Intrinsic Nucleoside Diphosphate Kinase-like Activity Is a Novel Function of the 20 S Proteasome*

Mihiro Yano, Sachie Mori, and Hiroshi Kido‡

From the Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan

The eukaryotic 20 S proteasome is the prototype of a new family of the N-terminal nucleophile hydrolases and is composed of numerous low molecular mass subunits arranged in a stack of four rings, each containing seven different α- or β-subunits. Among the β-type subunits in the yeast proteasome, three proteolytically active ones were identified, although the functions of the other β- and α-type subunits remain to be clarified. We report here that the purified 20 S proteasome exhibits intrinsic nucleoside diphosphate (NDP) kinase-like activity. The proteasome exhibited a preference for ATP and dATP as phosphate donors, and a broad specificity for NDPs, other than GDP, as phosphate acceptors, unlike conventional NDP kinase, which catalyzes the transfer of γ-phosphate between NDPs and nucleoside triphosphates. During the transfer of γ-phosphate, the proteasome formed acid-labile phosphohistidine as autoprophosphorylated intermediates, and NDP-dependent dephosphorylation of the latter then occurred. These enzymatic properties are similar to those of the molecular chaperone, Hsp70, which also exhibits intrinsic NDP kinase-like activity, instead of ATPase activity. C5 among the β-type subunits and C8 among the α-type subunits were autoprophosphorylated during the γ-phosphate transfer reaction and were photoaffinity labeled with 8-azido-[α-32P]ATP, suggesting that the C5 and C8 subunits of the proteasome are responsible for the NDP kinase-like activity.

The 20 S proteasome, representing a new family of the Ntn1 hydrolases (1, 2), is the central enzyme in protein degradation in both the cytosol and the nucleus and plays a role in the control of cellular processes, such as metabolism, the cell cycle, the immune response (by generating antigenic peptides), and the stress response (by removing abnormal proteins) (3). Eukaryotic proteasomes have multiple proteolytic activities that involve the 26 S proteasome, the core and proteolytic ubiquitin-independent protein degradation pathways, both of which involve the 26 S proteasome, the core and proteolytic chamber of which are formed by the 20 S proteasome. It is generally thought that the proteolytic activity of the 26 S proteasome is regulated by 19 S “cap” regulatory complexes (8, 9), which contain ATPasers and serve to unfold substrate proteins prior to translocation to the proteolytic 20 S core, although direct biochemical evidence of such a chaperone-like function of the 19 S cap is lacking (3, 6). The eukaryotic 20 S proteasome consists of 28 subunits with seven different α and β subunits (4, 10, 11), and that in animal cells contains additional nonessential subunits that are γ-interferon-inducible and contribute to antigen processing (12). The crystal structures of the yeast and archaebacterium Thermoplasma acidiophillum proteasomes revealed that the subunits are arranged in a particle comprising four heptamer rings, α-β1-β2-αγ, around a central catalytic cavity (4, 5). Recent mutational studies on the yeast 20 S proteasome revealed three proteolytically active β-type subunits, Pup1, Pre2, and Pre3, homologues of human subunits Z, X, and Y, respectively, that each have a catalytic N-terminal threonine residue and express trypsin-type, chymotrypsin-type, and peptidylglutamyl-peptide hydrolase activities, respectively (4, 13–15). However, the enzymatic activities of the 20 S proteasome other than proteolytic activities and the functions of the other β- and α-type subunits remain to be clarified.

Recently, we found the intrinsic NDP kinase-like activity, but not ATPase activity, of the molecular chaperone, Hsp70 (16), which catalyzes the transfer of γ-phosphoryl groups from NTPs to NDPs. The interaction of Hsp70 with protein or peptide substrates is regulated by the activities of ATP hydrolysis and ADP-ATP exchange. The latter reaction may be catalyzed by the intrinsic NDP kinase-like activity of Hsp70 or through cooperation between the NDP kinase-like activity of Hsp70 and a cytosolic GrpE-like cofactor, resulting in acceleration of the ATP-dependent reaction cycle in protein/peptide folding (16). In this paper, we first report that the eukaryotic 20 S proteasome exhibits a novel function, i.e. intrinsic NDP/NTP exchange activity other than proteolytic activity, the enzymatic properties being similar to those of the NDP kinase-like activity of Hsp70. The C5 subunit among the β-type and the C8 subunit among the α-type subunits of the 20 S proteasome give ATP-binding and autophosphorylated intermediates during the γ-phosphate transfer reaction. We discuss the possible role of this NDP kinase-like activity in the proteolytic function of the proteasome.

EXPERIMENTAL PROCEDURES

Materials—Various ribo- and deoxyribonucleoside tri-, di-, and monophosphates, AMP-NP, ATP-S, DEPC, NDP kinase (EC 2.7.4.6) from human erythrocytes and monoclonal antibody against Hsp70 were purchased from Sigma. [8-3H]ADP, [2-14C]CDP, [8-14C]ATP, and [γ-32P]ATP were obtained from NEN Life Science Products. 8-Azido-[α-
NDP Kinase-like Activity of the 20 S Proteasome

**32P**\(^{32}\)P-ATP was from ICN Biochemicals Inc. (Costa Mesa, CA). Synthetic peptide substrates, i.e. Boc-FSR-MCA, Z-LLL-MCA, and Suc-LLVY-MCA, were products of the Protein Research Foundation (Osaka, Japan). TSK-gel DEAE-SSW, HA-1000, and G-3000 SW columns were purchased from Toyo Soda Co. (Tokyo, Japan).

**Identification of the 20 S Proteasome—Human lymphoblastoma cell line Molt-4, clone 8 (6 × 10^6 cells) were homogenized in 60 ml of 10 mM Tris-HCl, pH 7, containing 135 mM NaCl, and then the homogenate was centrifuged at 100,500 × g for 1 h at 4 °C. The supernatant was brought to pH 5.5 with acetic acid and then centrifuged at 25,000 × g for 20 min. Polyethylene glycol 6000 was added to the supernatant to 10% with stirring, followed by centrifugation. The resulting precipitate was dissolved in a small volume of 25 mM Buffer A (Tris-HCl, pH 6.5) and then subjected to HPLC on a TSK gel DEAE 3SW column (7.5 × 75 mm) with a 65-min linear gradient of 25–300 mM Buffer A. The eluted fractions were monitored for amidolytic activity using Boc-FSR-MCA, Z-LLL-MCA, and Suc-LLVY-MCA as substrates. The proteasome was eluted with 200 mM Buffer A, concentrated with a YM-30 membrane, and then subjected to HPLC on a TSKgel HA-1000 column (7.5 × 75 mm) with a 70-min linear gradient of 10–30 mM Buffer B (potassium phosphate buffer, pH 6.7). The proteasome was eluted with 200 mM Buffer B, concentrated, and then subjected to HPLC on a double-linked TSKgel G3000 SW column (7.5 × 600 mm each) with 25 mM ammonium formate buffer, pH 5.5, containing 1 M urea (Fig. 1A). The purified enzyme was stored at −80 °C in the presence of 50% glycerol. The 20 S proteasome was purified in a 29% yield with 867-fold purification from the sample extract. Partially purified 20 S proteasome from yeast, which was a gift from Dr M. Groll (Max-Plank Institute of Biochemistry), was further purified by subjecting HPLC on a TSKgel HA-1000 column. The procedures used were similar to those for human proteasome.

**Assaying of Enzyme Activities and Substrate Specificities—Amidolytic activity was determined as described (17). The ATP synthesis and ATP hydrolysis activities were analyzed as described (16, 18). The reactions were carried out at 37 °C in Buffer C (100 mM Hepes-KOH buffer, pH 8), containing 5 mM ATP, 0.5 mM ADP, 6 mM MgCl₂, and 0.05 μCi of [\(^{32}\)P]ATP for the assaying of ATP hydrolysis or 0.02 μCi of [\(^{32}\)P]ADP for the assaying of ATP synthesis, in a total volume of 20 μl. The reaction was started by adding the enzyme preparation. After the incubation, ADP and ATP were separated on a polyethyleneimine-cellulose TLC plate.

**SDS-PAGE and Two-dimensional Gel Electrophoresis—**SDS-PAGE was carried out by the method of Laemmli (19). Two-dimensional PAGE was carried out by the method of O'Farrell (20) with a slight modification. A purified enzyme was separated in the first dimension by isoelectric focusing on a gel containing carrier Ampholine producing a pH gradient of 3–10 in the presence of 8.5 M urea and run from the anode to the cathode; for separation in the second dimension, the materials were subjected to SDS-PAGE.

**Detection of Autophosphorylated Intermediates—**The 20 S proteasome (4 μg) was incubated at 37 °C with 10 μCi of [\(^{32}\)P]ATP, 100 μM ATP, 5 mM MgCl₂, and 25 mM Buffer C for 2 h in the absence and presence of 5 mM CDP, in a total volume of 20 μl (16, 18). After the incubation, both reactions were quenched by the addition of 6 mM EDTA, and half of each sample was then treated with the SDS sample buffer (pH 6.8) without boiling and subjected to 15% SDS-PAGE. After electrophoresis, the gel was dried without acid fixation and examined with an imaging analyzer. The other halves of the samples were analyzed by TLC. The acid and base stabilities of the phosphorylated intermediates were analyzed as described previously (16, 18, 21).

**Phosphoamino Acid Analysis—**The alkaline-stable autophosphorylated 20 S proteasome was subjected to SDS-PAGE, transferred to a polyvinylidene difluoride membrane and then subjected to alkaline hydrolysis in 3 M KOH for 100 min at 120 °C as described (22). Phosphoamino acid analysis of the acid-stable phosphorylated proteasome was also performed (23). The hydrolysates were then analyzed by TLC and autoradiography.

**Photoaffinity Labeling with 8-Azido-[\(^{32}\)P]ATP—**The photoprobe, 8-azido-[\(^{32}\)P]ATP (10 μCi), was preincubated with the 20 S proteasome (2 μg) on ice for 20 min in 20 μl of 100 mM Hepes-KOH buffer, pH 7, containing 6 mM MgCl₂, before photolabeling (24, 25). The samples were then irradiated at 254 nm for 5 min, at a distance of 5 cm above the surface of the solution, and then subjected to SDS-PAGE.

**Identification of Subunits with NDP Kinase-like Activity—**The proteasome (100 μg) was incubated with [\(^{32}\)P]ATP to yield autophosphorylated intermediates or with 8-azido-[\(^{32}\)P]ATP to identify the ATP-binding subunits as described above and then separated by reversed-phase HPLC on a Cosmosil 5C4-AR-300 column (4.6 × 150 mm; Nacarai Tesque Inc., Kyoto, Japan) with a linear gradient of 40–60% acetonitrile in 0.1% trifluoroacetic acid for 135 min at the flow rate of 1.0 ml/min. The peptide peak materials that exhibited both radioactivities were S-alkylated and then digested with lysyl endopeptidase (26), and the N-terminal amino acid sequences of the digested peptides were determined with an Applied Biosystems 492 protein sequencer.

![Figure 1](image)

**RESULTS**

**Identification of the ADP-ATP Exchange Activity of the 20 S Proteasome—**The purified 20 S proteasome from human lymphoblastoma Molt-4, clone 8 cells, gave a single symmetrical peak, which coincided with the exact position of the Boc-FSR-MCA hydrolysis and ATP synthesis activities, on gel permeation HPLC (Fig. 1A). The Z-LLL-MCA and Suc-LLVY-MCA

**TABLE 1**

| Subunit | Molecular Weight (kDa) | ATPase Activity (nmol/min/mg) |
|---------|-----------------------|-----------------------------|
| A       | 30                    | 20                          |
| B       | 25                    | 15                          |
| C       | 20                    | 10                          |
| D       | 15                    | 5                           |

**TABLE 2**

| Subunit | Molecular Weight (kDa) | ATPase Activity (nmol/min/mg) |
|---------|-----------------------|-----------------------------|
| E       | 35                    | 30                          |
| F       | 30                    | 25                          |
| G       | 25                    | 20                          |
| H       | 20                    | 10                          |
hydrolysis activities, as well as ATP hydrolysis activity, also coincided with this peak (data not shown). To exclude the possibility of contaminating proteins in our 20 S proteasome preparation with ATP synthesis and ATP hydrolysis activities, such as conventional NDP kinase (27–29) and Hsp70 (16), the purified proteasome was applied to a Mono Q anion-exchange column, which is able to separate conventional NDP kinase protein with a molecular mass of 16 kDa from Hsp70 and/or Hsc70 (16, 27). Conventional NDP kinase and Hsp70, which are eluted with approximately 0.05 m M and 0.1 m M KCl, respectively, in 25 m M Tris-HCl buffer, pH 7.2, containing 0.1 m M EDTA and 0.5 m M dithiothreitol, was not detected in the 20 S proteasome preparation, and the proteasome with the activities of ATP synthesis, ATP hydrolysis, and peptidases was eluted with the much higher concentration of KCl of 0.2–0.3 m on that chromatography (data not shown). The enzyme was apparently homogeneous, as judged by 5% PAGE at pH 8.3 under nondenaturing conditions (data not shown). When the enzyme was denatured with 0.1% SDS and subjected to SDSPAGE, it gave multiple bands in the range of 21–31 kDa, and no protein band with a molecular mass of over 32 kDa was observed (Fig. 1B). Furthermore, no immunoreactivity of the purified proteasome against anti-Hsp70 and anti-NDP kinase-A antibodies was observed. Purified enzyme from yeast also revealed multiple bands in a similar range, but the exact electric mobilities of separated subunits were somewhat different from those of the subunits from human proteasome (Fig. 1B, lane 2). These findings are in good agreement with those reported for eukaryotic 20 S proteasomes (30, 31). Two-dimensional PAGE of the human proteasome gave 15 or 16 characteristic separated components (Fig. 1C); this pattern is similar to that reported previously (30, 32, 33).

The activities of ATP synthesis and ATP hydrolysis of the 20 S proteasome were further characterized, and the results are shown in Fig. 2. Although many data for the ATPase activities of ATP-dependent proteases and molecular chaperone proteins reported were obtained by measurement of inorganic phosphate liberated from ATP, we found that the human 20 S proteasome exhibits ATP hydrolysis, as well as the reverse activity, i.e. ATP synthesis, and thus the amount of inorganic phosphate in the reaction mixture is not directly reflected by the ATP hydrolysis activity in the presence of ADP in the reaction mixture. In this regard, we analyzed the ATP hydrolysis activity by measuring the conversion of [14C]ATP to [14C]ADP in the presence of 0.5 m M ADP, and the ATP synthesis activity was analyzed by measuring the conversion of [14C]ADP to [14C]ATP (16, 18).

The human proteasome catalyzed the synthesis of ATP at a slow but linear rate in the presence of ADP (0.5 m M) and ATP (5 m M), but no activity was observed in the presence of AMP instead of ADP or in the presence of ADP and Pi (5 m M) instead of ATP in the reaction mixture (Fig. 2A). These results indicate that the ATP synthesis activity of the proteasome is entirely different in nature from those of ATP synthase and adenylate kinase, which catalyzes the reversible ATP-dependent synthesis of ADP from AMP. No activity was observed for the control protein, bovine serum albumin (data not shown). On the other hand, the 20 S proteasome exhibited weak and linear ATP hydrolysis activity (Fig. 2B). Although ADP is a product inhibitor of common ATPases, a limited amount of ADP, i.e. concentrations between 0.1 and 1.0 m M in the reaction mixture, stimulated the ATPase activity of the proteasome by 5–6-fold, and ADP in excess of 1 m M suppressed the stimulated ATP hydrolysis activity (data not shown). In contrast to the effect of ADP, AMP (0.5 m M) had no effect on the ATP hydrolysis. From the kinetic results for ATP hydrolysis and for ATP synthesis, the equilibrium constant of ATP synthesis was calculated to be 2.86 and 0.14 m M, respectively. These Km values are in a similar range to those reported for NDP kinase, Hsp70, and 14-3-3 proteins (16, 18, 28). Furthermore, because both the Km values are in a range slightly lower than the concentrations of ATP (5 m M) and ADP (0.5 m M) in the cytosol, changes in the levels of these nucleotides in the cytosol would be expected to significantly affect the in vivo enzyme activities. The pH optimum for ATP hydrolysis and ATP synthesis of the 20 S proteasome was determined using 100 m M Mes buffer (pH 5.5–6), Hepes buffer (pH 7–8), Chaps buffer (pH 9), and Caps buffer (pH 10). In the presence of 0.5 m M ADP, the optimum pH for the enhanced ATP hydrolysis of the proteasome was 7.5–9, and the pH optimum for ATP synthesis was 7–9. These values are identical to those of Hsp70 (16). It is noteworthy that at pH 7–8, the rate constants for ATP hydrolysis and ATP synthesis of the proteasome were in a range similar to the physiological concentrations of 5 m M ATP and 0.5 m M ADP in the reaction mixture. Therefore, the ATPase activity of the 20 S proteasome is hard to detect at neutral pH, as judged on measurement of the release of inorganic phosphate from ATP. The function of the proteasome, which catalyzes both ATP hydrolysis and ATP synthesis, is an ADP/ATP exchange reaction, and the catalytic properties are similar to those of NDP kinase reported previously (27, 28).

The ATP synthesis and ATP hydrolysis activities of the yeast

---

**NDP Kinase-like Activity of the 20 S Proteasome**

---

**Fig. 2. Characterization of the activities of ATP synthesis (A) and ATP hydrolysis of the proteasome (B).** The measurement of ATP synthesis and ATP hydrolysis activities of the human proteasome was carried out at 37°C for 1 h as described under “Experimental Procedures,” in the presence of 5 m M ATP and 0.5 m M ADP (●), and 0.05 μCi of [14C]ATP for the assaying of ATP hydrolysis or 0.02 μCi of [14C]ADP for the assaying of ATP synthesis, in a total volume of 20 μl. In the reaction mixture, the nucleotide was removed or replaced as follows: the activities in the absence of ADP in the reaction mixture (●), those in the presence of AMP (0.5 m M) instead of ADP (□), those in the presence of Pi (5 m M) instead of ATP for the assaying of ATP synthesis (○), and those in the absence of ATP for the assaying of ATP hydrolysis (○). The activities of ATP hydrolysis and ATP synthesis of the yeast proteasome in the presence (●) or the absence (○) of 0.5 m M ADP in the reaction mixture were also assayed.
proteasome were also observed in the presence of ATP (5 mM) and ADP (0.5 mM) in the reaction mixture, as shown in Fig. 2, but, unlike human proteasome, ATP hydrolysis activities of the yeast proteasome in the absence of ADP were significantly lower than those of human proteasome and little detected.

The 20 S Proteasome Functions as a NDP Kinase-like Enzyme—Although NDP kinase utilizes any NTP as a phosphate donor with nearly the same efficiency (27), the human proteasome utilized ATP most efficiently and dATP moderately, the other NTPs being utilized at rates of only 2–30% of that in case of ATP (Fig. 3A). The ATP analogs, ATPγS and AMP-PNP, were not utilized, suggesting that the enzyme transfers the terminal phosphate of a NTP to an NDP.

We next examined the specificity of each nucleotide as an acceptor for the transfer of the \([\gamma^{-32P}]\)phosphate of ATP, as catalyzed by the human proteasome. The enzyme converted all NDPs and dNDPs, except GDP, to the corresponding NTPs and dNTPs with nearly the same efficiency (28, 34, 35). Under these assay conditions, the enzyme converted all NDPs and dNDPs to the corresponding NTPs and dNTPs with almost the same efficiency (28, 34, 35). Under these assay conditions, the conversion of ATP and dATP to ATP and dATP, respectively, could not be analyzed, because the newly formed products overlapped the phosphate donor, \([\gamma^{-32P}]\)ATP. Nevertheless, it was confirmed that the rates of conversion by the proteasome of \([^{14}C]ATP\) and \([^{14}C]CDP\) to the corresponding NTPs exhibited similar efficiency (data not shown). These results indicate that the substrate specificities of the 20 S proteasome are distinctly different from those of conventional NDP kinase (27) and are similar to those of chaperones, such as the Hsp70 and 14-3-3 proteins (16, 18), and hence, this enzyme activity of the proteasome is referred to as chaperone-type NDP kinase-like activity.

Formation of Autophosphorylated Intermediate Subunits and Their CDP-dependent Dephosphorylation (A and B) and photoaffinity labeling of the proteasome with 8-azido-[\(\gamma^{-32P}\)]ATP (C). A, the purified human 20 S proteasome (4 \(\mu\)g) was analyzed by SDS-PAGE under denaturing and reducing conditions, followed by Coomassie Brilliant Blue staining (lane 1). The proteasome was incubated at 37 °C with 10 \(\mu\)Ci of \([\gamma^{-32P}]\)ATP, 100 \(\mu\)M ATP, and 6 mM MgCl\(_2\) in Buffer C for 2 h in the absence (lane 2) or presence (lane 3) of 5 mM CDP as a phosphate acceptor, in a total volume of 20 \(\mu\)l. After the incubation, both reactions were quenched by the addition of 6 mM EDTA, and half of each sample was then treated with the SDS sample buffer (pH 6.8) without boiling and subjected to 15% SDS-PAGE. After the electrophoresis, the gel was dried without acid fixation under alkaline conditions (pH 8) (lane 4) or fixed with 20% trichloroacetic acid followed by drying (lane 5) and analyzed with an imaging analyzer. B, after the incubation, the samples in the absence (lane 1) and presence (lane 2) of 5 mM CDP were analyzed by TLC as described (16). C, the proteasome (2 \(\mu\)g) was incubated with 8-azido-[\(\gamma^{-32P}\)]ATP (10 \(\mu\)Ci), in the presence of 6 mM MgCl\(_2\) without (lane 1) or with (lane 2) 5 mM cold 8-azido ATP in the reaction mixture, irradiated under the conditions given under “Experimental Procedures,” and then subjected to SDS-PAGE. Large arrowheads, 28–29-kDa proteins; small arrowheads, 26–27-kDa proteins.
The findings of NDP kinase-like activity and autophosphorylated intermediate subunits of the proteasome suggest that the proteasome has an ATP-binding site(s) other than catalytic sites. Furthermore, ATP binding is crucial for clarifying the intrinsic NDP kinase-like activity of the proteasome. The azido photoprobe, 8-azido-[α-32P]ATP, was used to identify the ATP-binding subunits of the proteasome. The covalent photoincorporation of 8-azido-[α-32P]ATP into the proteasome specifically occurred upon activation by UV light, and the incorporation of 32P was saturated after a 5-min exposure to UV light. No increase in the extent of photolabeling was observed at 7 or 10 min. As shown in Fig. 4C, specific covalent photoincorporation of 8-azido-[α-32P]ATP into two protein band materials of the proteasome was observed, and these bands corresponded to molecular masses of 28–29 and 26–27 kDa, respectively, being identical to those of autophosphorylated intermediates generated during the catalytic phosphate transfer reaction. No other protein labeled with 8-azido-[α-32P]ATP was detected in our preparation.

**Identification of the Subunits Responsible for the NDP Kinase-like Activity**—The peptidase activities of the proteasome are generated through the cooperation of the active β-type subunits (X, Y, and Z) and the inactive subunits in the fully assembled form (13–15, 41), and no peptidase activity is observed after dissociation and/or separation of the proteasomal subunits (7, 42). In analogy with the peptidase activities, the NDP kinase-like activity of the 20S proteasome was almost completely lost on separation of the subunits by reversed-phase fractionation bands corresponding to molecular masses of 28–29 and 26–27 kDa in the absence of a phosphate acceptor, CDP, in the reaction mixture. Under the conditions used, the formation of CTP from CDP was not observed (Fig. 4B, lane 1). In the presence of 5 mM CDP, however, the radioactivities of the phosphorylated intermediates decreased significantly with the concomitant formation of 32P-labeled CTP from CDP, as shown in Fig. 4, A, lane 3, and B, lane 2, respectively. These results indicate that the proteasome catalyzes the transfer of a γ-phosphate group from ATP to CDP and that this transfer involves phosphoester intermediates. The proteasome, however, was not autophosphorylated by [α-32P]ATP instead of [γ-32P]ATP (data not shown). These phosphorylation properties are similar to those of conventional NDP kinase, Hsp70, and 14-3-3 protein reported previously (16, 18, 36–38). To characterize the phosphorylation of the enzyme, the autophosphorylated proteasome was subjected to acid and alkali treatment, which allows evaluation of a high energy phosphate on an active site histidine residue in NDP kinase or histidine protein kinase (16, 18, 21, 37). The majority of the phosphorylated intermediates of the proteasome was stable to alkali but labile as to acid (Fig. 4A, lanes 4 and 5, respectively), and significant decreases in the radioactivities of the autophosphorylated proteins were observed under acidic conditions. Because phosphorylated serine, threonine, and tyrosine residues are acid-stable, the acid-labile phosphorylated residue(s) in the proteasome may be basic amino acid(s). Phosphoamino acid analysis of the remaining and acid-resistant phosphorylated proteasome in Fig. 4, lane 5, was performed as described (23). Following electrophoresis of the hydrolyzed materials to a cellulose gel TLC plate, autoradiography revealed only one weak spot migrating with exactly the same mobility as the standard phosphoserine (data not shown). These results suggest that the proteasome catalyzes the phosphotransfer reaction between NTPs and NDPs as well as the phosphotransfer reaction on proteins. The results suggest that autophosphorylation of phosphoester intermediates takes place on the active site basic amino acids, and then phosphorylation of serine residue(s) probably occurs downstream in the same manner as in the cases of NDP kinase and histidine protein kinase.

Because NDP kinase autophosphorylates the active site histidines of its intermediate in the catalytic phosphate transfer reaction (21, 36–38), we examined the modification of the histidyl residue in the proteasome by DEPC, which selectively acylates histidyl residues to yield N-carbethoxy-histidyl derivatives (39). DEPC inhibited the NDP kinase-like activity of the proteasome in a time- and concentration-dependent manner, as shown in Fig. 5A, but modification of lysyl residues by pyridoxal phosphate had little effect (data not shown). This inactivation by DEPC was prevented by preincubation with ATP (5 mM) for 30 min. Furthermore, DEPC inhibited the autophosphorylation of the proteasome, and hydroxylamine, which re-generates free histidyl residues (39), nearly completely restored the NDP kinase-like activity of the proteasome within 6 h after its addition (data not shown). To verify histidine autophosphorylation of the proteasome in the catalytic phosphate transfer reaction, alkaline hydrolysis of the phosphorylated proteasome was carried out in 3 M KOH for 100 min at 120 °C, and phosphoamino acids were separated by TLC. As shown in Fig. 5B, N1-phosphohistidine was detected in the proteasome hydrolysate in analogy with that from NDP kinase from human erythrocytes, as expected (22). Collectively, these data suggest that histidine residue(s) facilitates the phosphate transfer in the NDP kinase-like reaction of the proteasome.

**ATP Binding to the Proteasome**—The finding of NDP kinase-like activity and autophosphorylated intermediate subunits of the proteasome suggest that the proteasome has an ATP-binding site(s) other than catalytic sites. Furthermore, ATP binding is crucial for clarifying the intrinsic NDP kinase-like activity of the proteasome. The azido photoprobe, 8-azido-[α-32P]ATP, was used to identify the ATP-binding subunits of the proteasome. The covalent photoincorporation of 8-azido-[α-32P]ATP into the proteasome specifically occurred upon activation by UV light, and the incorporation of 32P was saturated after a 5-min exposure to UV light. No increase in the extent of photolabeling was observed at 7 or 10 min. As shown in Fig. 4C, specific covalent photoincorporation of 8-azido-[α-32P]ATP into two protein band materials of the proteasome was observed, and these bands corresponded to molecular masses of 28–29 and 26–27 kDa, respectively, being identical to those of autophosphorylated intermediates generated during the catalytic phosphate transfer reaction. No other protein labeled with 8-azido-[α-32P]ATP was detected in our preparation.

**Identification of the Subunits Responsible for the NDP Kinase-like Activity**—The peptidase activities of the proteasome are generated through the cooperation of the active β-type subunits (X, Y, and Z) and the inactive subunits in the fully assembled form (13–15, 41), and no peptidase activity is observed after dissociation and/or separation of the proteasomal subunits (7, 42). In analogy with the peptidase activities, the NDP kinase-like activity of the 20S proteasome was almost completely lost on separation of the subunits by reversed-phase fractionation.
The amino acid sequences of the peak 8 and 13 materials were determined after S-alkylation and lysylendopeptidase digestion. Sequence analyses of the digested fragments of the peak 8 material revealed the sequences\(^{79}\)VIGCSGFHD-CLTLTK\(^{84}\) and \(^{186}\)NMQNVHVPDLDRAML\(^{202}\), indicating that the peak 8 material corresponds to the human C5 subunit with a molecular mass of 26–27 kDa. Sequence analysis of the digested fragment of the peak 13 revealed the sequence\(^{16}\)IIVYIHEVR\(^{25}\), indicating that the peak 13 corresponds to human C8 subunit with a molecular mass of 28–29 kDa. In studies on CDP-dependent dephosphorylation of the autophosphorylated proteasome, almost complete suppression of the autophosphorylation of the peak 8 and 13 materials was observed on the addition of 1 mM CDP as an acceptor of phosphate at the beginning of the reaction (Fig. 6B). Significant decreases in the autophosphorylated radioactivities of the peak 8 and 13 materials were also observed on the addition of 1 mM CDP for 3 h after the first reaction for 2 h for the formation of autophosphorylated intermediates. These results indicate that the C5 among the β-type and C8 among the α-type subunits of the proteasome exhibit NDP kinase-like activity.

**DISCUSSION**

In the present study, we found a novel function, intrinsic ADP/ATP exchange activity, of the proteasome; this activity is similar to that of NDP kinase. The exchange reaction exhibited a preference for ATP and dATP as phosphate donors and a broad specificity for NDPs other than GDP as phosphate acceptors, and yielded autophosphorylated intermediates during the transfer of γ-phosphate from NTPs to NDPs. Judging from the kinetic results for ATP hydrolysis and ATP synthesis of the proteasome, the rate constants for the two reactions were almost the same at neutral pH. Consequently, the ATP hydrolysis activity of the 20S proteasome determined as the release of \text{P}_i from ATP at neutral pH was hard to detect, and no ATPase activity of the proteasome has been reported so far. However, the activities of ATP hydrolysis and ATP synthesis of the proteasome can be analyzed as the conversion of \text{[\text{14C}]ATP} to \text{[\text{14C}]ADP} and of \text{[\text{14C}]ADP} to \text{[\text{14C}]ATP}, respectively, in the presence of 5 mM ATP and 0.5 mM ADP in the reaction mixture. The nucleotide specificity and other enzymatic properties of the NDP kinase-like activity of the proteasome are similar but not identical to those of conventional NDP kinase (27, 28) and show a striking resemblance to those of an intrinsic NDP kinase-like activity (\text{not ATPase activity}) of the molecular chaperones such as Hsp70 (16), DnaK,\(^{2}\) and the mitochondrial import stimulation factor 14-3-3 protein (18, 43). In the mechanism for the Hsp70-protein/peptide interaction, ADP-ATP exchange is rate-limiting in the cycle of substrate binding and release, and the intrinsic ADP-ATP exchange activity of Hsp70 may responsible for the ATP-dependent reaction cycle in protein folding (16), although this remains to be proven. We found that the C5 among the β-type and the C8 among the α-type subunits of the proteasome exhibit intrinsic chaperone-type NDP kinase activity, which catalyzes ATP hydrolysis as well as ATP synthesis.

NDP kinase autophosphorylates the active site histidine residue in the catalytic phosphate transfer reaction, and hence, NDP-dependent dephosphorylation of the autophosphorylated intermediate occurs (36–38). In addition, NDP kinase also exhibits histidine-dependent protein phosphotransfer activity, \textit{i.e.} downstream phosphorylation of serine residues from the acid-labile phosphohistidine residue (22). The C8 and C5 subunits of the proteasome gave autophosphorylated intermediates during the transfer of γ-phosphate from NTPs to NDPs. Most of the autophosphorylated intermediates of the protea-

---

\(^{2}\) Kido, H., Yano, M., and Kanesaki, Y., manuscript in preparation.
some were acid-labile, and phosphoamino acid analysis of the proteasome revealed an alkaline-stable histidine autophosphorylation (Fig. 5B). In addition, chemical modification of the histidine residues of the proteasomal subunits by DEPC inhibited the NDP kinase-like activity (Fig. 5A) and their autophosphorylation (data not shown). These results suggest the high possibility of a high energy phosphate on histidine residues of these subunits. The minor acid-stable phosphorylation, which was indicated by the remaining radioactivity after acid treatment, was determined to be due to phosphoserine residue(s) on phosphoamino acid analysis. These results are consistent with those of autophosphorylation and histidine-dependent protein phosphorylation of conventional NDP kinase and histidine protein kinase reported (22, 36–38). Furthermore, ATP binding of the C8 and C5 subunits of the proteasome was evident on specific photoaffinity labeling with 8-azido-ATP. Previously, the specific phosphorylation of serine residues of the C8 subunit by casein kinase II and those of C9 by cGMP-dependent protein kinase and/or casein kinase II in vitro was reported (44–46). In our purified proteasome preparation, however, no protein kinase activity toward casein was present (data not shown). The phosphorylation of serine residue(s) of the C8 subunit reported may be caused in part by protein phosphotransfer activity of the C8 subunit with NDP kinase-like activity.

The photoaffinity labeling studies involving 8-azido-ATP revealed that ATP binds to the NDP kinase-like C5 and C8 subunits. Many MgATP-binding proteins contain the consensus sequences of the two conserved domains designated as the Walker segment A and B motifs (47). Segment A (ATP-binding motif) consists of GXXXXG (where X is any amino acid) (48–50). Although the original Walker sequence for segment B (Mg$^{2+}$-binding motif) is rather long (2R/KXXXXXXXV/Y/X-hXAD, where Xh represents any hydrophobic residue), others have reported several shorter, modified versions, the key residue in all these sequences being the aspartic acid that lies at the end of the predicted b-sheet domain (51, 52). Although the ATP-binding site of the C5 subunit has not yet been clarified experimentally, we found the sequences 140GLDEEGK146 and 132GLDDEGK138 in the C5 subunits of the human and yeast proteasome, respectively, which closely resemble the Walker segment B motif. The C8 subunit of the human and yeast proteasome, however, does not contain any known ATP-binding motifs, and thus it may contain some other, more distant Walker motif homologues. NDP kinase has a three-dimensional ATP-binding domain structure (53) and exhibits no sequence similarity to Walker motifs. Further studies on the three-dimensional structure similarity between the proteasome C5 and C8 subunits and NDP kinase or ATP-dependent chaperones Hsp70 and Hsc70 are required to elucidate the nucleotide-binding structures of these subunits.

Although ATP and ADP had no effect on the proteolytic activity of the 20 S proteasome (data not shown), the enzyme catalyzes the activities of both ATP hydrolysis and ATP synthesis, resembling Hsp70 (16). There is strong evidence that the b-type subunits act in pairs, i.e. an “active” subunit must interact with an “inactive” subunit to generate peptidase activity (2, 4, 6, 13–15). Furthermore, the NDP kinase-like activity of the proteasome may be such a case, because the proteasome lost its NDP kinase-like activity after separation of the subunits. Recent structure analysis revealed that protease subunits Z (PUP1) and X (PRE2 or MB1) are neighbors of subunit C5 and that protease subunit Y (PRE3) and NDP kinase-like subunit C5 are neighbors of subunit C8 (4, 54). Although the C5 and C8 subunits do not directly participate in the mechanism of protein and peptide hydrolysis, these subunits may play some role in assisting the interactions between specific pairs of subunits to generate the peptidase activity or in supporting the assembly of the proteasomal subunits. It was recently reported that the human C8 subunit, but not other a-type subunits, forms hetero-oligomeric ring complexes with co-expression of neighboring a-type subunits (55). In the case of a heat-shock and ATP-dependent protease complex, HsIIVU, from Escherichia coli, ATP binding is required for oligomerization of HsIIVU, which is required for the association with HsIV peptidase (56, 57). Judging from these results, although the function of the chaperone-type NDP kinase-like activity of the C8 and C5 subunits has not been elucidated, the activity may participate in the subunit interactions. The intrinsic chaperone-like NDP kinase activity constitutes an important clue for a better understanding of the unknown mechanism of proteolysis by the proteasome. Further studies on the role of the NDP kinase-like activity in the proteolytic activity of the proteasome and in subunit interactions are currently in progress.

Acknowledgments—We thank Drs. R. Huber and M. Groll (Max Plank Institute of Biochemistry) for helpful discussions.

REFERENCES
1. Seemüller, E., Luras, A., Zühl, F., Zwickl, P., and Baumeister, W. (1995) Science 268, 578–582
2. Heinemeyer, W., Fischer, M., Krummen, T., Stachon, U., and Wolf, D. H. (1997) J. Biol. Chem. 272, 25200–25209
3. Coux, O., Tanaka, K., and Goldberg, A. (1996) Annu. Rev. Biochem. 65, 841–847
4. Moller, D., Ditzel, L., Løwe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Nature 386, 463–471
5. Löwe, J., Stock, D., Bap, Z., Zwickl, P., Baumeister, W., and Huber, R. (1995) Science 268, 533–539
6. Baumeister, W., Cejka, Z., Kania, M., and Seemüller, E. (1997) Biol. Chem. 378, 121–130
7. Orlovski, M., Cardozo, C., and Michaud, C. (1993) Biochemistry 32, 1563–1572
8. Dubiel, W., Fehling, H. J., and Sefton, B. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 526–530
9. von Boehmer, H. (1994) Science 265, 1241–1246
10. Heinemeyer, W., Kleinschmidt, L. A., Sadowsky, J., Escher, C., and Wolf, D. H. (1991) EMBO J. 10, 555–562
11. Freier, J. M., Brey, P., MacDonald, N. J., Mann, R. E., and Steeg, P. S. (1995) J. Biol. Chem. 270, 5525–5532
12. Heinemeyer, W., Grubh, A., Mohrle, V., Mahe, Y., and Wolf, D. H. (1993) J. Biol. Chem. 268, 5115–5120
13. Hijikura, M., Yano, M., Mori, H., Inoue, M., and Kido, H. (1998) J. Biol. Chem. 273, 5435–5438
14. Kido, H., Fukutomi, A., and Katunuma, N. (1990) J. Biol. Chem. 265, 20379–20385
15. Yano, M., Mori, S., Inoue, Y., and Kido, H. (1997) FEBS Lett. 419, 244–248
16. Lasken, U. K. (1979) Nature 280, 689–685
17. O’Farrell, P. H. (1975) J. Biol. Chem. 250, 4007–4021
18. Parks, R. E., and Agarwal, R. P. (1973) Enzymes 8, 307–334
19. Freier, J. M., Brey, P., MacDonald, N. J., Mann, R. E., and Steeg, P. S. (1995) J. Biol. Chem. 270, 5525–5532
20. Hunter, T., and Sefton, B. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 77, 1311–1315
21. Czarnecki, J., Geahlen, R., and Haley, B. (1979) Methods Enzymol. 56, 412–453
22. Kon, N., and Unah, R. J. (1996) J. Biol. Chem. 271, 19883–19990
23. Kido, H., Yokogoshi, Y., and Katunuma, N. (1988) J. Biol. Chem. 263, 3104–3107
24. Leung, S. M., and Hightower, L. E. (1997) J. Biol. Chem. 272, 2607–2614
25. Roisin, M., and Kepes, A. (1978) Biochim. Biophys. Acta 526, 418–428
26. Ingram, J. L., and Ginter, C. L. (1978) Methods Enzymol. 52, 371–375
27. Tanaka, K., Yoshihurra, T., Ichihara, A., Iki, A., Nishigai, M., Morimoto, Y., Sato, M., Tanaka, N., Katsu, K., and Takagi, T. (1988) J. Biol. Chem. 263, 585–596
28. Tanaka, K., Yoshimura, T., Numata, A., Ichihara, A., Iki, A., Nishigai, M., Kameyama, K., and Takagi, T. (1990) J. Biol. Chem. 265, 16209–16217
29. Schmidtke, G., Schmidt, M., and Klotz, P.-M. (1997) J. Biol. Chem. 268, 95–106

NDP Kinase-like Activity of the 20 S Proteasome
33. Kristensen, P., Johnsen, A. H., Uerkvitz, W., Tanaka, K., and Hendil, K. B. (1994) Biochem. Biophys. Res. Commun. 205, 1785–1789
34. Agarwal, R. P., Robison, B., and Parks, R. E., Jr. (1978) Methods Enzymol. 52, 376–386
35. Shikata, H., Egi, Y., Koyama, S., Yamada, K., and Kawasaki, T. (1989) Biochem. Int. 18, 943–949
36. Biondi, R. M., Waltz, K., Issinger, O.-G., Engel, M., and Passeron, S. (1996) Anal. Biochem. 242, 165–171
37. Deville-Bonne, D., Sellam, O., Merola, F., Lascu, I., Desmadril, M., and Veron, M. (1996) Biochem. Int. 35, 14643–14650
38. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
39. Miles, E. W. (1977) Methods Enzymol. 47, 431–442
40. Wei, Y.-F., and Matthews, H. R. (1991) Methods Enzymol. 200, 388–414
41. Fenteany, G., Standaert, R. F., Lane, W. S., Choi, S., Corey, E. J., and Schreiber, S. L. (1995) Science 268, 726–731
42. Arendt, C. S., and Hochstrasser, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7156–7161
43. Hachiya, N., Mihara, K., Suda, K., Horst, M., Schatz, G., and Lithgow, T. (1995) Nature 378, 705–709
44. Ludemann, R., Lerea, K. M., and Etlinger, J. D. (1993) J. Biol. Chem. 268, 17413–17417
45. Mason, G. G. F., Hendil, K. B., and Rivett, A. J. (1996) Eur. J. Biochem. 238, 453–462
46. Castano, J. G., Mahillo, E., Arizti, P., and Arribas, J. (1996) Biochemistry 33, 3782–3789
47. Walker, J. E., Saraste, M., Runswick, M. L., and Gay, N. J. (1982) EMBO J. 1, 945–951
48. Moeller, W., and Amon, R. (1985) FEBS Lett. 186, 1–7
49. Reinstein, J., Brune, M., and Wittinghofer, A. (1988) Biochemistry 27, 4712–4720
50. Black, M. E., and Hruby, D. E. (1992) J. Biol. Chem. 267, 6801–6806
51. Chin, D. T., Goff, S. A., Webster, T., Smith, T., and Goldberg, A. L. (1988) J. Biol. Chem. 263, 11718–11728
52. Myles, G. M., Hearst, J. E., and Sancar, A. (1991) Biochemistry 30, 3824–3834
53. Williams, R. L., Oren, D. A., Munoz-Dorado, J., Inouye, S., Inouye, M., and Arnold, E. (1993) J. Mol. Biol. 234, 1230–1247
54. Dahlmann, B., Kopp, P., Kristensen, P., and Hendil, K. B. (1999) Arch. Biol. Biophys. 363, 296–300
55. Gerards, W. L. H., de Jong, W. W., Bloemendal, H., and Boelens, W. (1998) J. Mol. Biol. 275, 113–121
56. Yoo, S. J., Sied, J. H., Seong, I. S., Kang, M.-S., and Chung, C. H. (1997) Biochem. Biophys. Res. Commun. 238, 581–585
57. Goldberg, A. L., Akopian, T. N., Kisselev, A. F., Lee, D. H., and Rohrwild, M. (1997) Biol. Chem. 378, 131–140