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DELTA-AMINOLEVULINIC DEHYDRATASE IS A PROTEASOME INTERACTING PROTEIN

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SUMMARY

The proteasome interacts with a large number of proteins which regulate specific cellular functions. The focus of this study is to examine the proteasome interaction with Delta aminolevulinate dehydratase (ALAD). ALAD is involved in the heme biosynthesis pathway and was co-isolated, with the 20S proteasome using several chromatographic purification steps. The MALDI-TOF mass spectrometry analysis identified this proteasome co-isolated protein as ALAD. When the proteasome was isolated using density-gradient centrifugation, ALAD was also found in the 26S proteasome fractions. It co-isolated with the 20S more than with the 26S proteasome. Furthermore, immunoprecipitated ALAD stained positive with antibodies to proteasome subunits. These results indicate that ALAD might interact with the proteasome. It is possible that ALAD is involved in modulating proteasome activity. When purified proteasomes were incubated with ALAD it was found that ALAD changes proteasome activity in a dose dependent manner. This indicates that ALAD may play a significant role in regulating proteasome activity. The data supports the hypothesis that ALAD, an important enzyme for heme synthesis, is also important as a proteasome interacting protein.

Keywords

Proteasome- interacting-proteins; PIPs; ALAD

INTRODUCTION

Proteasomes 20S and 26S are large protein complexes formed by several subunits responsible for the degradation of altered and oxidized proteins. They represent the major proteolytic pathway responsible for the degradation of proteins that regulate transcription factors, cell cycle, immune response and apoptosis. Proteasome inhibition or failure of the ubiquitin proteasome system (UPS) in the cell causes severe dysfunction of cellular function.

The enzyme δ-aminolevulinate dehydratase (δ-ALA-D), also called porphobilinogen synthase, is a cytosolic sulfhydryl-containing enzyme that catalyzes the condensation of two molecules of aminolevulinic acid (ALA) in order to form porphobilinogen, which is the precursor of heme, cytochromes and cobalamines. ALAD is a 280 kDa protein that is

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composed of 8 identical subunits. When this enzyme is inhibited, aminolevulinic acid accumulates which may impair heme biosynthesis. It increases protoporphyrin accumulation in the liver and pro-oxidant activity in the brain (Demasi et al, 1996; Emanueli et al., 2000). The protein, ALAD, has been reported previously to interact with the proteasome (Guo et al., 1994). However, the role of interaction between proteasomes and ALAD has not been clearly determined.

The aim of the present study is to investigate ALAD interaction with the proteasome and to define ALAD's role in regulating the proteolytic activity of the proteasome. Proteasome 20S was isolated using the chromatography FPLC system. The co-isolated ALAD in the 20S fraction was identified by MALDI TOF mass spectrometry analysis and confirmed by Western blot. The 26S proteasome was isolated using multiple centrifugations and a final density-gradient centrifugation to protect all proteasome interaction. The co-isolated ALAD in the 26S fractions was identified by Western blot analysis.

MATERIAL & METHODS

Animals

Animals were obtained from Harleco (Hollister, CA). They were maintained according to the Guidelines of Animal Care, as described by the National Academy of Sciences and published by the National Institute of Health (1996). In this study freshly cut male Wistar rat livers were used to purify the proteasomes.

Proteasome 20S Purification

The whole rat liver was homogenized in 20 mM Tris-HCl (pH 7.8), 0.1 mM EDTA, and 1 mM DTT, 1 mM EGTA, 50 mM Na-Fluoride, 50 μM Na-orthovanadate, 5 μM benzamidine, 20 mM p-Nitrophenyl phosphate. The homogenates were centrifuged for 2 hours at 25,000 g. The supernatants were subjected to an ammonium sulfate precipitation (40% and 60% saturation). The 60% ammonium sulfate pellets were resuspended in 20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, and 0.5 mM DTT and dialyzed against the same buffer overnight at 4°C. 20S proteasomes were then isolated using ion exchange chromatography over Q Sepharose Fast Flow, Mono Q HR 5/5 columns, and gel filtration chromatography over Superose 6 column (Amersham Biosciences, Piscataway, NJ). The protein concentration was determined using the Bio-Rad (Hercules, CA) assay, based on the Bradford method (Bradford, 1976).

Proteasome 26S Purification

The whole rat liver was homogenized in standard buffer (50 mM Tris-HCl (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$ and 1 mM DDT, 1 mM EGTA, 50 mM Na-Fluoride, 50 μM Na-orthovanadate, 5 mM benzamidine, 20 mM p-Nitrophenyl phosphate (Bardag-Gorce et al., 2004) based on the ratio 1g/20ml (w/v). Liver homogenates and were used for the first centrifugation (100,000 g for 1 hr.) to obtain the cytosolic fraction. The next centrifugation at 71,000 g for 6 hr. was used to precipitate the 26S proteasomes. The pellets were resuspended in 1 ml of standard buffer before they were loaded on top of the 32 ml glycerol density gradient (10-40% glycerol) and centrifuged (100,000 g for 22 hr.). The fractionation was performed using a peristaltic pump and 33 fractions of 1 ml were then collected. Protein concentration measurements were determined using the Bio-Rad assay (Hercules, CA), based on the Bradford method (Bradford, 1976). The same amount of protein from each fraction analyzed was used to perform proteasome enzyme assays and Western blots.

MALDI-TOF MS and MS/MS Analysis

Purified ALAD fractions were separated on a 4–12% Bis-Tris acrylamide gel (Bio-Rad). The bands around 40 kDa that correspond to the ALAD subunit were cut following Coomassie Blue staining. Trypsin endoprotease digestion of the bands was done. Samples were prepared for MALDI-TOF MS using a Millipore ZipTipC18 according to the manufacturer's protocol. To crystallize the samples, 1 μl of 20 mg/ml alpha-cyano-4-hydroxy-trans-cinnamic acid (CCA) in 0.1% TFA/50% acetonitrile (Sigma-Aldrich, St. Louis, MO) was added to the eluted peptides. The peptides were then spotted onto the MALDI target and dried. Full scans of the peptide mixture from 500–3500 m/z and tandem mass spectral data of select ions were collected on a QSTAR XL (Applied Biosystems Inc., Foster City, CA) quadrupole time-of-flight mass spectrometer with an orthogonal MALDI source. The TOF region acceleration voltage was 4 kV and the injection pulse repetition rate was 6.0 kHz. Laser pulses were generated with a nitrogen laser at 337 nm, ~9 μJ of laser energy using a laser repetition rate of 20 Hz. Mass spectra were the average of approximately 50–200 laser shots collected in positive mode. External calibration was performed using human angiotensin II (monoisotopic [MH+] m/z 1046.5417; Sigma) and adrenocorticotropin hormone (ACTH) fragment 18–39 (monoisotopic [MH+] m/z 2465.1989; Sigma). Mass accuracy was calculated in parts per million (ppm) by dividing the difference between the experimental and theoretical m/z values by the theoretical m/z value then multiplying by 106. MS/MS was performed by gating the peptide of interest (Westfalische-Wilhelms-Universitat Munster, Institut fur Medizinsche Physik und Biophysik.

Immunoprecipitation

The liver cytosolic fraction was used to perform immunoprecipitation. It was precleared by incubation rocking for 30 min at 4°C with iron beads conjugated with proteinG (DYNAL Biotech, Inc. Lake Success, NY). Rabbit antibody against ALAD (Gift from Dr Etlinger, New York Medical College) was then added to the cleared supernatant. The mixture was incubated over night racking at 4°C. The next day the iron beads were added to the mixture then incubated 4 hrs rocking at 4°C. The mixture was then washed 4 times with PBS and elution was done using magnetic device. The eluted immune complex was freed from the iron beads by adding Leammli sample buffer (Laemmli, 1970) and boiling for 5 min at 100°C.

Western Blots

The proteins were separated by SDS-PAGE electrophoresis using 12% polyacrylamide gels. The proteins were transferred to a PVDF membrane (Bio-Rad, Hercules, CA) for 1 hour in 25 mM Tris-HCl (pH 8.3), glycine 192 mM and 20% methanol. Primary antibodies were used over night at 4°C. Goat anti-mouse monoclonal or anti-rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used as the second antibodies. Immunodetection was done using an alkaline phosphatase kit from Bio-Rad or ECL plus (Amersham Bioscience Corp., Piscataway, NJ). Densitometric measurements of the bands were done using the GS-700 imaging densitometer (Bio-Rad, Hercules, CA).

Proteasome–ALAD Incubation

ALAD (Sigma, Saint Louis, MO) and 20S proteasome were incubated 10 min at 37°C in Tris 50 mM and DTT 1mM pH 8, prior to adding the fluorogenic substrates for chymotrypsin-like activity measurement as described below.

Chymotrypsin-like and Trypsin-like Assay

The chymotrypsin-like (ChT-L) activity was measured as follows: The reaction mixtures contained 0.1 μg of purified proteasome, 50 mM Tris-HCl pH 8, 1 mM DTT, and 40 μM Suc-LLVY-AMC. The mixture was incubated for 30 min at 37°C and then stopped by adding 100 μM monochloroacetate and 30 mM sodium acetate pH 4.3. Fluorescence was determined by measuring the release of AMC (λ excitation: 370 nm, λ emission: 430 nm) using a Perkin Elmer LS 30 spectrofluorometer. The concentration of liberated products was calculated using a standard curve for AMC.

RESULTS

20S proteasomes from the livers have been routinely purified in our laboratory. When the purification at the last chromatographic stage was fine-tuned using a narrow KCl gradient, the Px fraction (Figure 1A) was successfully separated from the 20S proteasome fraction. The fraction corresponding to the Px peak and the 20S proteasome peak were then analyzed by SDS-PAGE electrophoresis and stained with Coomassie Blue (Figure 1B and 1C respectively). The protein ALAD subunits each had a molecular weight of about 44 kDa on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The subunits were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry with a high score as ALAD protein (Figure 2).

Immunoprecipitation was performed on rat liver homogenates to analyze the interaction between proteasome and ALAD using the ALAD antibody. The eluates were analyzed by Western blot and stained with antibodies against two proteasome subunits: Rpt4, a 19S regulatory complex ATPase subunit, and 20S alpha 2 subunit. Figure 3A shows that immunoprecipitation with ALAD antibody pulled down the proteasomes because proteasome were detected in the analyzed eluates. Figure 3B shows an immunoblot stain of the Px fraction collected from the 20S isolation using the ALAD antibody. These results support the identification by mass spectrometry of ALAD as a proteasome interacting protein.

26S proteasomes were isolated with zonal centrifugation using ATP and glycerol that protects the 26S structure and also preserves to a maximum the proteasome interacting proteins (Figure 3C). The immunoblot analysis of the fractions collected from the 26S proteasome isolation showed that the 20S richest fractions (beta 5 stain (Figure 3D) stained strongly with the ALAD antibody and the 26S richest fraction stained weakly with ALAD antibody (TBP7, Figure 3D). This result suggested that ALAD co-isolated with low molecular weight proteasomes such as the 20S proteasome more than with high molecular weight proteasomes such as the 26S proteasome.

To further study the role played by ALAD in proteasome activity, purified ALAD from Sigma was incubated with the proteasome prior to adding the proteasome substrates. Commercially available ALAD was chosen due to the high degree of purity of this ALAD fraction. Figure 4A shows that the highest concentration of ALAD stimulated significantly the proteasome chymotrypsin-like and trypsin-like activity and this was reproducible among the different 20S purifications. Although the proteasome activity varied between the three preparations, the activation by the addition of ALAD was similar. Inhibition of proteasome activity was also sometimes observed when a low ALAD concentration (1 μg) was used (Figure 4A).

In order to verify the nonspecific effect of protein-proteasome interaction on the proteasome activity, albumin was incubated with the proteasomes the same way as with ALAD. Figure

4B shows an increase of the proteasome activity due to a nonspecific protein interaction factor; however this increase was less significant (25%) than that caused by ALAD (75%).

DISCUSSION

The ubiquitin-proteasome pathway is a vital pathway for cellular homeostasis. It is involved in the cell cycle, regulation of transcription factors, assurance of cellular quality control, immune response and many other vital cellular functions (Ciechanover, 2006). The 26S proteasome, a protein complex formed by 32 subunits, performs multiple important proteolytic activities in the cell interacting with the ubiquitination system (E1s, E2s, E3s and deubiquitinases) and with various proteasome interacting proteins (PIPs) (Bousquet-Dubouch MP, et al., 2009). These PIPs differ in their biological roles and interact with the proteasome according to the specific cellular function required. For instance, there are chaperones which assist in the unfolding of the proteins destined for degradation by the proteasome. Hsp70 and Hsp25 are the most known Hsps that interact with the proteasome (Bercovich et al., 1997; Yao et al., 2002). The chaperone system Bip/PDI associated with the endoplasmic reticulum associated degradation (ERAD) (Molinari et al., 2002) is also know to interact with the proteasome. Proteins containing a UBA domain such as Rad23 and p62 are also involved in carrying and docking of the protein substrates at the proteasome (Elsasser et al., 2002). Also, there are kinases and phosphatases that interact with the proteasome system which regulate the activity of the proteasome such as casein kinase II (Bose et al., 2004), PP2A and PKA (Gomes AV et al., 2006) (Pereira et al., 1990).

In the present study, the new finding is the identification of ALAD as a new proteasome interacting protein. The association of a heme synthesis enzyme and the proteasome pathway is intriguing. The ALAD enzyme catalyzes the asymmetric condensation of two molecules of aminolevulinic acid to form porphobilinogen in the initial steps of heme biosynthesis (Gibson et al., 2001). It has been reported previously that ALAD is a part of the 26S complex and acts as a proteasome inhibitor (Guo et al., 1994). The results of the present study showed further evidence that the proteasome and ALAD indeed co-isolated and may be interacting proteins. Immunoprecipitation and pull down assay documented the interaction of ALAD with the proteasome. However, the mechanism of this interaction needed to be further investigated. The proteasome is a dynamic structure and exist in different forms. There is the 26S (20S and two regulatory complexes 19S), the 20S by itself, the immunoproteasome (20S + two 11S regulating complexes), the proteasome hybrid (20S +11S+19S). Each of these proteasomes has several specific functions involved in different important cellular functions. It is possible that ALAD and the catalytic core 20S form a complex which performs a specific activity, which could influence the rate of degradation of specific protein substrates. 20S-ALAD interaction is more likely than 26S-ALAD interaction, since the protocol used for 20S purification did not perturb the interaction of 20S-ALAD. The 20S purification procedure has drastic conditions that do not preserve most of the proteasome interaction with its associating proteins compared to the 26S purification procedure.

The incubation of proteasomes with ALAD caused an increase in the proteasome activity as indicated by using chromogenic substrates to measure the chymotrypsin-like and the trypsin-like activity. These results indicated that there was a high affinity between the 2 interacting proteins and suggested that ALAD is not a substrate for the proteasomes but rather a 240 kDa eight subunits regulator complex of the 20S proteasome. Guo and co-authors (Guo et al., 1994) had reported evidence that ALAD was a proteasome inhibitor based on the fact that ALAD inhibited the proteasomes activity to degrade [^{14}C]CH3-a-casein. In the present study fluorogenic substrates were used to evaluate proteasome activity. These substrates are specific for each proteasome activity (Chymtrypsin-like and trypsine-like activity) and were

not specific substrate for ALAD activity. The fraction that contained the co-purified ALAD was not used in the incubation experiments because as shown in Figure 1 by the Coomassie Blue gel stain other unidentified PIPs were also coisolated in this fraction. However the incubation of the proteasome with the ALAD showed a significantly higher increase in the proteasome activity than the increase caused by the commercially available ALAD 9data not shown). This may be due to the species of origin or simpely due to the other unidentified components that co-isolated with ALAD proteins. These results document the existence of multiple PIPS and indicate that proteasome activity undergo a sophisticated regulation by the PIPs.

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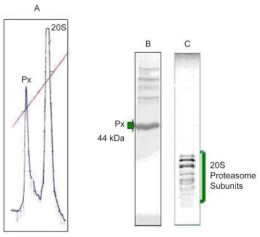

Fig. 1.

Panel A shows: (A) Chromatogram of 20S proteasome purified from rat liver. The spectra represents the protein measurement at 280nm using the optical unit linked to the FPLC system used for the proteasome purification. The columns used for this purification are described in MM. The red line is the KCl gradient applied for the 20S isolation. Note the peak preceding the 20S peak was an unknown protein Px, which co-isolated with the 20S, and, therefore, was isolated along with the 20S proteasome in the purification process. (B) and (C): SDS-Page electrophoresis and Coomassie Blue stained gels of Px Peak fractions (B) and 20S peak fraction (C). Note that the 44 kDa Px protein was highly concentrated and purified with the 20S. The band was cut out of the gel and analyzed by mass spectrometry.

Fig. 2.

The 20S co-isolated protein Px was analyzed by matrix-assisted laser desorption/ ionization-time of flight (MALDI-TOF) mass spectrometry. The trypsin digested peptides showed a high score of identification with the Delta aminolevulinic acid dehydratase sequences (a). The protein was identified in the database as Heme2 mouse Delta-aminolevulinic acid dehydratase (b)

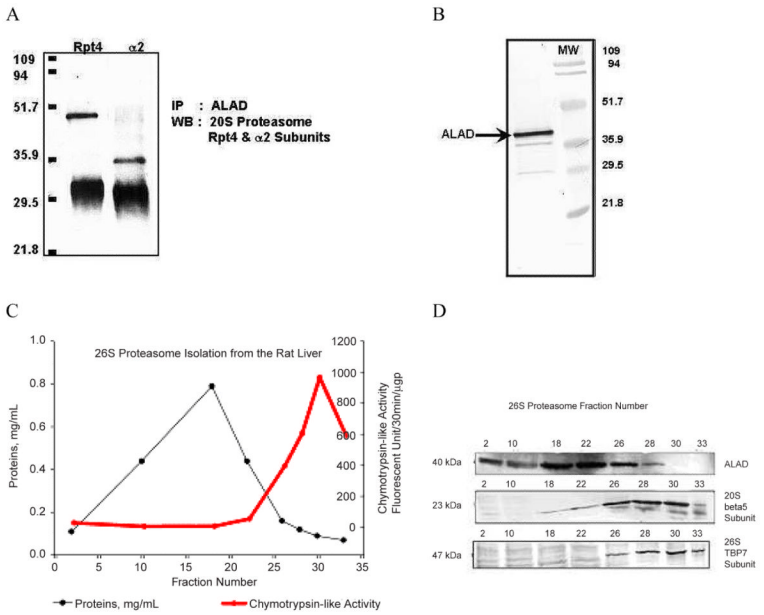

Fig. 3.

ALAD and proteasome interaction. (a) Immunoprecipitation of ALAD and Western blot of the eluates using antibodies to the proteasome subunits Rpt4, a 19S ATPase subunit and to the 20S alpha 2 subunit. (b) Western blot analysis of the Px fraction collected from the 20S isolation using ALAD antibody. (c) 26S proteasome isolation. (d) Western blot of 26S proteasome fraction analyzed with ALAD antibody. (a) The “higher MW” corresponds to the unspecific binding of the 2nd antibody against the mouse monoclonal specific to Rpt4 or to α2. Possibly this binding is due to the protein-G that co-isolate with the immunoprecipitated complex. The protein-G has high affinity for the mouse monoclonal antibody. (b) The lower MW is certainly antigens detected by the antibody used to detect ALAD. However these lowers MW are not at the right size of ALAD that was detected efficiently with this antibody. The Markers are purchased from Bio-Rad and they are Blue when transferred to the membrane in the Western blot analysis. The detection method was done using Alkaline phosphatase Kit (colorimetric method). Therefore there is no need to visualize the markers.

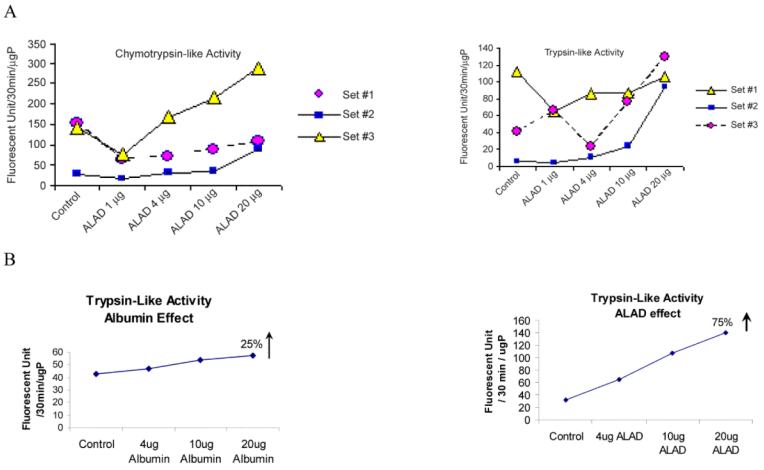

Fig. 4.

ALAD modulated proteasome activity. (a) Chymotrypsin-like and trypsin-like activities were measured after incubating the proteasomes with ALAD for 10 min at 37°C. The three traces are measurements using three different proteasome preparation. (b) Control for ALAD-proteasome interaction effect. Bovin albumin was used as a nonspecific protein control.