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Identity of the 15S ATPase with the Eukaryotic Mg^{2+}-ATPase and Its Immunoreactivity to the 26S protease Complex from Chick Skeletal Muscle

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Partial internal amino acid sequences of the 15S ATPase from chick skeletal muscle were determined and found to be identical to the corresponding regions of the Mg^{2+}-ATPase from *Xenopus laevis* oocytes, that is a close homolog of N-ethylmaleimide-sensitive factor (called NSF) in hamster and Sec18p in yeast, both of which are believed to play an essential role in vesicle fusion in secretory process. Thus, the 15S ATPase in chick skeletal muscle may also belong to a protein family of the "vesicle fusion proteins". Unlike the Mg^{2+}-ATPase with an isoelectric point (pI) of 5.5, however, the 15S ATPase was separated into four spots with pIs of 4.9, 6.4 and 6.9 upon analysis by two-dimensional gel electrophoresis. In addition, the anti-15S ATPase IgG was found to be capable of interacting with the 26S protease complex upon analysis by immunoprecipitation. Moreover, immunoblot analysis revealed that the anti-15S ATPase IgG recognizes three subunits, two of which show the same mobilities as the 510-kDa subunit 4 and 48-kDa subunit 7 of the 26S protease complex that are known to contain a highly conserved ATP-binding motif. These results sugest that a common antigenic site, likely the consensus nucleotide-binding site, exists in the 15S ATPase and the 26S protease complex and hence both the enzymes may also be related ATPases.

KEY WORDS: 15S ATPase, Mg^{2+}-ATPase, 26S protease complex

A high molecular weight ATPase that absolutely requires Mg^{2+} for activity has been purified from erythrocytes (White and Ralston, 1980). This ATPase has been suggested to be involved in the energy-requiring endocytotic process, since it is sensitive to inhibition by Cd^{2+}, Zn^{2+}, *p*-chloromercuribenzoate and N-ethylmaleimide (NEM), known inhibitors of membrane endocytosis. A Mg^{2+}-ATPase with the structure resembling a cylinderic ring-shaped particle has also been purified from *Xenopus laevis* oocytes (Peters *et al.*, 1990, 1992) and shown to have a strong sequence homology with NSF in hamster (Block *et al.*, 1988; Beckers *et al.*, 1989; Diaz *et al.*, 1989) and Sec18p in yeast (Eakle *et al.*, 1988), both of which are believed to be essential for vesicle fusion in secretory process (Block *et al.*, 1988; Wilson *et al.*, 1990). In addition, the amino acid sequence of the Mg^{2+}-ATPase has been shown to contain two highly conserved sequences for ATP-binding (Peters *et al.*, 1990), one of which can also be found in certain subunits of the regulatory component of the ATP/ubiquitin-dependent 26S protease complex (Dubiel *et al.*,

1992; Rechsteiner *et al.*, 1993).

During purification of the 26S protease complex from chick skeletal muscle, we fortuitously found an ATPase activity that is initially coeluted with the 26S protease complex upon gel filtration but can be dissociated from it by later purification steps. In the preceding paper (Shim *et al.*, 1994), therefore, we purified the ATPase to apparent homogeneity using conventional column chromatographies and glycerol density gradient centrifugation and showed that the ATPase has a sedimentation coefficient of 15S and is a hexameric complex of 95-kDa subunits. In the present studies, we performed partial amino acid sequence analysis of the purified 15S ATPase to examine its identity with the Mg^{2+}-ATPase, since the latter enzyme also is an oligomer consisting of six identical subunits of 97 kDa. We also performed immunochemical analysis using the antibody raised against the purified 15S ATPase to determine whether any functional relationship may exist between the 15S ATPase and the ATPase component of the 26S protease complex.

Materials and Methods

Materials

The 15S ATPase, 20S proteasome and 26S protease complex were purified from chick skeletal muscle as described in the preceding paper (Shim *et al.*, 1994). For preparation of anti-15S ATPase antiserum, the purified 15S ATPase was subjected to electrophoresis on a 8% (w/v) polyacrylamide slab gel containing sodium dodecyl sulfate (SDS). After the electrophoresis, the bands corresponding to the major 95-kDa and minor 83-kDa polypeptides were excised out separately from the gel. Each was minced and injected into albino rabbits. IgGs were then isolated from the antisera by sodium sulfate fractionation (Johnston and Thrope, 1982). [γ-^{32}P]ATP (3 Ci/mmol) was purchased from Amersham and Ampholytes were from LKB-Pharmacia. All other chemicals were obtained from Sigma unless otherwise indicated.

Peptide sequencing

The purified 15S ATPase (20 μg) was digested by incubation at 37°C for 7 hr with Lys-endopeptidase in 50 mM Tris-HCl buffer (pH 9) containing 4 M urea. The cleavage products were subjected to separation by reverse phase HPLC using a μRPC C_2/C_{18} column (21 × 100 mm). The peptides bound to the column were eluted with a linear gradient of 1-60% acetonitrile containing 0.065% trifluoroacetic acid. Elution of the peptides was monitored by their absorbance at 214 nm. Four peptide fragments were selected and degraded with an automated gas-phase protein sequencer (Applied Biosystems, model 470A) according to operation program 02RPTH provided for the sequencer. phenylthiohydantoin (PTH)-amino acids liberated were identified by HPLC using an Ultrasphere ODS column (2 × 250 mm) at 49°C. Solvents used for the elution of PTH-amino acids were composed of acetonitrile/0.1% trifluoroacetic acid adjusted to pH 4.9 at a ratio of 1/10 or 1/1 (v/v). The eluates were monitored simultaneously at 269 and 322 nm.

Electrophoretic analysis

Two-dimensional gel electrophoresis was carried out by following the method of O'Farrell (1975) with some modification. In the first dimension, the purified 15S ATPase was subjected to isoelectric focusing on 5% (w/v) polyacrylamide rod gels (0.2 × 13 cm) containing 8 M urea and 1% (v/v) each of pH 3.5-10 and pH 4-6 Ampholytes. The resulting gels were equilibrated in 2% SDS and subjected to gel electrophoresis in 8% slab gels as described by Laemmli (1970). Proteins in the gels were visualized by silver staining (Harlow and Lane, 1988).

Immunochemical analysis

Immunoprecipitation of the 26S protease complex was performed by incubation for 2 hr at room temperature with anti-15S ATPase IgG. After the incubation, the samples were gently shaken with 0.1 ml of protein A-Sepharose (0.1 mg/ml) for 2 hr at 4°C followed by centrifugation at 10,000 ×g for 10 min. The supernatants were then assayed for the 26S protease complex by measuring its ability to hydrolyze ATP as described in the preceding paper (Shim *et al.*, 1994).

For immunoblot analysis, the purified 26S protease complex and the 15S ATPase were electrophoresed on 10% slab gels containing SDS as above. After the electrophoresis, proteins in the gels were transferred onto nitrocellulose membranes and reacted with anti-15S ATPase IgG and then with ^{125}I-labeled-protein A (Towbin *et al.*, 1979). The membranes were then dried and exposed in X-ray films (Fuji).

Results

Determination of partial amino acid sequence of the 15S ATPase

In order to determine partial amino acid of the 15S ATPase, the purified enzyme was digested with Lys-endopeptidase in the presence of 4 M urea. The cleavage products were then separated by reverse phase HPLC using a μRPC C2/C18 column (Fig. 1). Four peptide fragments eluted from the column were randomly selected and subjected to Edman degradation for their sequence analysis. Fig. 2 shows the amino acid sequence of the selected peptide fragments numbered F1 to F4. On computer analysis using the data base of NCBI/BLAST (The U.S. National Center for Biotechnology Information), the partial amino acid sequences of the 15S ATPase were found to be completely identical to those of the Mg^{2+}-ATPase isolated from *Xenopus laevis* oocytes. The Mg^{2+}-ATPase has been shown to have a strong sequence homology with NSF in hamster and Sec18p in yeast (Peters *et al.*, 1990), both of which are believed to be essential for vesicle fusion in secretory process (Wilson *et al.*, 1990). Thus, the 15S ATPase isolated from chick skeletal muscle seems to also belong to a member of a protein family of the "vesicle fusion proteins" (Peters *et al.*, 1990), which includes NSF, Sec18p and the Mg^{2+}-ATPase.

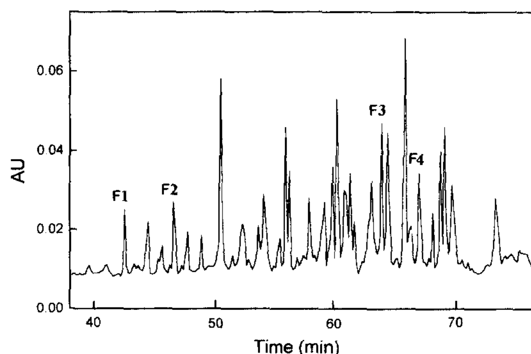

Fig. 1. HPLC-separation of the peptide fragments of the 15S ATPase. The purified 15S ATPase was digested with Lys-endopeptidase and its cleavage products were separated by reverse phase HPLC as described under Materials and Methods. The peptide fragments chosen for their N-terminal amino acid sequence analyses (see Fig. 1) were numbered as F1 to F4.

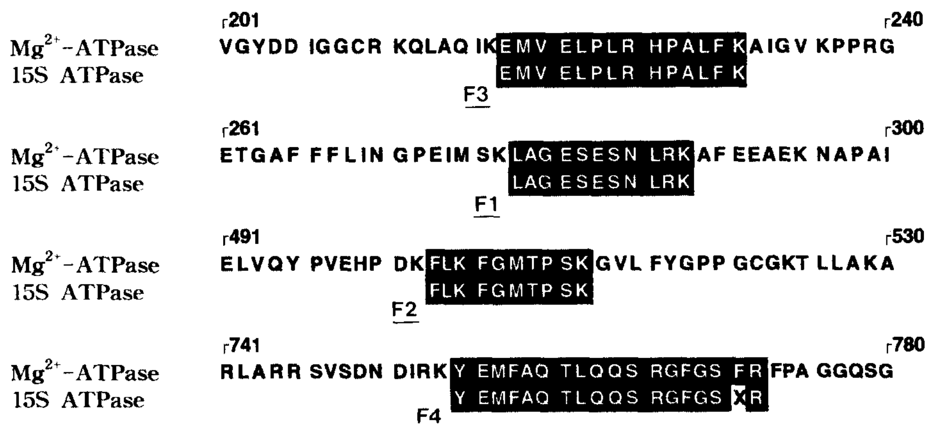

Fig. 2. Determination of the amino acid sequences of the peptide fragments of the 15S ATPase. The peptide fragments (numbered as F1 to F4; see Fig. 1) were subjected to Edman degradation for their sequence analysis. The letter X indicates the amino acid that could not be identified by the analysis.

Two-dimensional gel electrophoretic separation of the 15S ATPase subunits

The Mg^{2+}-ATPase from *Xenopus* oocytes has been reported to have a pI of 5.5 (Peters *et al.*, 1990). In addition, the same enzyme has been shown to migrate as a single band upon two-dimensional gel electrophoresis in which the first dimensional separation was isoelectric focusing and the second dimension was SDS-polyacrylamide gel electrophoresis, despite the fact that the Mg^{2+}-ATPase has several potential sites for myristoylation and for phosphorylation by calmodulin kinase II, casein kinase II and protein kinase C types (Peters *et al.*, 1990).

To clarify further the identity of the 15S ATPase from chick muscle with the Mg^{2+}-ATPase from *Xenopus* oocytes, we carried out two-dimensional gel electrophoresis of the 15 ATPase using the same protocol. As shown in Fig. 2, however, the 15S ATPase was separated into four spots which were referred to as spots 1 to 4. Of these, the spots 1, 2 and 3 have nearly the same molecular mass of about 95 kDa but different pIs of 4.9, 6.4 and 6.9, respectively. These results suggest that the spots may have been covalently modified but to different extents, such as by phosphorylation. Therefore, further studies, such as treatment of protein phosphatase to the 15S ATPase and determination of its complete sequence, are of necessity for clarification of its identity with the Mg^{2+}-ATPase and the nature of covalent modifications.

On the other hand, the spot 4 has a size of 83 kDa and pI of 6.4. Incubation of the purified 15S ATPase preparation itself at 4°C resulted in initial accumulation of the spot 4, and prolonged incubation under the same condition caused the further processing of the spot into smaller-sized fragments (data not shown). Thus, it appears possible that the spot 4 is the product of limited proteolysis of the 15S ATPase subunits, perhaps of the spot 2, by the action of certain unknown protease that may be contaminated in the purified enzyme preparation.

Cross-reactivity of anti-15S ATPase IgG to the 26S protease complex

The Mg^{2+}-ATPase has been shown to contain two highly conserved internal repeats for ATP-binding (Peters *et al.*, 1990). By cDNA sequence analysis, it has also been reported that putative transcritpion factors including MSS1, SUG1 and TBP1 contain one of the two highly conserved ATP-binding motifs and therefore grouped as a member of newly found ATPase family (Dubiel *et al.*, 1992). Furthermore, subunit 7 that is an integral component of the ATP/ubiquitin-dependent 26S protease complex has been identified as MSS1 upon comparison of their primary structures (Dubiel *et al.*, 1993). Assuming that the 15S ATPase is a very close homolg of the Mg^{2+}-ATPase or they are identical molecules, it appears likely that the 15S ATPase also contains the consensus ATP-binding sites and may be related with the ATP-interacting subunit(s) of the 26S protease complex.

Therefore, we examined whether the antibody raised against the 15S ATPase could interact with

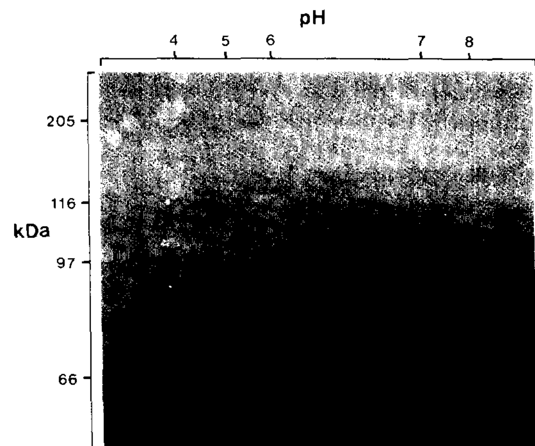

Fig. 3. Two-dimensional gel electrophoresis of the 15S ATPase. The subunits of 15S ATPase was subjected to isoelectric focusing on a 5% rod gel as the first dimensional separation and then to electrophoresis on a 8% slab gel containing SDS as the second dimension as described under Materials and Methods. The pH values shown on top of the gel were determined by running a duplicate gel containing the marker proteins with known pIs. The protein spots were visualized by silver staining.

the 26S protease complex by immunoprecipitation experiments. The 26S protease complex was incubated with the anti-15S ATPase IgG and then with protein A-Sepharose. After the incubation, the samples were centrifuged to remove precipitates and their supernatants were assayed for the ATPase activity of the 26S protease complex. As shown in Fig. 3, the purified 26S protease complex could successively precipitated by the anti-15S ATPase IgG. Furthermore, antibody raised against the 26S protease complex could also precipitate the purified 15S ATPase. On the other hand, the anti-15S ATPase IgG did not interact with the 20S proteasome, which is the proteolytic core but lacking the ATP-binding regulatory component of the 26S protease complex (data not shown).

We then examined whether the antibody against the 15S ATPase can recognize any subunits of the 26S protease complex, particularly the one having a similar size as the 95-kDa subunit of the 15S ATPase. The purified 26S protease complex and the 15S ATPase were electrophoresed in duplicate on 10% slab gels containing SDS. One of the gels was stained with silver nitrate (Fig. 4A), and the other was subjected to immunoblot analysis using the anti-15S ATPase IgG (Fig. 4B). At least three bands clearly interacted with the anti-IgG, and of these, the second and third bands seemed to migrate in the gel to similar locations to subunits 4 and 7 of the 26S protease complex that are known to contain the consensus ATP-binding motif. However, no immunoreactive band could be seen in the regions of 95 kDa in the gel lane for the 26S protease complex, suggesting that the 95-kDa subunit of the 26S protease complex is distinct from that of the 15S ATPase. These results suggest that the immunoreactivity of the 26S protease complex with the 15S ATPase is conferred by their consensus ATP-binding motifs and hence they also are related ATPases.

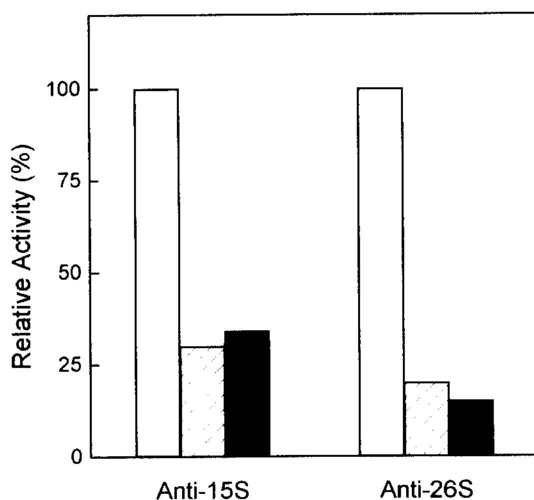

Fig. 4. Effects of anti-IgGs against the 15S ATPase and the 26S protease complex on the ATPase activities of the enzyme complexes. Each of anti-IgGs (20 μg) was incubated with the 26S protease complex (▨) and 15S ATPase (■) and then with protein A-Sepharose as described under Materials and Methods. The incubation mixtures were centrifuged, and the resulting supernatant fractions were assayed for their ability to hydrolyze [γ-^{32}P]ATP as described in the preceding paper (Shim *et al.*, 1994). The ATPase activities seen after incubation with the preimmune IgGs were expressed as 100% activity (□), and the others were as their relative values.

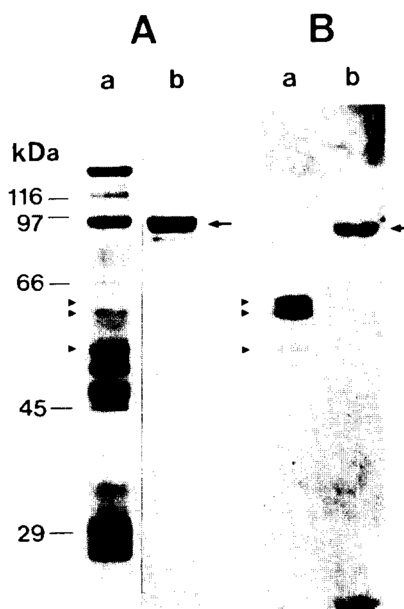

Fig. 5. Cross-reactivity of anti-15S ATPase IgG to the 26S protease complex. The purified 26S protease (lanes a) and the 15S ATPase (b) were electrophoresed in duplicate on 10% slab gels. After electrophoresis, one of the gels was stained with silver nitrate (A) and the other was subjected to immunoblot analysis (B) as described under Materials and Methods. The arrow heads indicate the subunits of the 26S protease complex which interacted with the anti-15S ATPase IgG, and the arrows show the 15S ATPase.

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15S ATPase와 진핵세포에 존재하는 Mg^{2+}-ATPase의 동질성 및 계 골격근에서 분리된 26S 단백질 분해효소와의 면역반응에 관한 연구

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계 골격근에서 분리된 15S ATPase의 부분적인 아미노산 서열결정 결과, *Xenopus laevis* oocyte에서 분리된 Mg^{2+}-ATPase의 아미노산 해당부위와 정확히 일치하였다. 이 Mg^{2+}-ATPase는 hamster에서 분리된 N-ethylmaleimide-sensitive factor (NSF)와 효모에서 분리된 Sec18p와 homologous한 것으로, 이 두 효소는 모두 세포내 분비과정에서 vesicle fusion에 필수적인 역할을 담당하는 것으로 생각되는 단백질이다. 따라서, 계 골격근에서 분리된 15S ATPase 또한 이러한 "vesicle fusion protein"들로 구성된 family에 포함된다고 할 수 있다. 그러나, 등전점이 5.5인 Mg^{2+}-ATPase와는 달리, 15S ATPase는 2차원 전기영동 상에서 4.9, 6.4, 6.9의 등전점을 갖는 네개의 서로 다른 spot으로 분리되었다. 그리고, immunoprecipitation 실험 결과, 15S ATPase에 대한 항체가 26S 단백질 분해효소 복합체와 반응하였다. 또한, 동일 항체를 이용한 immunoblot 분석 결과, 이 항체는 highly conserved ATP-binding motif를 갖는 26S 단백질 분해효소 복합체의 51-kDa의 S4와 48-kDa의 S7의 크기에 해당하는 두개의 단위체와 또 다른 한개의 단위체를 인식하는 것으로 나타났다. 이러한 결과는, ATP-binding motif와 같은 antigenic site가 15S ATPase와 26S 단백질 분해효소 복합체에 공통적으로 존재하고 있으며, 따라서 두 효소는 서로 연관된 ATPase임을 추측케 하였다.