Nα-Acetylation and Proteolytic Activity of the Yeast 20 S Proteasome*

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Nα-Acetylation, catalyzed co-translationally with Nα-acetyltransferase (NAT), is the most common modifications of eukaryotic proteins. In yeast, there are at least three NATs: NAT1, MAK3, and NAT3. The 20 S proteasome subunits were purified from the normal strain and each of the deletion mutants, nat1, mak3, and nat3. The 20 S proteasomes derived from either the normal strain or the mutants indicated that the α1, α2, α3, α4, α7, and β3 subunits were acetylated with NAT1, the α5 and α6 subunits were acetylated with MAK3, and the β4 subunit was acetylated with NAT3. Furthermore, the Ac-Met-Phe-Leu and Ac-Met-Phe-Arg termini of the α5 and α6 subunits, respectively, extended the known types of MAK3 substrates. Thus, nine subunits were Nα-acetylated, whereas the remaining five were processed, resulting in the loss of the N-terminal region. The 20 S proteasomes derived from either the nat1 mutant or the normal strain were similar in respect to chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing activities in vitro, suggesting that Nα-acetylation does not play a major functional role in these activities. However, the chymotrypsin-like activity in the absence of sodium dodecyl sulfate was slightly higher in the nat1 mutant than in the normal strain.

Nα-Acetylation that occurs co-translationally is one of the most common protein modifications, occurring on 50–90% of all eukaryotic proteins (1, 2). In eukaryotes, protein synthesis is initiated with Met. The initiator Met is co-translationally removed from proteins by methionine aminopeptidase if proteins have the penultimate residue with short radii of gyration (3). After the removal of Met, the N-terminal residues such as Ser, Ala, Gly, or Thr, in the context of special sequences, are acetylated with an Nα-acetyltransferase (NAT),¹ called NAT1 (or Nat1p) (4–6). When proteins have the penultimate residue, such as charged amino acids and amino acids with large side chains, the initiator Met is not removed. In this case, the N-terminal Met of proteins with the penultimate residue of Asp or Glu is acetylated with the second NAT, NAT3 (also called Nat3p) (6), whereas certain other proteins with Met at the N termini are acetylated with the third NAT, MAK3 (also called Mak3p) (6, 7). Although NAT1, NAT3, and MAK3 are not essential for viability (6), NAT1 deletion mutants are known to express phenotypes such as slow growth, lack of repression of a silent mating type locus (HML), and failure to sporulate and to advance G0 phase in the cell cycle (5, 8, 9). The pleiotropic phenotypes of nat1 indicate that NAT1 may be required for the function of protein(s) involved in the regulation of the cell cycle and the repression of HML. Furthermore, mak3 mutants exhibited nearly complete lack of growth on YPG medium at 37 °C (6, 7), and the growth of nat3 strains were severely retarded on both YPD and YPG media at both temperatures, but especially on YPG medium at 37 °C and on NaCl-containing media (6). Also, the mating efficiency of MATα nat3 cells, but not MATα nat3 cells, was reduced by nearly three orders of magnitude. The viability of the NAT mutants allows the determination of which proteins are acetylated by each of the NATs.

In eukaryotic cells, ubiquitin-dependent proteolysis underlies the bulk of non-lysosomal protein degradation (10), and the ubiquitin-proteasome pathway is a major mediator of post-translational control, which functions in the control of cell proliferation, cell cycle, and other processes (11). Naturally short-lived as well as damaged or otherwise abnormal proteins are recognized by the ubiquitin system and marked for degradation by the attachment of multi-ubiquitin chains. The ubiquitinated proteins are destined to be degraded by the 26 S proteasome in an ATP-dependent manner.

The 26 S proteasome consists of a pair of 19 S regulatory complexes and a single 20 S proteasome (12, 13). The 20 S proteasome has no protease activity in a latent form that can be activated in vitro by mild chaotrophic agents such as SDS and heat treatment without the 19 S regulatory complexes (13). The 20 S proteasome is a barrel-like particle appearing as a stack of four rings made up of two outer α-rings and two inner β-rings, being associated in the order of αββα (14). The α- and β-rings are each made up of seven structurally related subunits. Because the entrance of the latent 20 S proteasome cavity is closed, proteins to be degraded cannot penetrate into the cavity. The activation of the 20 S proteasome by SDS and heat is suggested to be due to the opening of the entrance of the cavity (15).

In the eukaryotic 20 S proteasome, among seven distinct β subunits, only three, β1, β2, and β5, contain the保守
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N-terminal Thr, which acts as a nucleophile in catalyzing the hydrolysis of peptide bonds of polypeptide substrates. The β3, β2, and β1 subunits have chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing (PGPH) activities, respectively. The N-terminal Thr is located on the surface of cavity formed by two rings of the β subunits (14, 16). These subunits are synthesized as an inactive precursor containing a propeptide that is considered to be autocatalytically cleaved, yielding the mature β subunits with the N-terminal Thr (14–19). In yeast, these three subunits are not N-terminally modified (18, 20–22). However, it is unknown whether the other subunits of the yeast 20 S proteasome are N-terminally modified.

In this study, the yeast 20 S proteasome subunits were purified from the nat1, mak3, and nat3 mutants, and the normal strain and N-terminal modifications of these subunits were investigated. The concomitant chymotrypsin-like, trypsin-like, and PGPH activities of the 20 S proteasome were compared between the nat1 mutant and the normal strain to investigate the effect of N*-acetylation on functions of the 20 S proteasome.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—The following strains of yeast were used in this study: the normal strain, B-3852 (MATα ura3-52 CYC1-963 cyc7-67 lys5-10); the nat1 mutant, B-3830 (MATα nat1::URA3 ura3-52 CYC1-963 cyc7-67 lys5-10); the mak3 mutant, B-9074 (MATα mak3::URA3 CYC1-963 cyc7-67 lys5-10); and the nat3 mutant, B-11974 (MATα nat3::kanMX2 CYC1-963 cyc7-67 lys5-10). Yeast cells were grown in YPD medium (1% (w/v) Bacto-yeast extract, 2% (w/v) Bacto-peptone, and 2% (w/v) glucose) at 30 °C for two days.

**Purification of the 20 S Proteasomes**—Yeast cells were disrupted by homogenization in 50 mM Tris-HCl buffer (pH 7.5) containing 1 μg/ml leupeptin, 1 μM ethylene-diamine-N,N,N,N*-tетраэтиловая кислота, 1 mM di-thiothreitol, and 20% (v/v) glycerol with the Bead-Beater (BioSpec Products, Bartlesville, OK). The 20 S proteasome from yeast extracts was purified by sequential chromatography on octyl-Sepharose CL-4B, Bio-Gel A-1.5 m, DEAE-Sepharose CL-6B, PD-10, and FPLC Mono-Q columns. The purification was performed according to the method reported previously (23), except that Bio-Gel A-1.5 m gel filtration was used instead of dialysis (24) and FPLC with a Mono-Q column was used as the final purification step (25).

**Protein Assay**—Protein concentration was determined by the Bio-Rad protein assay, based on the method of Bradford (26), with bovine serum albumin as a standard.

**Assay of Peptidase Activity**—Peptidase activity was assayed according to the method of Tanaka et al. (27) with slight modification. The proteasome (2 μg) was incubated at 30 °C for 1 h in the presence or absence of SDS in 100 mM Tris-HCl (pH 7.5) containing 0.1 mM fluorescent synthetic peptide substrates such as succinyl-Leu-Leu-Val-Tyr 4-methyl-coumaryl-7-amide (Suc-LLVY-MCA), t-butoxy-carbonyl-Leu-Arg-Arg-MCA (Boc-LRR-MCA), or carbobenzoxy-Leu-Leu-Glu-MCA (Z-LLE-MCA) (Peptide Institute, Osaka, Japan) to detect chymotrypsin-like, trypsin-like, or PGPH activities, respectively. The reaction was stopped by adding SDS at a final concentration of 0.5% (w/v) and 100 mM Tris-HCl (pH 9.0). The hydrolytic activities on various fluorescent substrates were determined by measuring the fluorescence of groups liberated from these peptides and values were calibrated to standard curves using 7-amino-4-methyl-coumaryl-7-amide (MCA).

**Non-denaturing Polyacrylamide Gel Electrophoresis**—Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed by the method as described previously (28).

**Two-dimensional Polyacrylamide Gel Electrophoresis**—The purified 20 S proteasome was subjected to two-dimensional (2D) PAGE. Proteins were separated by isoelectric focusing in the first dimension followed by SDS-PAGE as described by Hirano (29) with the following modifications: the cathodic and anodic electrode solutions used for isoelectric focusing were 0.02 M H3PO4 and 2% N,N,N,N*-tetramethylphenylenediamine, respectively. After electrophoresis, proteins in the gels were detected by Coomassie Blue R-250 staining.

**Analysis of the N-terminal Amino Acid Sequence**—The proteins separated by 2D-PAGE were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Fluorotrans, Pall, NY) as described previously (30). The proteasome subunits were cut from the PVDF membranes and sequenced by automated Edman degradation on a gas phase sequencer (Perkin-Elmer, Applied Biosystems 491).

**Analysis of the Internal Amino Acid Sequence**—After 2D-PAGE, Coomassie Blue-stained spots of the proteasome subunits were cut out of the gel and digested in the gel with 0.1 μg of lysylendopeptidase (Wako Pure Chemicals, Osaka, Japan) in 20 μl of 100 mM Tris-HCl (pH 9.0) containing 0.1% (w/v) SDS. The digests were separated by high-performance liquid chromatography using a microbore reverse-phase C18 column (Wakopack, 100A, 100 mm in length, 1 mm in internal diameter). Partial amino acid sequences of the peptides were determined with the gas phase sequencer.

**RESULTS**

**Purification of the 20 S Proteasome**—The final preparation of purified proteasome from the nat1, mak3, and nat3 mutants and the normal strain gave a single band in 4.5% polyacrylamide gel under non-denaturing conditions. The 20 S proteasome, isolated in a latent form, is known to have proteolytic activity in the presence of mild chaotropic agents such as SDS (13). The band detected on the 4.5% gel showed chymotrypsin-like activity (Suc-LLVY-MCA hydrolytic activity) in the presence of 0.02% SDS (data not shown).

When the purified proteasome was subjected to SDS-PAGE under dissociated condition, 14 protein bands having molecular weights ranging from 21,000 to 32,000 were observed by Coomassie Blue staining, revealing a typical electrophoretic pattern for the 20 S proteasome subunits. The electrophoretic pattern indicated the purified protein complex to be more than 90% pure (data not shown).

**Identification of the 20 S Proteasome Subunits**—The proteasome subunits of the normal strain of yeast were separated by 2D-PAGE, electroblotted onto PVDF membranes, and stained with Coomassie Blue (Fig. 1). The stained subunits on PVDF membranes were subjected to automated Edman degradation with the gas-phase sequencer. N-terminal sequences of β1, β2, β3, β5, β6, and β7 were obtained (Table 1), but sequence information could not be obtained for the other nine subunits, suggesting that these proteins were N-terminally blocked. The subunits, detected by Coomassie Blue staining after 2D-PAGE, were digested in gels with lysylendopeptidase, and the peptides were separated by reverse-phase high-performance liquid chro-
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The underlined segment in the N-terminal sequence of pro-proteins denotes the N-terminal sequence of the mature protein. The slash (/) in the N-terminal sequence of pro-proteins indicates the site of cleavage in the processed β1, β2, β5, β6, and β7 subunits.

| Subunit | Internal sequence | N-terminal sequence of proprotein | Results of Edman degradation | Normal | nat1 | mak3 | nat3 | Normal N-terminal sequence |
|---------|-------------------|----------------------------------|-----------------------------|--------|------|------|------|----------------------------|
| a1 (Scp1, C7) | TDPAGYEV GYK | MSGAGAASAGYDKTTSFPEGRGQYEVE | Blocked SGAASAC Blocked Blocked | Blocked SGAASAC | Blocked SGAASAC | Blocked SGAASAC | Ac-SGAASAC |
| a2 (Pre8p, Y7) | VSLTPIDIG AVS | MDRTYSSFSLTFPSGKLQDIALYATVQK | Blocked TDRYSLITT Blocked Blocked | Blocked TDRYSLITT | Blocked TDRYSLITT | Blocked TDRYSLITT | Ac-TDRYSF |
| a3 (Pre9p, Tsa) | AISVGAN MGRS | RISRTTTFPSFPEGRGLQVEALEISIS | Blocked GSRVDSRT Blocked Blocked | Blocked GSRVDSRT | Blocked GSRVDSRT | Blocked GSRVDSRT | Ac-GSRVDSRT |
| a4 (Pre6p) | SLLVEQVT GAK | MGDYRALIFSPDHIGQVEALEAKVREK | Blocked SYDRSIF Blocked Blocked | Blocked SYDRSIF | Blocked SYDRSIF | Blocked SYDRSIF | Ac-SYDRSIF |
| a5 (Pup2p) | EALLVLRK | MFLTRSEYDRGFVFPSRGLQVEALESEA | Blocked Blocked MFLTRSEYDR Blocked | Blocked Blocked | Blocked Blocked | Blocked Blocked | Ac-MFLTRSEYDR |
| a6 (Pre5p) | DTPFTYD GEAVAK | MSGRVVGLRVS | Blocked Blocked Blocked Blocked | Blocked Blocked Blocked Blocked | Blocked Blocked Blocked Blocked | Blocked Blocked Blocked Blocked | Ac-MFRNVY |
| a7 (Pre10p, C1) | IYYLHED | MTSQTVGDLSNSVSPFQRQVEYAKEVAY | Blocked TSIGTVGD Blocked Blocked | Blocked TSIGTVGD | Blocked TSIGTVGD | Blocked TSIGTVGD | Ac-TSIGTVGD |
| β1<sup>*</sup> (Pre3p) | LTSQIND RREAL | MNGIQVDNVRLKGEVSLTGSMATVF | TSITAVTF TSITAVTF TSITAVTF TSITAVTF | TSITAVTF | TSITAVTF | TSITAVTF | TSITAVTF |
| β2<sup>ε</sup> (Pup1p) | ND<sup>ε</sup> | MAGLSFDONGQRRNLFANSHQPKATEG | TTVGVKFR TTVGVKFR TTVGVKFR TTVGVKFR | TTVGVKFR | TTVGVKFR | TTVGVKFR | TTVGVKFR |
| β3 (Pup3p) | ERATAPE TF | MDPPSSINGGIVMGTVGNDCAICDLRSQ | Blocked SDPPSINGG Blocked Blocked | Blocked SDPPSINGG | Blocked SDPPSINGG | Blocked SDPPSINGG | Ac-SDPPSINGG |
| β4 (Pre1p, C11) | VELPYGA HGY | MGIIILGIRVQGDSVHASSVAGTRGQKVD | Blocked Blocked Blocked MDIILGIQV | Blocked Blocked | Blocked MDIILGIQV | Blocked MDIILGIQV | Ac-MDIILGIQV |
| β5<sup>α</sup> (Pre2p) | EGPTIIY VDDGTLR | MQAAIADSFSVNRVLKVQDNEQNLSEDFT | TTTLARFR TTTLARFR TTTLARFR TTTLARFR | TTTLARFR | TTTLARFR | TTTLARFR | TTTLARFR |
| β6<sup>ε</sup> (Pre7p, C5) | YLGVEEVI | MATIASEYSESSANPICH/QFNPYGDONGTI | QFNPYGDN QFNPYGDN QFNPYGDN QFNPYGDN | QFNPYGDN | QFNPYGDN | QFNPYGDN | QFNPYGDN |
| β7<sup>α</sup> (Pre4p) | WDFAK | MNNHPDSSWGRPADTSYCAMYQIANAGASPMV | TQQPIVVTG TQQPIVVTG TQQPIVVTG TQQPIVVTG | TQQPIVVTG | TQQPIVVTG | TQQPIVVTG | TQQPIVVTG |

<sup>*</sup> Cleaved.
<sup>ε</sup> Active Thr N terminus.
<sup>α</sup> Not determined.

matography. Representative peptides corresponding to internal regions were sequenced. The amount of β2 subunit purified by 2D-PAGE was insufficient for analyzing the internal sequence. On the basis of the N-terminal and internal amino acid sequences determined, all 14 subunits of the 20 S proteasome could be identified on 2D-PAGE gels (Fig. 1, Table I).

**Difference of the Electrophoretic Patterns**—Purified proteasome subunits from the normal strain and each of the three mutants were separated by 2D-PAGE. It was found that six subunits in the nat1 mutant, α1, α2, α3, α4, α7, and β3, differed in electrophoretic mobility from those in the normal strain (Fig. 1). These subunits of the nat1 mutant shifted toward the alkaline side of the gels in comparison with those of the normal strain. This shift corresponds to the pI change by acetylation of the α-amino group. The N-terminal amino acid sequences of these subunits could be obtained with Edman degradation in the nat1 mutant but not in the normal strain (Table I). In addition, two subunits, α5 and α6, of the mak3 mutant and the β4 subunit of the nat3 mutant shifted toward the alkaline side of the gels (Fig. 1), and these subunits could be sequenced by Edman degradation. These results suggest that the α1, α2, α3, α4, α7, and β3 subunits are acetylated with NAT1, the α5 and α6 subunits with MAK3, and the β4 subunit with NAT3.

**Determination of the Proteolytic Activities**—The 20 S proteasome has three latent proteolytic activities, chymotrypsin-like, trypsin-like, and PGPH activities. These latent proteolytic activities are known to be activated by SDS and heat (13). We investigated the effect of SDS on protease activities of the latent 20 S proteasome with three types of substrates, Suc-Leu-MCA for chymotrypsin-like activity, Boc-LRR-MCA for trypsin-like activity, and Z-LLE-MCA for PGPH activity in the nat1 mutant and the normal strain. The optimal concentration of SDS was determined to be 0.02% for stimulation of both chymotrypsin-like and PGPH activities, whereas SDS did not stimulate the trypsin-like activity, confirming the results of others (13).

The difference of protease activities between the nat1 mutant and the normal strain was investigated in the presence or absence of 0.02% SDS (Fig. 2). In the presence of SDS, chymotrypsin-like activity was highly induced in both strains, compared with trypsin-like and PGPH activities. In the absence of SDS, the activities of all three proteases were low in 20 S proteasomes derived from both strains, although the chymotrypsin-like activity of the nat1 mutant was significantly higher (Fig. 2).

**Effect of pH on Proteolytic Activity**—The effect of pH on the chymotrypsin-like activity of the 20 S proteasome derived from the nat1 mutant and the normal strain is shown in Fig. 3. In the presence of 0.02% SDS, the optimal pH value for chymotrypsin-like activity of the 20 S proteasome was between 7.5 and 9.0, and the activities of both the normal and nat1 20 S proteasome were essentially identical (Fig. 3a). In the absence of SDS, the activity levels were extremely low, and no pronounced optimum pH was observed. However, as observed with the experiment shown in Fig. 2, which was carried out at pH 7.5, chymotrypsin-like activity of the nat1 20 S proteasome was significantly higher than the normal 20 S proteasome at all pH levels (Fig. 3).

**DISCUSSION**

The yeast 20 S proteasome contains 14 different but structurally related subunits, encoded by seven α-type and seven β-type subunit genes. The present study revealed that 9 of 14 subunits are modified at the N terminus, but 6 subunits have
Met acetylated with NAT3, according to the modification rule in which Met-Glu-, Met-Asp-, and a subset of Met-Asn-proteins serve as substrates (6). In the present study, the β4 subunit was confirmed to be Nα-acetylated with NAT3.

On the other hand, the α6 subunit of the rat liver proteasome contains Met and the penultimate residue Phe in the N-terminal region and is blocked by an acetyl group (30). The yeast 20 S proteasome α5 and α6 subunits also contain the N-terminal Met and the penultimate residue Phe. The Met of these subunits was found to be Nα-acetylated with MAK3 in the present study. Subsets of proteins with Met-Ile-, Met-Leu-, and Met-Trp- termini were previously reported to be substrates for MAK3 (6, 32). The results in this study have extended the known substrates to at least some proteins with Met-Phe-Leu- and Met-Phe-Arg- termini.

The remaining subunits, β1, β2, β5, β6, and β7, are processed, resulting in the loss of the N-terminal region (Table I).

The yeast proteasome quaternary structure resembles that of Thermoplasma, except that the N-terminal residues of the α subunits, which are disordered in the structure of the Thermoplasma proteasome, form tightly closed α-ring contractions with several layers of interdigitating subunits (20). Namely, within the α rings, intimate α-cis contacts are made by the intertwined N-terminal segments of α1, α2, α3, α6, and α7 subunits in the center of the heptameric rings in yeast. This suggests that the eukaryotic 20 S proteasomes, but not the Thermoplasma proteasome, are purified in a latent state and can be activated by mild chaotropic agents such as SDS and heat. Presumably, these agents selectively denature the N-terminal region, resulting in an opening of the channel of the 20 S proteasome cavity (15). The present report shows that in the absence of SDS, chymotrypsin-like activity of the 20 S proteasome in the nat1 mutant, lacking N-terminal acetylation of the subunit, was slightly higher than that from the normal strain. Therefore, it is considered that the 20 S proteasome in the nat1 mutant results in a change of the higher order structure of the 20 S proteasome, possibly opening the channel without activation by mild chaotropic agents.

Our results on the lack of a substantial effect of acetylating the α1, α2, α3, α4, α7, and β3 subunits differs from the results of Arendt and Hochstrasser (33) and Jäger et al. (34), who demonstrated that the abnormal N-acetylation of N-terminal threonine residues in specially engineered β1, β2, or β5 subunits led to diminished function. These β1, β2, and β5 subunits are normally synthesized with an N-terminal propeptide that is autocatalytically cleaved during particle assembly, resulting in termini with threonine residues (Table I). As described above, the free α-amino group of the N-terminal threonine residue serves as a nucleophile in substrate hydrolysis.

Thus, as discussed by Sherman et al. (35), the lack of Nα-acetylation, or even abnormal Nα-acetylation, can lead to a wide range of consequences depending on the protein in question, from innocuous or undetectable changes to complete lack of function.

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