an unrelated carrier protein, production of the epitope was profoundly influenced by its position (43). It can be concluded that the flanking regions of SIINFEKL in ODC-ova (especially at the C terminus) are inert, thus further contributing to the ability of the epitope to be excised.

The effects of PA28 on antigen processing are unclear. It has been reported that the PA28α modifies the cleavage pattern by 20 S proteasomes by promoting double cleavages in the substrate so as to enhance the excision of antigenic epitopes (17, 18). Other reports indicated that PA28α stimulates antigen processing by the 20 S proteasome without changing the cleavage pattern (8). It is also not yet known whether PA28 functions in the context of 20 S-PA28 to edit peptides initially produced by the 26 S proteasome or whether it modulates 26 S activity directly as part of a hybrid PA700–20 S-PA28 (26 S-PA28). We therefore tested the effect of PA28α on the processing of ODC-ova by comparing the yield of the antigenic peptide produced by the 26 S alone with that obtained when the reaction was supplemented with either preformed 20 S-PA28 or PA28 particles. Our initial results show that none of the complexes significantly affected the yield of SIINFEKL (Table II). If 20 S-PA28 stimulates secondary processing as previously suggested, then it is likely to increase the yield of antigenic peptides when processing by the 26 S is incomplete. According to this model, the minor effect of the 20 S-PA28 complex on the processing of ODC-ova may be explained by the finding that the 26 S proteasome had already produced the final epitope rather efficiently. However, we cannot rule out the possibility that in the cell or under different experimental conditions in vitro, PA28 may have a significant stimulatory effect on antigen processing. To investigate the possibility that PA28 directly regulates 26 S activity, it will be necessary to isolate 26 S-PA28 complexes devoid of free 26 S, something we were, thus far,
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FIG. 7. Processing of $^{35}$S-labeled ODC-ova by purified 26 S proteasome. $^{35}$S-Labeled ODC-ova (4.3 µg of protein, 87 pmol, 120,000 cpm) was incubated for the indicated time periods with purified 26 S proteasome and MBP-AZ in a volume of 350 µl in a standard reaction mixture. At each time point, generation of SIINFEKL (A) and degradation of $^{35}$S-ODC-ova (B) were quantified. To determine the percentage of degradation of $^{35}$S-ODC-ova, duplicate aliquots of 50 µl were withdrawn at each time point. The amount of $^{35}$S-ODC-ova degraded was then determined by measuring the amount of soluble radioactivity after the addition of trichloroacetic acid as described under “Experimental Procedures” and computed as described in the legend to Table I. The numbers in parentheses indicate the percentage of ODC-ova degraded at each time point. To quantify the amount of SIINFEKL, peptides were isolated from the remaining reaction mixture (250 µl) and then incubated with RMA/S cells. The cells were then tested for recognition by mAb 25-D1.16 as described under “Experimental Procedures.” The amount of SIINFEKL produced from ODC-ova was calculated based on the reactivity of RMA/S cells that were incubated in parallel with known amounts of synthetic SIINFEKL.

Unable to accomplish.

The precise role of PA28 is only one of the fundamental, yet unresolved, questions concerning the mechanism and regulation of the 26 S proteasome and the possible auxiliary role of the 20 S in antigen processing. For example, it remains unclear how key residues either within or flanking antigenic epitopes affect the initial processing by the 26 S proteasome. It is also unknown whether, in fact, 26 S digests can serve as substrates for secondary processing by either the 20 S proteasome or possibly by amino peptidases (44). Having established a quantitative antigen processing system in which production of the antigenic epitope and the rate of degradation of the protein-antigen by the 26 S proteasome can be independently measured, we can now directly explore questions of that kind.

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### Table II

| Proteasome complex | SIINFEKL generated $^a$ (fmol) |
|-------------------|---------------------------------|
| 26 S              | 56                              |
| 26 S + PA28       | 54                              |
| 26 S + 20 S       | 53                              |
| 26 S + 20 S-PA28 $^b$ | 73                              |

$^a$ Results are the average of two independent experiments.

$^b$ Formation of 20 S-PA28 complex was verified by a 15-fold stimulation of 20 S peptidase activity in the presence of PA28. Peptidase activity was measured with the fluorogenic peptide sLLY-AMC as described under “Experimental Procedures.”

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