ATPase II, an ATP-dependent Mg$^{2+}$-transporting ATPase, is a member of a subfamily of P-type ATPases and is presumably responsible for aminophospholipid translocation activity in eukaryotic cells. The aminophospholipid translocation activity plays an important physiological role in the maintenance of membrane phospholipid asymmetry that is observed in the plasma membrane as well as the membranes of certain cellular organelles. While the preparations of ATPase II from different sources share common fundamental properties, such as substrate specificity, inhibitor spectrum, and phospholipid dependence, they are divergent in several characteristics. These include specific ATPase activity and phospholipid selectivity. We report here the identification of four isoforms of ATPase II in bovine brain. These isoforms are formed by a combination of two major variations in their primary sequences and show that the structural variation of these isoforms has functional significance in both ATPase activity and phospholipid selectivity. Furthermore, studies with the phosphoenzyme intermediate of ATPase II and its recombinant isoforms revealed that phosphatidylserine is essential for the dephosphorylation of the intermediate. Without phosphatidylserine, ATPase II would be accumulated as phosphoenzyme in the presence of ATP, resulting in the interruption of its catalytic cycle.

The phospholipid distribution in the plasma membrane (PM) of eukaryotic cells is asymmetric. The outer leaflet consists predominately of phosphatidylcholine (PC) and sphingomyelin, whereas aminophospholipids, including phosphatidylethanolamine (PE) and phosphatidylethanolamine (PE), are present almost exclusively in the inner leaflet (1, 2). The asymmetric nature of phospholipid composition is found in several other intracellular membrane systems as well, and the physiologic importance of phospholipid asymmetry is multifold. The asymmetric distribution of lipids provides the two sides of the membrane with different characteristics that are necessary for their respective physiologic function. For instance, the tight packing of the outer leaflet of the PM is important for membrane stability in circulating blood cells and the resistance of cells to attack by cytotoxic T lymphocytes (3). On the other hand, the enrichment of aminophospholipid in the inner leaflet of the PM and the cytosolic leaflet of endocytic and exocytic vesicle membranes may contribute to these surfaces being in a fusion-competent state (4). In addition, the regulated disruption of phospholipid asymmetry in the PM provides a pathway for cellular signaling in certain physiologic events. The surface exposure of PS, for example, has been demonstrated in activated platelet to promote the reaction cascade of blood coagulation (5, 6) and in apoptotic cells to trigger the recognition between these cells and macrophages (7, 8). Furthermore, the dynamic process of phospholipid translocation may play important roles in cellular events such as membrane budding and endocytosis (2).

While a thorough understanding of the mechanism for membrane asymmetry and phospholipid translocation has yet to emerge, one of the enzymes that plays a major role in these processes has been identified as aminophospholipid translocase (9, 10). This activity catalyzes an energy-dependent aminophospholipid translocation requiring Mg$^{2+}$ and ATP (10), and it is sensitive to the sulhydryl group reagent N-ethylmaleimide and vanadate, an inhibitor of P-type ATPases (9, 11). The primary candidate protein for this activity is ATPase II, a PS-dependent and vanadate-sensitive Mg$^{2+}$-ATPase. This ATPase has an apparent molecular mass of about 116 kDa and has been isolated and purified from several sources including chromaffin granules (12), clathrin-coated vesicles (13), and the plasma membrane of erythrocytes (14). This enzyme is a P-type ATPase with characteristics strikingly similar to those of the aminophospholipid translocase activity measured in situ (9). Comparison of the sequence data of ATPase II from bovine chromaffin (15), mouse muscle, and human tissues (18) indicates that this enzyme contains 10 putative transmembrane domains and several P-type ATPase consensus sequences. An

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Identification and Functional Expression of Four Isoforms of ATPase II, the Putative Aminophospholipid Translocase

EFFECT OF ISOFORM VARIATION ON THE ATPase ACTIVITY AND PHOSPHOLIPID SPECIFICITY*

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ATPase II Isoforms

ATP binding site and a phosphoenzyme formation site are located within the largest cytosolic loop, whereas a sequence implicated in the coupling to transport activity was identified in another hydrophilic loop. The sequence of mammalian ATPase II is homologous to a yeast ATPase encoded by the DRS2 gene, of which the null mutant of a yeast strain lacks a specific PS internalization activity that is otherwise present in wild type yeast strains (15). This observation provides further evidence indicating the involvement of ATPase II in PS translocation. Furthermore, proteoliposomes that were purified with ATPase II from erythrocytes were shown to transport fluorescent PS, and to a lesser extent PE, but not PC (19).

The ATPase II from different mammalian sources, however, is divergent in several characteristics. The specific ATPase activity of these preparations, for instance, is reported to range from 0.8 (μmol P/min/mg of protein) for the enzyme isolated from human erythrocyte (14) to 8.0 for that from bovine chromaffin granules (12) and to 42 for the preparation from clathrin-coated vesicles (13). Furthermore, the stimulating effect of PS over PE on these preparations varies from 2- to 20-fold. This disparity may be of physiological importance in fulfilling the requirement of different tissue- and/or organelle-specific processes in which aminophospholipid translocation is involved. Differences in the distribution of aminophospholipids in various cellular compartments may arise from intrinsic functional differences between isoforms of ATPase II, different co-factors that are required for the function of ATPase II, or entirely different enzymes. Alternatively, differences in the activity profile of ATPase II preparations from various sources may be due to variations in the purification protocols used to isolate these ATPases from various sources. Clarification of these issues would facilitate the study of aminophospholipid translocation system, a very important but poorly understood enzyme system.

Although certain differences between the cDNA sequences of ATPase II from different sources have been found, their correlation to biochemical activity has not been established because of the limited characterization of these preparations at a protein level.

ATPase II, when purified from clathrin-coated vesicles, appears as a doublet of 116 kDa by SDS-PAGE (13). The two proteins have proven difficult to separate biochemically, although gel filtration chromatography indicates that the two proteins do not exist as a complexed dimer (13). These results, in composite, suggest the possibility that the two proteins represent different isoforms of ATPase II. The current studies were undertaken to characterize basic functional properties of the bovine brain form(s) of ATPase II and to determine if structurally and functionally distinct isoforms of the enzyme exist in brain.

In this paper, we report the identification of four isoforms of ATPase II from bovine brain. These isoforms are characterized with respect to structural and functional differences in both ATPase activity and phospholipid selectivity. In addition, studies with the phosphoenzyme intermediate of ATPase II and its recombinant isoforms revealed that PS is essential for the dephosphorylation of the intermediate. The catalytic cycle of ATPase II would be stopped without PS or PE, resulting in the accumulation of its phosphoenzyme intermediate. This observation provides a biochemical explanation for the aminophospholipid dependence of this enzyme, as well as an important clue for further understanding of the mechanism of how aminophospholipid translocate transports PS across biological membranes.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and T4 DNA ligase were purchased from Roche Molecular Biochemicals; the GeneAmp polymerase chain reaction (PCR) reagent kit with Thermus aquaticus Top DNA polymerase and DNA sequencing materials and reagents were from Perkin-Elmer. A bovine brain cDNA library in λAzor was the kind gift of Dr. Richard A. F. Dixon (University of Texas Health Science Center at Houston). The host Escherichia coli cells BB4 and XL1-Blue and helper phage R408 were from Stratagene; an oligolabeling kit was from Amersham Pharmacia Biotech; the Sulfolinik kit was from Pierce; the Bac-to-Bac from Life Technologies, Inc. All phospholipids were from Avanti Polar lipids, Inc. Radioactive materials were from Amersham Pharmacia Biotech. All other reagents were obtained from Sigma.

Enzyme Preparation —The vanadate-sensitive Mg2+-ATPase (ATPase II) was purified basically as described previously (13), with the following modifications. Instead of purified clathrin-coated vesicles, we used a fraction that contained all membranes except nucleus and mitochondria. A higher concentration of C12E9 was used than previously reported, and the last two glycerol gradient centrifugation steps with SDS were replaced by FPLC. In brief, the modified procedure consists of 1) washing the P2 membranes twice, once with 0.75 M Tris-HCl, pH 8.5, and once with 1% sodium cholate, followed by extraction of the enzyme with 0.5% C12E9; 2) hydroxyapatite chromatography; 3) ammonium sulfate fractionation; 4) glycerol gradient centrifugation; 5) FPLC with ceramic hydroxyapatite; and 6) FPLC with a superdex gel filtration column. The resulting preparation was stored at −70 °C.

Mass Spectrometry Analysis of the 116-kDa Doublet—The purified enzyme appears in SDS-PAGE as a doublet with an apparent molecular mass of 116 kDa (13). The two bands were separated by SDS-PAGE (8% acrylamide) with extended running time (30 min after the dye front reached the lower edge of the gel). The two bands were localized by staining with Coomassie Blue following SDS-PAGE and were subjected to in-gel digestion with trypsin (20). Aliquots (1 μl) of the resulting peptide mixtures were each mixed with 1 μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 50% water, 0.1% trifluoroacetic acid (v/v/v), and the mixture was dried on the target of a Voyager DE mass spectrometer (PE Biosystems, Foster City, CA) for matrix-assisted laser desorption/ionization. Time-of-flight mass spectra were acquired (21). The data were searched against the NCBI protein database issue number 10.04.99, using the MS-Fit search routine accessed through Protein Prospector on the Internet.

Isolation of cDNA Sequences Encoding Isoforms of ATPase II—A PCR-based approach was used to isolate cDNAs encoding for different isoforms using a bovine brain cDNA library as a source for DNA template. Because of the size of the protein, two pairs of primers were designed to independently clone the 5′- and 3′-halves of the cDNA, based on published sequence data of the bovine chromaffin granule ATPase II (15). The first pair of primers (A-1, 5′-CACTCGAGATGC-CCACCATGCGAGAC-3′; A-2, 5′-GAATTCAATTCCTAGGTC-3′) annealed to the base positions 175–1955 and 1700–1727 of the cDNA sequence, respectively. A XhoI restriction site was introduced immediately before the starting codon in primer A-1, and a ClaI site was introduced at the 5′-end of the second primer, as indicated by italics, for further cloning purposes. The second pair of primers (B-1, 5′-ATGTTATCTGATTCACATCGGCG-3′; B-2, 5′-AGGAGTTCTTCCACACCTCATGACGCTCTG-3′) annealed to positions 1717–1741 and 3600–3622, respectively. Restriction sites ClaI and HindIII were introduced at the 5′-end of primer B-1 and B-2, respectively. A high fidelity PCR kit (Roche Molecular Biochemicals) was used, and the PCR products were cloned into pCR-Blunt II-TOPO Plasmid using an empty pCR-Blunt II-TOPO PCR Cloning Kit, following manufacturer’s instructions. DNA sequencing was performed using an ABI Prism DNA sequencer. To generate full-length clones for expression studies, the PCR-amplified 5′-half and 3′-half clones were gel-purified and cleaved with restriction enzymes: XhoI and ClaI for 5′-half fragment and ClaI and HindIII for the 3′-half fragment. The enzyme-treated fragments were ligated through a three-way ligation by T4 DNA ligase into pFastBacHF vector that was cleaved by XhoI and HindIII.

4. Ding and X.-S. Xie, unpublished observation.

5. 175 is the position of the start codon, shown in boldface type within the sequence of the primer.
Screening of the bovine brain cdNA library was also performed in order to obtain clones covering the middle part of the coding sequence as well as clones containing the 5'- and 3'-end noncoding sequences. Two DNA probes for library screening were generated by PCR, of which one is 444 base pairs (bp) in length extending from the start codon and the other is 456 bp encoding for the 15-amino acid insert from the samples were dot-blotted to nitrocellulose paper directly. Immunode- PAGE and transferred to nitrocellulose paper, and synthetic peptide the corresponding peptide, following the manufacturer's instructions. For Western blot analysis, protein samples were separated by 8% SDS-PAGE and transferred to nitrocellulose paper, and synthetic peptide samples were dot-blotted to nitrocellulose paper directly. Immunode-

Generation of Anti-peptide Antibodies and Western Blot Analysis—

Five epitopes of interest were selected and synthesized. These include two conserved sequences near either N or C termini and three isoform-specific sequences that are discussed in detail under “Results.” A cysteine was added to the N terminus of each peptide to facilitate covalent linkages with sulfhydryl-reactive reagents. Antiserum was raised in New Zealand White rabbits to peptides linked to keyhole limpet hemocyanin as described previously (23). All antibodies were affinity-puriﬁed by Sufolink column chromatography after covalent attachment of the corresponding peptide, following the manufacturer’s instructions.

For Western blot analysis, protein samples were separated by 8% SDS-PAGE and transferred to nitrocellulose paper, and synthetic peptide samples were dot-blotted to nitrocellulose paper directly. Immunode-

Expression of the ATPase II Isoforms—A Bac-to-Bac baculovirus expression system (Life Technologies, Inc.) was used for the generation of recombinant baculovirus and expression of recombinant ATPase II isoforms, following the manufacturer’s instructions. The coding region of α1 and α2 isoforms was ampliﬁed by PCR and inserted into the polylinker sites of baculovirus vector pFastBacHTb in compliance with its open reading frame. The construct for β isoforms was generated by removing the 45 bp encoding for the 15-amino acid insert from the corresponding α isoforms. To do so, two primers with overlapping se-

quences were designed. Primer F (5′-GTCCTTACGGCGACGTCGTACAG-3′) anneals to bp 1284–1354 but excluding the 45 bp encoding for the 15-amino acid insert. Primer R (5′-TGTAGCTGCTCGCCGTA-AGCCACTCUC-3′) anneals to bp 1281–1351, also excluding the same 45 base pairs. With previously cloned α isoforms as templates, two PCR fragments were produced with primers A-1 and R for the 5′-half frag-

ment and primers F and B-2 for the 3′-half fragment. These two frag-

ments were gel-puriﬁed and mixed together. The mixture was then used as the template for the PCR to clone the full-length β isoforms with A-1 and B-2 as primers using a high ﬁdelity PCR kit (Roche Molecular Biochemicals). The full-length PCR product was gel-puriﬁed and cleaved with Xhol and HindIII and subsequently cloned into pFast-

BacHTb vector, which was also cleaved by Xhol and HindIII. The removing of the 45-bp was conﬁrmed by DNA sequencing. The expression vector pFastBacHTb generates a histidine tag at the N terminus of recombinant proteins for puriﬁcation purposes. The recombinant pFastBacHTb donor plasmid was then transformed into DH10Bac-competent E. coli cells, which contain bacmid and helper, to transform the recombinant bacmid. The recombinant bacmid was isolated from E. coli cells and transfected into Spodoptera frugiperda (SF9) ovaly cells with CellFECTIN reagent to produce recombinant baculovirus. SF9 cells were propagated in suspension culture at 27 °C in either Grace’s or IPI-41 medium supplemented with 8% heat-inacti-

vated fetal bovine serum plus 0.1% pluronic polyol F68. 4 liters of SF9 cells were transfected with recombinant virus for 40–48 h and har-

vested by centrifugation. The cells were resuspended in buffer A (50 mm Tris-HCl (pH 7.50), 0.1 m NaCl, 2 mm DTT, 10% glycerol, 1 mm phenylmethylsulfonyl ﬂuoride, 1 µg/ml leupeptin, and 1 µg/ml pepstatin A) and were broken by sonication. The membrane fraction was separated from cytosol by centrifugation at 100,000 × g for 1 h and analyzed by SDS-PAGE and Western blot.

Preparation of Phospholipids for Enzymatic Assays—

A fraction of ATPase II, either native or recombinant, was incubated with 0.5 µm (γ-32P)ATP (106 P/mmol of ATP) at room temperature for designated periods of time in a solution containing 25 mm Tris/MES, pH 7.5, 30 mM NaCl, 1 mm DTT, 10% glycerol, and a 3 mm concentration of either MgCl2 or EDTA, in a ﬁnal volume of 15 µL. The reaction was stopped by the addition of SDS sample buffer, followed by SDS-PAGE and autoradiograph.

RESULTS AND DISCUSSION

Identification of the Two Bands in the 116-kDa ATPase Dou-

blet as Isoforms—Because of the aforementioned indistinguish-

able characteristics of the two bands of the 116-kDa doublet, we considered the possibility that these two bands were isoforms of this enzyme, and we performed mass spectrometry analysis of tryptic fragments of these polypeptides to investigate this is-

FIG. 1. Sequence variations in the isoforms of ATPase II from different sources. Sequences of isoform determinant are printed in boldface or italic type.

eluted by a linear gradient of sodium phosphate up to 200 mm. Each active fraction from HTP column was directly loaded on top of a glycerol gradient from 15 to 30% prepared in buffer B and centrifuged in a SW 60 rotor at 60,000 rpm for 20 h. Fractions were collected by piercing the centrifuge tube from the bottom and were used for assays. Usually, a total of 14 fractions, at 0.3 ml each, were collected from each gradient. The phospholipids were then suspended in the solution by soaking overnight and vortexing. The phospholipid suspensions were then son-

icated in a bath-type sonicator three times for 5 min each, until the milky suspensions became completely clear, which would be the stock solutions. The stock solution for each phospholipid was then diluted 10-fold with buffer B (the same buffer used for the ﬁnal step of enzyme preparation) to form the working solution at concentration of 0.5 mg of phospholipid/ml.

ATPase Assay—ATPase activity was measured as the liberation of 32P from [γ-32P]ATP as described (13). In general, samples were pre-
inuculated with or without 2.5 µg of designated phospholipid, prepared as described above, for 10 min at 25 °C. ATPase assay was started by the addition of 0.2 ml of assay solution consisting of 50 mm Tris-MES, pH 7.0, 30 mM MgCl2, 3 mM MnCl2, and 3–5 mM (γ-32P)ATP (400 cpm/)

nm), with or without 0.1 mm sodium vanadate as an inhibitor, and incubated at 37 °C for 1 h. The reaction was terminated by the addition of 1 ml of 1.25 N perchloric acid, and phosphate was extracted and counted in a scintillation counter. The results are expressed as nmol of P/min/assay, or calculated as µmol of P/min/mg of protein.

Detection of Phosphoenzyme Intermediate—A fraction of ATPase II, either native or recombinant, was incubated with 0.5 µm (γ-32P)ATP (106 P/mmol of ATP) at room temperature for designated periods of time in a solution containing 25 mm Tris/MES, pH 7.5, 30 mM NaCl, 1 mm DTT, 10% glycerol, and a 3 mm concentration of either MgCl2 or EDTA, in a ﬁnal volume of 15 µL. The reaction was stopped by the addition of SDS sample buffer, followed by SDS-PAGE and autoradiograph.

Miscellaneous—SDS-PAGE was performed according to Laemmli (24), and protein concentration in general was determined by the the Amido Schwarz method (25) using bovine serum albumin as a standard. The content of recombinant enzyme was determined by Western blot analysis and densitometry scanning using a titration of native ATPase II as a standard curve.

RESULTS AND DISCUSSION

Identification of the Two Bands in the 116-kDa ATPase Dou-

blet as Isoforms—Because of the aforementioned indistinguish-

able characteristics of the two bands of the 116-kDa doublet, we considered the possibility that these two bands were isoforms of this enzyme, and we performed mass spectrometry analysis of tryptic fragments of these polypeptides to investigate this is-
**ATPase II Isoforms**

| Bovine brain | MPRRSRVKQ IRGHAQRY RGTQEXVRG AQGKIQYF IQQPEKFRK DHHVTFKVR VVYFVYKRV YKHFVYKRV
| Human        | MPRRSRVKQ IRGHAQRY RGTQEXVRG AQGKIQYF IQQPEKFRK DHHVTFKVR VVYFVYKRV YKHFVYKRV
| Mouse        | MPRRSRVKQ IRGHAQRY RGTQEXVRG AQGKIQYF IQQPEKFRK DHHVTFKVR VVYFVYKRV YKHFVYKRV

| Bovine brain | 1TTPPLPFLY SQPRAANSF FLJIALAQI EPSQPSFPY TVLQALLLA YAAEKKED
| Human        | 1TTPPLPFLY SQPRAANSF FLJIALAQI EPSQPSFPY TVLQALLLA YAAEKKED
| Mouse        | 1TTPPLPFLY SQPRAANSF FLJIALAQI EPSQPSFPY TVLQALLLA YAAEKKED

**FIG. 2.** Alignment of ATPase II sequences from different sources. Sequences of isoform determinant are printed in **boldface** or italic type. Other variable nucleotides are denoted by a *dot* under each position.
The two proteins were individually identified using a peptide mass fingerprinting approach in which the mass values for tryptic peptides derived from each protein were measured by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry and then matched against the masses expected from all available proteins from mammalian species. Searches specified a mass accuracy of 300 ppm and allowed up to one missed cleavage by trypsin in any peptide sequence. 25 of 25 peptide masses measured for the smaller of the two "116-kDa" polypeptides and 28 of 28 peptide masses for the larger polypeptide of the doublet matched bovine chromaffin granule ATPase II (U51100). Secondary matches with ATPase II from another mammalian species (mouse, U75321) were also recorded. The data from the smaller protein spanned 21% of the amino acid sequence of bovine ATPase II, and the data from the larger protein covered 24%. All major peaks in the spectra could be assigned to bovine ATPase II or to known background signals. It could therefore be concluded that the two proteins represent different forms of ATPase II. We named the longer and shorter forms as ATPase II isoform α and β, respectively, for convenience.

It was noticed that a mass spectral signal for peptides spanning residues 1108–1136 and 1144–1149, at the C terminus of the ATPase II sequence, was recorded in spectra from the α isoform but was absent from the β isoform. This suggested the possibility that these two proteins of the doublet differed in length due to the removal of amino acids from the C terminus of the shorter form. This notion was confirmed by Western blot analysis with sequence-specific antibodies, as discussed below.

**Isolation of cDNA Clones Encoding the ATPase II Isoforms**—Once the existence of isoforms was confirmed, we performed both PCR-based cloning and cDNA library screening using a bovine brain cDNA library as described under "Experimental Procedures," to isolate the cDNAs encoding these isoforms. 15 positive clones were obtained by a PCR-based approach including seven for the 5'-half and eight for the 3'-half of the cDNA. In addition, eight positives were isolated by library screening. Collectively, these clones constitute an open reading frame of 3492 base pairs, coding for a protein of 1164 amino acids. This is 15 residues longer than that of chromaffin granule ATPase II (15) as the result of a 45-nucleotide insertion between base pair positions 1296 and 1297, as shown in Fig. 1A. A corresponding insert has been previously found in human cDNA clones isolated from both brain (17) and skeletal muscle (18). It appears that all of our clones are coding for the α isoform of ATPase II, since we did not find any clone that is without the 45-base pair insertion or is missing the 3'-end. To summarize the cloning results, all clones are identical in their overlapping sequences except for variations in two regions. The first is a divergent sequence located from bp 454 to 513 (numbering begins with the start codon), resulting in an alteration of the 20-amino acid sequence between residues 152 and 171. As shown in Fig. 1B, the first sequence, designated isoform α1, is identical to that of bovine chromaffin granule ATPase II and to the mouse ATPase II homologue. The second, designated isoform α2, is identical to that of the human sequence. The ratio for our clones containing the signature sequence of isoform α1 versus that of isoform α2 is 2:11.

The second variation among the sequences of the clones consisted of a single base difference at nucleotide 1598 to be either T or A. As a result, residue 533 is predicted to be either Asp or Val, with a 50:50 ratio among all clones covering this region. Because all available sequences of ATPase II from different sources have Asp at this position, we used the clones with Asp at this position in our current expression study.

It is of particular note that the 15-amino acid insert is located in the catalytic domain, positioned between the ATP binding site and the aspartic acid residue that is phosphorylated in the phosphoenzyme intermediate. The divergent sequence of 20 amino acids (signature sequence for isoforms 1 and 2) is located 12 amino acids upstream from a P-type ATPase consensus sequence implicated in the coupling of ATP hydrolysis to transport. The functional ramification of these unique locations of the isoform variations is discussed below.

In addition, there are 12 single base differences between the bovine brain sequences and the published sequence of the bovine chromaffin granule ATPase II (revised version (January 7, 1999)).5 Five of these changes resulted in amino acid alteration, namely Val¹⁰⁹⁸ to Glu, Arg⁶⁶⁵ to Leu, Gly⁶⁴⁰ to Val, Tyr⁵⁵⁴ to Asn, and Arg⁶⁵⁸ to Lys. While some of these single amino acid changes are between amino acids with differing polarity or different functional groups, their functional significance remains to be determined. The alignment of amino acid sequence of ATPase II from different sources is shown in Fig. 2.

**Identification of Four Isoforms of ATPase II in Bovine Brain at the Protein Level**—In order to determine if these putative isoforms exist at the protein level and to identify the structure for the β isoform, antibodies were generated against five synthetic peptides corresponding to the informative region of the protein. Peptide A is identical to the 15-amino acid insert (Fig. 1A). Peptides B and C are sequences located either near the N terminus (residues 19–32) or C terminus (residues 1148–1162), respectively. Peptides D and E represent the 20-residue "signature" sequences for isoforms 1 and 2 (Fig. 1B), respectively. The corresponding antibodies generated against these peptides are named antibodies A, B, C, D, and E, respectively. As can be seen in Fig. 3A, antibody A recognizes only the larger band (isoform α) of the doublet, indicating that the shorter protein (isoform β) does not contain this insert. Antibody B (Fig. 3B) recognizes both bands, but antibody C (Fig. 3C) only blotted the larger protein. As noted before, the C terminus was not recovered from the shorter band of the doublet in mass spectrometry analysis. Based on that observation and the experiments of Fig. 3, it appears that the reduced molecular mass of isoform β is due to the combination of lack of the 15-amino acid insertion sequence and the truncation of the C terminus of the enzyme. The fact that no cDNA clone that is missing the 3'-end has been found suggests that the C-terminal truncation in isoform may be due to proteolysis as a post-translational

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*Fig. 3. Western blot analysis with sequence-specific antibodies.* Western blot analysis was performed as described under "Experimental Procedures." A sample of purified native ATPase II was resolved by SDS-PAGE at 10 μl (0.15 μg) in each lane, electrotransferred to nitrocellulose paper, and analyzed by blotting with antibodies against the 15-amino acid insert (A), the N terminus (B), the C terminus (C), isoform 1 (D), and isoform 2 (E). Both peptides D and E (10 ng each spot) were dot blotted directly to two stripes of nitrocellulose paper and immunanalyzed by antibody D or E under the same condition.

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*F. Williamson, GenBank™ accession number U51100, version U51100.1.*
modification. Because of the general consistency and stability of the doublet pattern in our enzyme preparations, it is less likely that the proteolysis occurred during the enzyme preparation procedure.

Also shown in Fig. 3, antibody D or E can blot both bands, indicating the presence of both α1 and α2 isoforms and β1 and β2 isoforms. However, the signal intensity for antibody D is much weaker than that for antibody E. This comparison was further validated by immunoanalysis of the synthetic peptides D and E, which shows similar intensity between these peptides and their respective antibodies under this condition and shows no cross-reaction. This observation coincides with our cDNA cloning results, which shows that the majority of positive clones covering this region are isoform 2 (11 of 13). Taking together, these results indicate that there are at least four isoforms of ATPase II present in bovine brain, with α2 and β2 being the major isoforms. Fig. 4 is a schematic representation of the four isoforms and antibody recognition sites. Comparison of our results with the sequence reported for human ATPase II indicates that it is an α2 isoform, whereas those of bovine chromaffin granules and mouse muscle are the equivalent of β1 isoform but with a complete C terminus. Since it is not known at present how many residues are actually removed from the native β isoforms of bovine brain ATPase II, we used the β isoforms with a complete C terminus in our subsequent expression study and labeled them as isoforms β1 and β2 to indicate the difference.

Expression of ATPase II Isoforms in Sf9 Cells Using Baculovirus Vectors—The coding region of the ATPase II α1 isoform was amplified by PCR and cloned into pFastBacHTb vector as described under “Experimental Procedures.” This expression vector provides a tag containing six histidines for protein purification purposes. The histidine-tagged recombinant proteins were expressed in Sf9 cells as described under “Experimental Procedures.” A membrane fraction from both transfected and control cells was prepared and analyzed by Western blot. The expression of α1 isoform is demonstrated in Fig. 5. As can be seen, antibody against the histidine tag (Fig. 5A) gave strong signals at around 120 kDa in the membrane fraction from transfected cells (lane 2) but not in the control cell (lane 1) nor the purified native enzyme (lane 3). This demonstrates the expression of a recombinant 120-kDa protein. Furthermore, this protein is recognized by antibodies A, B, and C, indicating that this protein is a full-length recombinant ATPase II α isoform. The recombinant ATPase II was extracted from the membrane of transfected cells by 1.2% C12E9 and partially purified by Ni2+-NTA chromatography. The nickel column fraction (lane 4) was blotted by anti-histidine tag antibody as well as by antibodies A, B, and C. In addition, there is a band of about 35 kDa in this fraction that was blotted only by the anti-histidine antibody and antibody B, indicating that it is a fragment of recombinant ATPase II resulting from either incomplete translation or proteolysis. As shown in Fig. 6, the nickel column fraction has a PS-dependent and vanadate-sen-
recognizes only α1 isoform of ATPase II, the recombinant proteins of all four isoforms were expressed, partially purified by Ni²⁺-NTA chromatography, and further purified by hydroxyapatite chromatography and glycerol gradient centrifugation to remove the smaller fragments of recombinant proteins. As expected, both antibodies B and C (against the conserved sequences near either the N or C terminus, respectively) blotted all four isoforms (data not shown). As shown in Fig. 7, antibody D (isoform 1-specific) recognizes only α1 and β₁ isoforms, antibody E (isoform 2 specific) recognizes only α2 and β₂ isoforms, and antibody A (against the 15-amino acid insertion) has a strong reaction with both α isoforms. However, while antibody A recognizes only α isoforms with native enzyme preparation, it has a cross-reaction with the recombinant β₁ isoforms. A contamination of α isoforms in the β₁ isoform preparations was excluded by repeating DNA sequence and the expression experiments. Analysis of ATPase II sequence revealed that the sequence of the last four amino acids of the 15-aa insert (PDEW) is identical to that of the C terminus of the enzyme. Because a peptide sequence of 3–5 amino acids is generally considered an antigenicity unit, and since the antibody is polyclonal, the identity in the last four amino acids between the insert and the C terminus appears to be the reason for the cross-reaction. The fact that the β isoform in native enzyme preparation from bovine brain lacks a complete C terminus explains why antibody A has no cross-reaction with it.

All four recombinant proteins exhibit PS-dependent and vanadate-sensitive ATPase activity, demonstrating for the first time the functional expression of recombinant ATPase II.

Based on the expression conditions established with the α1 isoform of ATPase II, the recombinant proteins of all four isoforms were expressed, partially purified by Ni²⁺-NTA chromatography, and further purified by hydroxyapatite chromatography and glycerol gradient centrifugation to remove the smaller fragments of recombinant proteins. As expected, both antibodies B and C (against the conserved sequences near either the N or C terminus, respectively) blotted all four isoforms (data not shown). As shown in Fig. 7, antibody D (isoform 1-specific) recognizes only α1 and β₁ isoforms, antibody E (isoform 2 specific) recognizes only α2 and β₂ isoforms, and antibody A (against the 15-amino acid insertion) has a strong reaction with both α isoforms. However, while antibody A recognizes only α isoforms with native enzyme preparation, it has a cross-reaction with the recombinant β₁ isoforms. A contamination of α isoforms in the β₁ isoform preparations was excluded by repeating DNA sequence and the expression experiments. Analysis of ATPase II sequence revealed that the sequence of the last four amino acids of the 15-aa insert (PDEW) is identical to that of the C terminus of the enzyme. Because a peptide sequence of 3–5 amino acids is generally considered an antigenicity unit, and since the antibody is polyclonal, the identity in the last four amino acids between the insert and the C terminus appears to be the reason for the cross-reaction. The fact that the β isoform in native enzyme preparation from bovine brain lacks a complete C terminus explains why antibody A has no cross-reaction with it.

All four recombinant proteins exhibit PS-dependent and vanadate-sensitive ATPase activity, similar to that shown in Fig. 6 for isoform α1. However, there are significant differences in both specific activities and phospholipid selectivities between the four isoforms of ATPase II. The consolidated results are shown in Fig. 8, where all four isoforms are compared side by side for their ATPase activities in the presence or absence of PS, PE, and PC, a nonsubstrate phospholipid for this enzyme. For the experiments shown in Fig. 6, the amounts of recombinant isoforms of ATPase II were determined by Western blot and densitometry scanning, using a titration of purified native ATPase II as a standard curve. By doing so, differences in degree of purification are excluded in the comparison of the ATPase activity between these isoforms. Thus, the ATPase activity is expressed as μmol/min/mg of recombinant protein. As can be seen, isoforms α1 and 2 show higher activities than do isoforms β₁ and 2, with the order of α2 > α1 >> β₁, β₂. While both isoform variations seem to contribute to the differences in phospholipid selectivity as determined by comparing the ratio of stimulation by PS over that of PE, it appears that the altered 20-residue sequence has a greater impact than does the 15-amino acid insert. As a result, the α2 isoform has the least response to PE, whereas PE stimulates β₁ isoform up to the 75% level of PS. In earlier studies with ATPase II from different sources, significant difference has been observed for both its specific ATPase activity and phospholipid selectivity, as discussed previously. Thus, the experimental results we present here provide a possible explanation for the observed differences in both specific ATPase activity and phospholipid selectivity reported for preparations of native enzyme from various sources.

It should be noted that the β isoforms we used in the expression studies differ from the native β isoforms of ATPase II in terms of having a complete C terminus. In addition, there may be other minor differences, since the constructs of β isoforms are derived from their corresponding α isoforms. For instance, the native β isoforms of bovine brain may be more homologous to the β₁ isoform isolated from bovine chromaffin granules, which has additional changes in the positions of five single amino acids compared with the brain α isoforms as discussed earlier. Thus, the comparison of the α isoforms with the β isoforms in these experiments does not completely reflect that with the native β isoform but is instead limited to a narrower scope. It has the advantage, however, of attributing the observed effects to more defined factors, i.e. only the 15-amino acid insert and the 20-amino acid signature sequence for isoforms 1 and 2. On the other hand, the difference between the recombinant β₁ isoform and the native β isoforms raised a question about the physiological relevance of this analysis. To address this issue, we did an immunodepletion experiment using anti-C terminus antibody to remove the α isoforms from a native preparation of purified ATPase II and compared the change in its ATPase activity. As shown in Fig. 9, α isoforms were no longer detectable after incubating twice with the antibody/protein A-agarose beads, left only β isoforms in the preparation. The resulting enzyme shows a decrease in specific ATPase activity but an increase in the ratio of stimulation by PE over that by PS. Considering that the original sample is a mixture of both α and β isoforms, the result is in very close agreement with the analysis obtained with recombinant isoforms.

![ATPase II Isoforms](image1)

**Fig. 7. Western blot analysis of four isoforms of recombinant ATPase II.** Western blot analysis was performed as described under “Experimental Procedures.” Different isoforms of recombinant ATPase II were purified by Ni²⁺-NTA column, hydroxyapatite chromatography, and glycerol gradient as described under “Experimental Procedures.” Enzyme samples (10 μl in each lane) were resolved by SDS-PAGE as triplicate sets and were blotted by antibodies D, E, and A, respectively.

**Fig. 8. Comparison of ATPase activity and phospholipid selectivity of different isoforms of recombinant ATPase II.** Recombinant ATPase II was expressed, solubilized, and purified by combined techniques of Ni²⁺-NTA column, hydroxyapatite chromatography, and glycerol gradient centrifugation as described under “Experimental Procedures.” To measure ATPase activity, 20 μl of each fraction was incubated with or without 2.5 μg of PC, PE, or PS for 10 min prior to the addition of ATPase assay solution, and the assay was proceeded as described under “Experimental Procedures.” The content of recombinant protein of each isoform was determined by Western blot analysis and densitometry scanning with a titration of purified native ATPase II as a standard curve.
It was noticed in our expression study that the recombinant ATPase II requires higher detergent concentration for effective solubilization. While this may be partially due to the difference of lipid composition between bovine brain and insect cells, improper folding of the recombinant protein, at least a portion of it, cannot be ruled out. This may also be a contributing factor for the lower specific ATPase activity of recombinant ATPase II, compared with the native enzyme from bovine brain.

ATPase II is a P-type ATPase that forms a phosphoenzyme intermediate during its catalytic cycle. With native enzyme preparation, we have observed that both α and β isoforms are quickly phosphorylated when incubated with 0.5 μM [γ-32P]ATP in the presence of Mg2⁺ but without the addition of any phospholipid. The phosphoenzyme, under these conditions, is slowly dephosphorylated following the exhaustion of the low concentration of ATP, as shown in Fig. 10A, lanes 1–3. The formation of phosphoenzyme of ATPase II can also be observed in the absence of Mg2⁺, but with a much slower rate. As shown in lanes 4–6 of Fig. 10, however, the phosphoenzyme formed in the presence of EDTA does not dephosphorylate, resulting in the accumulation of the phosphoenzyme intermediate, until Mg2⁺ is added (lanes 7 and 8). It is also observed that the more purified the enzyme is, the slower the rate of dephosphorylation in the presence of Mg2⁺ (data not shown), suggesting that the slow but continued catalytic turnover of this preparation is due to the presence of a trace amount of endogenous phospholipids. This point was further confirmed by the experiments with recombinant ATPase II as shown in Fig. 10B. With recombinant enzyme, we were able to remove virtually all endogenous phospholipids by extensive wash of the Ni²⁺-NTA column during purification (usually 200 times column volume). It is clearly shown in Fig. 10 that Mg2⁺ is not sufficient by itself to cause the dephosphorylation of the intermediate that probably requires phospholipids as well. To address this issue, we next added Mg2⁺ and different phospholipid to the accumulated phosphoenzyme, as shown in Fig. 11. Fig. 11A was created with a native ATPase II preparation, which, not surprisingly, shows that PS (lane 1) induces a fast release of 32P from the phosphoenzyme intermediate in the presence of Mg2⁺ compared to Mg2⁺ alone (lane 2). The effect of adding PE (lane 3) is interesting because the partial dephosphorylation it induced is primarily at the lower band, i.e. the β isoforms of the enzyme. This differential effect of PE is further demonstrated in the experiments with recombinant isoforms (Fig. 11B), in which two isoforms, α2 and β1, were compared for their dephosphorylation induced by different phospholipids. The former has the lowest PE/PS stimulation ratio (0.09:1) on ATPase activity, among the four isoforms, and the latter has the highest (0.75:1). In these experiments, the effect of PC was also measured as an additional control of a nonsubstrate phospholipid, which indeed does not induce any detectable decrease of phosphorylation (lane 4). While the two isoforms behave in the same fashion when PS (lane 2) or PC is added, they have an apparent

**FIG. 9.** Immunodepletion of α isoforms from native preparation of ATPase II. Purified native ATPase II, 20 μg/ml, was diluted 5-fold with a buffer consisting of 20 mM Tris-HCl, pH 7.5, 0.1% C₁₂E₉, and 1 mM DTT. To 1 ml of this diluted ATPase, 10 μl of affinity-purified IgG against the C terminus of ATPase II (antibody C) was added, and the mixture was rotated at 4 °C for 1 h. Protein A-agarose beads (50 μl) were then added to the enzyme-antibody mixture, and the rotation continued at 4 °C for an additional 2 h, followed by centrifugation to remove the agarose beads. To the resulting supernatant, antibody was added again, and the procedure described above was repeated until the second supernatant was obtained. The agarose bead pellets were combined, and the bound proteins were eluted by 200 μl of SDS sample buffer. The ATPase II samples either before (lane 1) or after (lane 2) immunodepletion and the sample eluted from the protein A-agarose beads (lane 3)(10 μl in each lane) were either purified native ATPase II (A) or recombinant isoforms (B). The incubation conditions and time are indicated at the bottom.

**FIG. 10.** The formation of phosphoenzyme intermediate of ATPase II and its recombinant isoforms. The detection of phosphoenzyme intermediate was performed as described under “Experimental Procedures.” Enzyme samples (0.2 μg in each lane) were either purified native ATPase II (A) or recombinant α2 isoform (B). The incubation conditions and time are indicated.
difference in their response to PE (lane 3) in terms of the rate of dephosphorylation. These studies of the formation and dephosphorylation of the phosphoenzyme intermediate of ATPase II demonstrate, from a different angle, that the isoform variation has a significant effect on the phospholipid selectivity of ATPase II. In addition, the results of these experiments provide a biochemical explanation for the PS dependence of ATPase II; i.e. aminophospholipid, PS in particular, is essential for the dephosphorylation of the phosphoenzyme intermediate of ATPase II and therefore the continuation of its catalytic cycle.

Sequence analysis revealed that the 15-amino acid insertion constitutes a PEST site, which is a primary sequence motif that is highly susceptible to proteolytic action that is usually found in proteins with short half-lives (26, 27). Thus, it is possible that the α isoforms of ATPase II, while having a higher ATPase activity than β isoforms, are less stable and subject to down-regulation by proteolysis. The reverse transcriptase-PCR analysis by Mouro et al. (18) has shown that tissues with only mRNA for α isoforms have a much higher messenger level than those with only β isoforms. Furthermore, in brain tissue that has detectable mRNA for both isoforms, the level of messenger that contains the insertion is much higher, whereas the abundance of the two isoforms is almost equal at the protein level in our preparation from bovine brain. All of these data support the notion that the α isoforms of ATPase II have a higher turnover rate and may be subject to regulation by cellular signals.

Although both isoform variations affect the phospholipid selectivity of ATPase II, they are not necessarily directly involved in PS binding. Studies are under way in our laboratory to identify the phospholipid-binding domain of the enzyme in order to understand the molecular mechanism of ATPase II as the aminophospholipid translocase. Furthermore, the success of recombinant ATPase II expression has paved the way for site-directed mutagenesis studies of this enzyme, which will certainly provide further information for this enzyme with far reaching physiological importance.

It was recently reported that, using a separately constructed yeast DRS2 null mutant, the deletion of the DRS2 gene shows no defect in the uptake or distribution of fluorescence-labeled PS and PE (16). In addition, it was also reported that the mRNA of ATPase II was either not detected or very weakly expressed in several tissues and cultured cells including lung, liver, trachea, bone marrow, and K562 cells (18). There has been no report showing any abnormality in aminophospholipid translocation in these tissues, and an active PS transport has been described in K562 cells (17). These observations challenge the exclusiveness of ATPase II or Drs2p as aminophospholipid translocase and suggest that an additional gene product is also responsible for aminophospholipid transport activity. It is possible that the multiple enzymes involved in aminophospholipid transport may play roles in different physiologic events or in different subcellular locations. This issue cannot be solved until the other activity is identified and the subcellular localization of both ATPase II and the unknown protein responsible for the other activity is determined.

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