# Control of Cell Membrane Ecto-ATPase by Oligomerization State: Intermolecular Cross-Linking Modulates ATPase Activity†

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ABSTRACT: The extracellular ATPase (ecto-ATPase) is a divalent cation-dependent nucleoside triphosphatase with an unusually high specific activity. Monoclonal antibodies, described previously [Stout, J. G., Strobel, R. S., & Kirley, T. L. (1995) *J. Biol. Chem. 270,* 11845−11850], and newly generated polyclonal antibodies, both raised against the chicken gizzard ecto-ATPase, were evaluated for their ability to cross-react with mammalian ecto-ATPases and were used as specific immunochemical probes to identify non-cross-linked and cross-linked ecto-ATPase. Unlike previous results obtained with the rabbit skeletal muscle ecto-ATPase enzyme, cross-linking the chicken gizzard smooth muscle ecto-ATPase with 3,3′-dithiobis-(sulfosuccinimidylpropionate) (DTSSP) and dithiobis(succinimidylpropionate) (DSP) increased the activity of the enzyme which corresponded to an increase in a ≈130 kDa immunoreactive band, proposed to be a ecto-ATPase homodimer, and a concomitant decrease in a ≈66 kDa immunoreactive band, the ecto-ATPase monomer. Ecto-ATPase was immunochemically identified in chicken, rat, mouse, rabbit, and pig. Interestingly, under nonreducing conditions, the ecto-ATPase activity in rat and pig (unlike chicken and rabbit) was evident on Western blots as an immunoreactive band at ≈200 kDa, proposed to be an intermolecularly disulfide-linked ecto-ATPase homotrimer. Nonreducing Western blot analysis of various rat tissues with three different monoclonal antibodies that recognize the 66 kDa chicken gizzard ecto-ATPase monomer strengthened the hypothesis that this 200 kDa band indeed represents the trimeric ecto-ATPase. After reduction, ecto-ATPase monomers were found to be ≈66 kDa in all species examined. The differences in ecto-ATPase quaternary structure stability may account for the observed species differences in ecto-ATPase enzymatic properties. Intermolecular disulfide bonds appear to be one of the species-specific ways to stabilize the native, active ecto-ATPase quaternary structure (the homotrimer). Based on the data obtained, as well as previous data from this and other laboratories, a hypothesis was developed to explain the modulation of ecto-ATPase activity by a variety of agents, including detergents, chemical cross-linkers, lectins, antibodies, and small molecule inhibitors. It is proposed that agents and conditions stabilizing ecto-ATPase oligomers stimulate enzyme activity, whereas agents and conditions destabilizing ecto-ATPase homooligomers would inhibit the ecto-ATPase.

The extracellular ATPases (ecto-ATPases) are integral membrane glycoproteins that, in the presence of millimolar concentrations of divalent cations, hydrolyze extracellular nucleoside triphosphates with a very high specific activity. These ATPases distinguish themselves from the other more characterized classes of ATPases via a unique enzymology that includes sensitivity to inactivation by detergents, insensitivity to ouabain, oligomycin, vanadate, and other "classic" ATPase inhibitors, modulation of activity by lectins, resistance to proteolysis, and lack of substrate specificity (hydrolysis of any nucleoside triphosphate). The ecto-ATPases are the subject of a recent review which describes the enzymology of these proteins in more detail (Plesner, 1995). These enzymes are present in a variety of species, tissues, and cell types (Plesner, 1995, and references contained therein), but difficulties associated with the isolation of these proteins, including inactivation by most detergents, low abundance, and lack of specific inhibitors/probes, have hindered structural and functional characterization of these enzymes. The ecto-ATPase has been postulated to be associated with a number of interesting physiological processes, including cell adhesion (Cunningham et al., 1993; Dzhandzhugazyan & Bock, 1993; Lin & Guidotti, 1989), cancer (Knowles, 1988; Knowles & Leng, 1984), purine recycling (Che et al., 1992), and the termination of purinergic receptor mediated responses to extracellular ATP (Plesner, 1995). However, the determination of the physiological function of the ecto-ATPases has been elusive.

One very puzzling phenomenon observed with the ecto-ATPase has been the difference in enzymology observed between species, and especially between the avian (chicken) and mammalian (rabbit) enzymes. For example, the chicken skeletal muscle transverse tubule (t-tubule) ecto-ATPase exhibits non-Michaelis−Menton enzyme kinetics; however, in the presence of the lectin concanavalin A (Con A),[1] the enzyme is both stimulated and changes its kinetics to the classic Michaelis−Menton type (Moulton et al., 1986). On

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the contrary, the rabbit skeletal muscle t-tubule ecto-ATPase typically follows Michaelis−Menton kinetics (Hidalgo et al., 1983) and, after solubilization, is inhibited by Con A (Kirley, 1988). Other studies done on rat ecto-ATPase have shown a nonlinear time course of ATP hydrolysis and substrate-induced (ATP) inactivation (Beeler et al., 1983). In addition, unusual effects of lectins, detergents, membrane perturbants, and cross-linkers have been observed. We hypothesized that at least some of these properties may be explained by species dependent differences in the strength of the interactions between ecto-ATPase monomers in homooligomeric active complexes. In order to investigate this hypothesis, cross-linkers were used to explore cross-link effects on ecto-ATPase activity and oligomer formation. Previously characterized ecto-ATPase monoclonal antibodies (Stout et al., 1995b), as well as newly generated ecto-ATPase polyclonal antibodies, were used to probe for ecto-ATPase cross-linked complexes. The result of cross-linking was an increase in chicken gizzard ecto-ATPase activity corresponding to the formation of a higher molecular mass immunoreactive band proposed to be a ecto-ATPase homodimer. The antibodies were also used to detect differences in the quaternary structure (intermolecular disulfide bonds) of the ecto-ATPases from different species. We propose that the native, active ecto-ATPases of all species are homooligomeric (trimeric) complexes and that differences in the ecto-ATPase quaternary structure stability account for at least part of the enzymatic differences observed for different species. A unifying hypothesis was formulated to explain the effects of many classes of inhibitors and stimulators of ecto-ATPase activity on the basis of their ability to modify the strength of the monomer-to-monomer interactions within the ecto-ATPase oligomeric structure.

## EXPERIMENTAL PROCEDURES

*Materials.* Adult chicken gizzards were obtained fresh from a local slaughterhouse. All reagent grade chemicals were obtained from Fisher Scientific. Digitonin, sucrose, actin (cat no. A-4785), anti-actin antibody (cat no. A-2066), and MOPS were purchased from the Sigma Chemical Co. 4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was from Dojindo. The cross-linking agents, DTSSP [3,3′-dithiobis(sulfosuccinimidylpropionate)], DSP [dithiobis(succinimidylpropionate)], and $BS^3$ [bis(sulfosuccinimidyl)-suberate] and sulfo-NHS-acetate (sulfosuccinimidyl acetate) were from Pierce. PolyScreen PVDF transfer membrane and enhanced chemiluminescence (ECL) reagents were from DuPont. Colloidal gold solution (Quantigold) was from Diversified Biotech. All electrophoresis apparati, chemicals (including DTT), and standards were from Bio-Rad.

*Membrane Isolation and Purification.* Tissues were isolated immediately from freshly killed animals (adult male Sprague-Dawley rats, New Zealand white rabbits, adult pigs, or chickens) and placed in ice-cold tissue homogenization buffer (THB, 30 mM MOPS, 2 mM EDTA, 250 mM sucrose, pH 7.4). The tissues were excised and membranes prepared from these tissues as described previously (Stout & Kirley, 1994b). Rabbit skeletal muscle transverse tubule membranes (Kirley, 1991) and chicken gizzard membranes (Stout & Kirley, 1994a) were processed and prepared as described previously. Protein concentrations of all the resultant membranes were determined by the Bio-Rad dye binding assay, using the modification of Stoscheck (1990) and bovine serum albumin as the standard.

*Immunogen Preparation.* Approximately 82 mg of chicken gizzard smooth muscle membranes were solubilized with 1.0% Nonidet P-40 at 1.0 mg/ml protein concentration as described (Stout et al., 1995b). The solubilized proteins were diluted 5-fold into 20 mM Tris-HCl/150 mM NaCl, pH 7.4 (Tris buffered saline, TBS) and incubated overnight at 4 °C with the previously described Mab #6 (Stout et al., 1995b), covalently coupled to 2 mL of protein A-Sepharose. The immunoaffinity column and bound ecto-ATPase was washed, eluted, and quantitated as described (Stout et al., 1995b). Immunoaffinity chromatography was repeated using the unbound fraction until the amount of protein eluted from the immunoaffinity column decreased. This immunopurification procedure allows for a single-step purification of the 66 kDa ecto-ATPase (Stout et al., 1995b). The eluants were pooled and concentrated to ≈150 $\mu$L using Centriprep 50 and Centricon 30 microconcentrators (Amicon). The concentrated sample (≈170 $\mu$g of immunoprecipitated protein) was diluted 1:1 with SDS sample buffer (5% SDS, 125 mM Tris-HCl, pH 6.8, 25% glycerol, 0.0025% bromphenol blue) spiked with 10% $\beta$-mercaptoethanol. The sample was boiled for 5 min prior to loading onto a 5−15% gradient Laemmli (1970) gel. After SDS−PAGE, the gel was stained with 0.1% Coomassie Brilliant Blue for 30 min at room temperature and destained, and the band at ≈66 kDa was excised, washed with $H_2O$, and sent to Cocalico Biologics (Reamstown, PA) for polyclonal antisera production in rabbits.

*Polyclonal Antibody Purification.* The polyclonal antisera was purified to ensure specificity for the ecto-ATPase. Ten milligrams of chicken gizzard smooth muscle membranes were solubilized with 1.0% Nonidet P-40 and the ≈66 kDa ecto-ATPase purified by immunoaffinity chromatography on a Mab # 6 column as described above. Fractions containing the protein were pooled and concentrated as described above. The sample was diluted with SDS sample buffer (5% SDS, 20 mM DTT, 125 mM Tris-HCl, pH 6.8, 25% glycerol, 0.0025% bromphenol blue), and boiled for 5 min prior to SDS−PAGE on a 1.5 mm thick 5−15% gradient gel. After SDS−PAGE, the sample was electroblotted onto Immobilon $P^{sq}$ PVDF at 33 V for 3.5 h in 10 mM CAPS buffer, pH 11.0 (Matsudaira, 1987). The blot was stained with 0.1% Ponceau S in 1% acetic acid for 1 min, and the ecto-ATPase protein band at ≈66 kDa was excised. The band was briefly destained with 1 mM NaOH, washed thoroughly with $H_2O$, and cut into ≈2 $mm^2$ pieces. The PVDF/ecto-ATPase pieces were blocked with PVP-40 as described for proteolysis of proteins on blots (Fernandez et al., 1992). After washing thoroughly with water, the PVDF/ecto-ATPase pieces were incubated with 4 mL of polyclonal antisera containing 0.02% $NaN_3$ and incubated overnight at 4 °C. The polyclonal supernatant was removed, and the PVDF/ecto-ATPase pieces with bound polyclonal were washed five times with TBS. The bound polyclonal antibody was eluted twice with 0.5

[1] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; ABD-F, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); DSP, dithiobis(succinimidylpropionate); $BS^3$, bis(sulfosuccinimidyl)suberate; TBS, Tris buffered saline; DTT, dithiothreitol; DCB, digitonin column buffer; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Con A, concanavalin A; QMA, quaternary methyl ammonium; HPSEC, high-performance size exclusion chromatography; Mab, monoclonal antibody.

mL of 200 mM glycine buffer, pH 3.0, containing 0.05% $NaN_3$, and neutralized immediately with unbuffered 2.0 M Tris base. Eluted protein was quantitated by optical density at 280 nm. This process was repeated with the unbound fraction until a decrease in the optical density of the eluted protein was observed. The eluants containing the immunoaffinity purified polyclonal antibodies were pooled and the protein concentration determined by optical density at 280 nm (based on 1.0 $OD_{280}$ = 0.8 mg/mL for IgG).

*Ecto-ATPase Partial Purification*. Five milligrams of pig coronary artery membranes were solubilized with 1.0% digitonin at 1.0 mg/mL protein concentration as previously described (Stout et al., 1995b). The solubilized proteins were diluted 5-fold into digitonin-free column buffer (10 mM Tris-HCl, pH 8.2, 2 mM $MgCl_2$) and loaded onto a QMA MemSep (size 1000) anion exchanger equilibrated with pH 8.2 digitonin column buffer (DCB, 0.1% digitonin in 10 mM Tris-HCl, 2 mM $MgCl_2$, pH 8.2). The anion exchange column was washed with 5 mL of DCB and eluted with a 0−200 mM NaCl gradient in DCB at 1.0 mL/min. Twenty 1.0 mL fractions were collected and assayed for ecto-ATPase activity as described previously (Stout et al., 1994). Aliquots of fractions containing ecto-ATPase activity were acetone precipitated (Hager & Burgess, 1980), resolved by SDS-PAGE, and subjected to Western analysis with anti-ecto-ATPase polyclonal and monoclonal antibodies.

*Western Analysis*. Tissue microsomes were diluted in nonreducing SDS sample buffer (5% SDS, 125 mM Tris-HCl, pH 6.8, 25% glycerol, 0.0025% bromphenol blue) at a protein concentration of 1.0 mg/mL. The samples were boiled 5 min prior to SDS-PAGE according to Laemmli (1970). The resolved proteins were electroblotted onto PVDF membrane for 2 h at 33 V in 10 mM CAPS buffer, pH 11.0 (Matsudaira, 1987). The blot was stained with 0.1% Ponceau S in 1.0% acetic acid for 1 min. The standards were cut away and restained with Coomassie Blue. The remaining blot was briefly destained with 1 mM NaOH, rinsed several times with water, and blocked with 5.0% nonfat dry milk in pH 7.4 TBS containing 0.02% $NaN_3$ for 1 h at room temperature. The blocking buffer was removed and the blot incubated overnight at room temperature with the primary antibody diluted in blocking buffer. After washing with TBS containing 0.05% Tween 20 and incubation for 1 h with an anti-rabbit or anti-mouse horseradish peroxidase conjugated secondary antibody (in blocking buffer containing no $NaN_3$), immunoreactivity was detected by chemiluminescence with the DuPont NEN ECL reagents as described by the manufacturer.

*Cross-Linking/Mg-ATPase Assay/Western Analysis*. Solubilized chicken gizzard or rabbit t-tubule protein [membrane protein was solubilized with digitonin as described previously (Stout & Kirley, 1994a)] or membrane-bound chicken gizzard protein was cross-linked with DTSSP, DSP, or $BS^3$ basically as described previously (Kirley, 1991). As a control for the effects of lysine modification in the absence of cross-linking, sulfo-NHS-acetate was employed as a monofunctional reagent very similar in structure and specificity to each half of the divalent DTSSP cross-linker. Briefly, either membranes or solubilized protein (0.1 mg/mL) were incubated with various concentrations of crosslinker for 10 min at 22 °C. The reaction was stopped by addition of a final concentration of 5 mM lysine and incubated for 5 min at 22 °C. Each sample was then split, and 25% was diluted and assayed for Mg-ATPase activity, while the remaining 75% was further incubated with a final concentration of 2 mM ABD-F (Kirley, 1989) for 30 min at 22 °C (to block free sulfhydryls and prevent disulfide−sulfhydryl exchange as well as to label the proteins fluorescently to aid in visualization of the precipitation and resolubilization of the proteins). These samples were precipitated with four volumes of −20 °C acetone (Hager & Burgess, 1980), resuspended in nonreducing SDS−PAGE sample buffer, and resolved by SDS−PAGE under nonreducing conditions. The resolved proteins were electroblotted onto PVDF membrane as described above and subjected to Western analysis. Immunoreactive bands were detected by ECL.

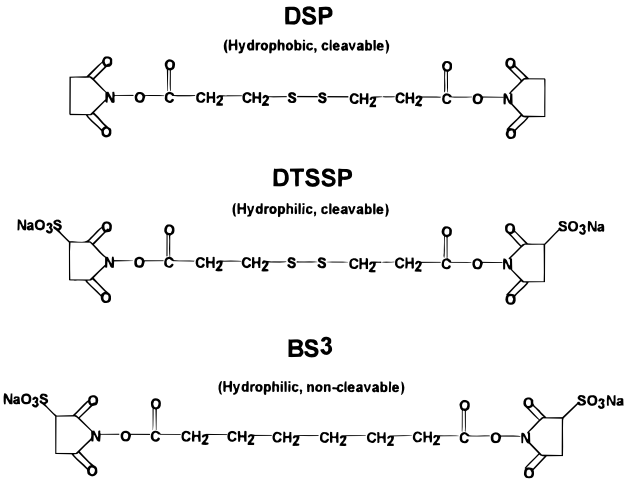


FIGURE 1: Structure of chemical cross-linkers used in this study.

In some experiments, the effect of adding DTT to reductively break the cross-link (of digitonin solubilized chicken gizzard ecto-ATPase), and thereby reverse the stimulation of ecto-ATPase activity induced by cross-linking, was measured (see Figure 4). DTT treatment was also performed on the non-cross-linked, digitonin-solubilized enzyme control.

## RESULTS

Chicken gizzard smooth muscle membranes and digitonin-solubilized proteins from gizzard membranes were cross-linked using the cleavable, lysine specific, primary amine cross-linkers, DTSSP (hydrophilic), and DSP (hydrophobic) (see structures of the cross-linkers in Figure 1) to investigate their effects on the ecto-ATPase. In addition to activity measurements, the ecto-ATPase protein was also examined by Western blot (after nonreducing SDS−PAGE) using Mab #6 as a specific probe for the ecto-ATPase (Stout et al., 1995b). After incubation with DTSSP, the membrane-bound and digitonin-solubilized ecto-ATPase activity increased with increasing concentrations of cross-linker (Figure 2). The increase in ecto-ATPase activity was accompanied by a decrease in immunoreactivity at ≈65 kDa and, in the case of the solubilized protein, an increase in immunoreactivity at ≈130 kDa (Figure 2). The increase in both ATPase activity and immunoreactivity at ≈130 kDa plateaued at high concentrations of cross-linker (Figure 2). Control experiments using the lysine specific monofunctional analogue of DTSSP, sulfo-NHS-acetate, demonstrated little stimulation of activity under the same conditions used to stimulate with DTSSP (data not shown).

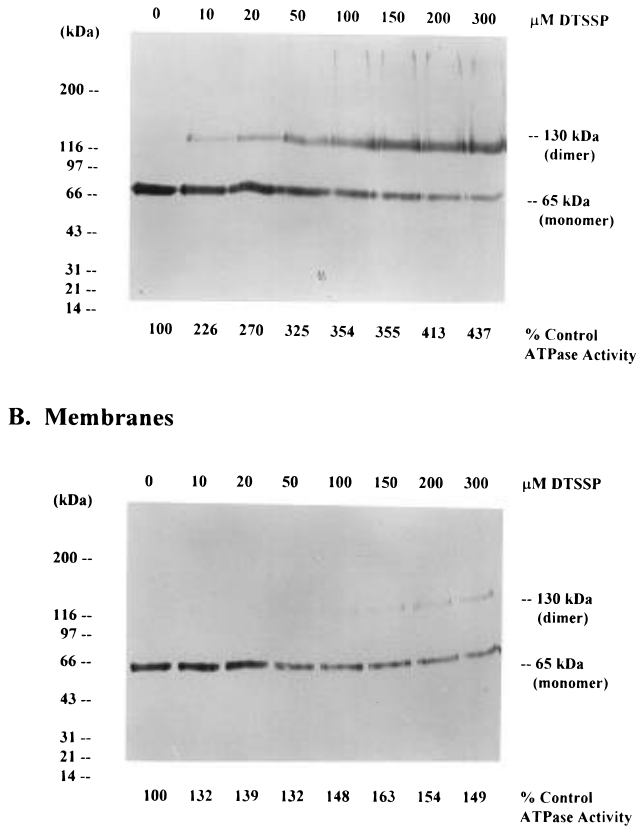


FIGURE 2: Effect of DTSSP cross-linking on the chicken gizzard smooth muscle ecto-ATPase. Cross-linking of the digitonin-solubilized **(A)** and membrane-bound **(B)** gizzard ecto-ATPase with DTSSP was assessed by measuring Mg-ATPase activity and Western blotting with anti-ecto-ATPase monoclonal antibody #6. The specific activity of the membranes used was 200−300 $\mu$mol of ATP hydrolyzed per mg of protein/h, and was not changed significantly by solubilization. Molecular weight markers are on the left, and ecto-ATPase markers are on the right of the Western blots. The micromolar concentration of cross-linker is indicated at the top of each lane, and the relative ATPase activity after treatment is indicated at the bottom. See Experimental Procedures for details.

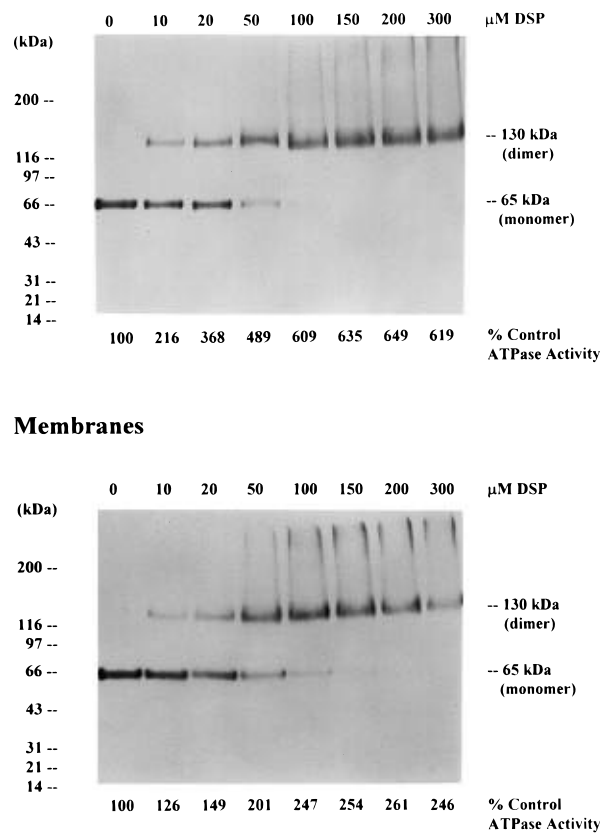


FIGURE 3: Effect of DSP cross-linking on the chicken gizzard smooth muscle ecto-ATPase. See the legend to Figure 2 for details.

Using DSP, the hydrophobic analog of DTSSP, treatment of both the membrane-bound and digitonin-solubilized ecto-ATPase also resulted in an increase of ATPase activity with increasing concentrations of cross-linker (Figure 3). Although this increase in activity was similar to that observed with DTSSP, the stimulation of ecto-ATPase activity was ≈1.6-fold greater for both membrane-bound and digitonin-solubilized cross-linked samples using the hydrophobic DSP (compare ATPase activities in Figures 2 and 3). Also, similar to DTSSP cross-linking, the DSP-increased activity followed an increase in immunoreactivity of a protein at ≈130 kDa and a decrease in immunoreactivity of a protein at ≈65 kDa (Figure 3). The homodimer formed upon cross-linking of the ecto-ATPase with DSP was visually more apparent than that observed with DTSSP (compare Figures 2B and 3B), a possible result of the hydrophilic DTSSP modifying or sterically hindering access to the epitope recognized by Mab #6, resulting in the decreased detectability of the 130 kDa dimer in the membranes cross-linked by DTSSP (see Figure 2B). Finally, with both cross-linkers, the cross-link induced stimulation of ecto-ATPase activity was 2−3 times greater for the solubilized versus the membrane-bound gizzard smooth muscle ecto-ATPase enzyme, and the corresponding Mab #6 immunoreactivity with the ≈130 kDa dimer was more pronounced with the solubilized gizzard membranes using either cross-linker (Figures 2 and 3). This greater cross-link-induced stimulation of the solubilized ecto-ATPase activity may result from more efficient cross-linking in the solubilized state or from a larger change in oligomer stability of the solubilized (and therefore destabilized) ecto-ATPase.

To determine if the cross-link-induced stimulation of activity could be reversed by reductive cleavage of the newly formed cross-links, the digitonin solubilized chicken gizzard ecto-ATPase was first treated with DTSSP or DSP, and then with DTT (at 11 min after initiation of cross-linking). As is evident from Figure 4, the stimulation resulting from cross-linking was partially reversed by reduction, although the interpretation is complicated by the demonstration of a significant inhibitory effect of DTT on the non-cross-linked control (Figure 4, control). In a similar control experiment, using 20 mM DTT for 30 min at 22 °C, digitonin solubilized rabbit t-tubule ecto-ATPase was inhibited 57%. These results indicate that there may be endogenous intramolecular disulfide bonds important for ecto-ATPase activity present in both rabbit and chicken enzymes.

Solubilized rabbit skeletal muscle transverse tubule ecto-ATPase was previously demonstrated to be inhibited, not stimulated, by cross-linking with DTSSP (Kirley, 1991). This previously reported effect was also not caused by simple lysine modification without cross-linking, as the monofunctional analogue of DTSSP, sulfo-NHS-acetate, was observed to be a much less effective inhibitor. We extended this

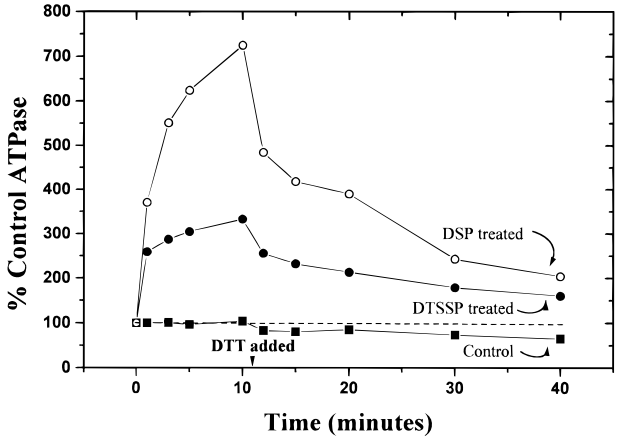


FIGURE 4: Reversal of cross-link-induced stimulation of ecto-ATPase activity by reductive cleavage of the cross-link. Digitonin-solubilized chicken gizzard membrane proteins at 0.1 mg/mL were reacted as described in Experimental Procedures with 150 $\mu$M DSP (open circles), 150 $\mu$M DTSSP (filled circles), or no cross-linker (filled squares, control) for 11 min at 22 °C, at which time DTT was added to a final concentration of 20 mM. The dashed line marks the level of the ecto-ATPase activity before any additions.

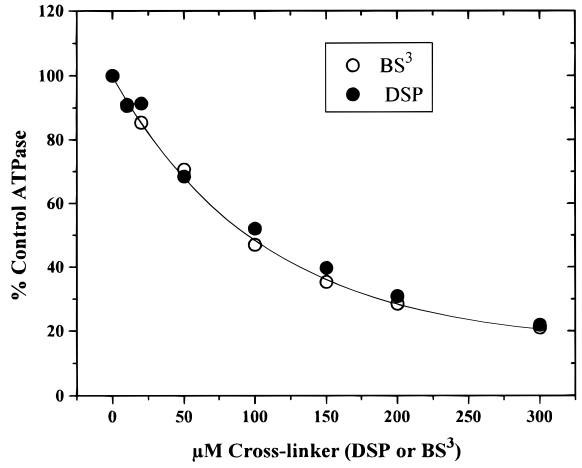


FIGURE 5: Effect of DSP and BS3 cross-linking on the Mg-ATPase activity of digitonin-solubilized rabbit transverse tubule membranes. Rabbit skeletal muscle t-tubule membranes were assayed for Mg-ATPase activity after reaction at room temperature for 10 min with various concentrations of cross-linkers. The relative ATPase activity expressed as percent of control is plotted versus increasing concentrations of cross-linker.

observation by using DSP and the noncleavable analogue of DTSSP, $BS^3$, to confirm the opposite results of cross-linking of the same enzyme from different species. In agreement with our earlier findings using DTSSP, the rabbit enzyme was also inhibited by the same cross-linkers reported here to stimulate chicken ecto-ATPase (Figure 5). We were unable to repeat the type of Western analysis done for the chicken enzyme (see Figures 2 and 3) on the rabbit enzyme, since the rabbit immunoglobulins present in the t-tubule preparation interfered with detection with the polyclonal antibodies (the concentration of endogenous IgG in the rabbit t-tubule membrane preparation is much greater than the ecto-ATPase, and therefore the reaction of secondary antibody with the endogenous rabbit IgG is so strong relative to the detection of the ecto-ATPase that the IgG is detected as a streak that covers a very broad molecular weight range, interfering with the weaker signal arising from the ecto-ATPase), and none of the monoclonal antibodies cross-reacted with the rabbit t-tubule enzyme (not shown).

The ability of the gizzard smooth muscle ecto-ATPase to form a more active homodimer upon cross-linking suggests that native ecto-ATPase may exist as a homooligomer, which is consistent with the previous report that the rabbit skeletal muscle transverse tubule ecto-ATPase may be a homodimer (Treuheit et al., 1992). In order to address this possibility, however, a specific probe(s) for the mammalian ecto-ATPase(s) was needed. Since, under reducing conditions, Mabs #6, #10, and #15 [raised against the chicken gizzard enzyme (Stout et al., 1995b)] were found not to cross-react with mammalian ecto-ATPases on Western blots, polyclonal antisera were generated in rabbits against the immunopurified chicken gizzard smooth muscle 66 kDa ecto-ATPase in an attempt to obtain a universal ecto-ATPase probe with more epitope heterogeneity for cross-reacting with ecto-ATPases from many tissues and species. Also, various pre-electrophoresis conditions prior to SDS−PAGE were screened in an attempt to optimize cross-reactivity of the Mabs. It was found that the Mabs reacted much more strongly with nonreduced gizzard ecto-ATPase, yielding a single band at 66 kDa, as was also observed under reducing conditions (Stout et al., 1995b). Upon reexamination of mammalian tissue cross-reactivity after nonreducing SDS−PAGE, it was discovered that the Mabs did, in fact, cross-react with some mammalian ecto-ATPases. The monoclonal and polyclonal antibodies were found to cross-react with the ecto-ATPase from a variety of species including chicken, rat, mouse, and pig. The Mabs failed to cross-react with rabbit and cow ecto-ATPase membrane preparations, but the polyclonal antibodies cross-reacted with the same rabbit membranes (data not shown). However, in several tissues and species (all mammalian) a 200 kDa band, and not the expected 66 kDa band, was detected by all of the Mabs after nonreducing SDS−PAGE.

To examine the relationship of the polyclonal reactivity, the monoclonal reactivity, and the ATPase activity, Western analysis (with Mab #6 and the affinity purified polyclonal antibodies) of a partially purified ecto-ATPase from pig coronary artery was performed (Figure 6), since this tissue provided an interesting and possibly physiologically relevant source of ecto-ATPase. Under nonreducing conditions, an immunoreactive band at ≈200 kDa was detected by both the monoclonal and polyclonal antibodies that correlated quantitatively with the ecto-ATPase activity measured in the same ion-exchange column fractions (Figure 6). The ecto-ATPase activity and corresponding immunoreactivity "tailed" on the ion-exchange column (Figure 6) due to the charge heterogeneity of the enzyme which presumably arises from glycosylation differences. This same pattern of immunoreactivity was observed with the partially purified ecto-ATPase from rat lung under the same conditions (data not shown). In addition, analysis of the digitonin-solubilized rat lung, chicken gizzard, and rabbit skeletal muscle ecto-ATPase by high-performance size exclusion chromatography (HPSEC) as described earlier for the rabbit enzyme (Treuheit et al., 1992) revealed that the ecto-ATPase activity and immunoreactivity eluted before intact IgG (data not shown), indicating that the digitonin-solubilized, active ecto-ATPases from all species migrate with an apparent molecular size greater than 150 kDa. The data are consistent with the ecto-ATPases of all species forming a active homooligomer (trimer) under native conditions.

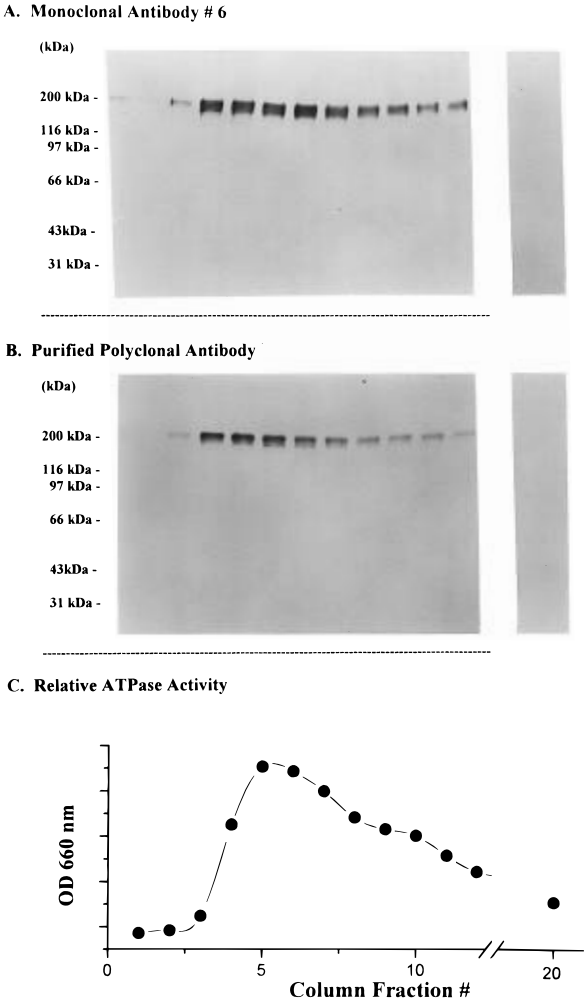


FIGURE 6: Immunoreactivity of the 200 kDa band with both monoclonal antibody and the purified polyclonal antibody correlates with ATPase activity. Digitonin-solubilized proteins from pig coronary artery membranes were partially purified by chromatography on a QMA Mem-Sep, pH 8.2, column eluted with a 0−200 mM NaCl gradient. The fractions were assayed for Mg-ATPase activity **(C)** as described in Experimental Procedures. Equal volume aliquots across the peak of activity were acetone precipitated and analyzed by Western blot using anti-ecto-ATPase monoclonal antibody #6 **(A)** and an anti-ecto-ATPase affinity purified polyclonal antibody **(B)**. Molecular weight markers are shown to left of the Western blots, and the column fraction numbers are aligned vertically with the corresponding Western blot lanes. See Experimental Procedures for further details.

To further investigate the quaternary structure of the ecto-ATPase from a variety of species, samples were examined by Western blot under reducing and nonreducing conditions in order to identify any disulfide linkages that might exist between monomers. Western analysis of four different tissues and species with the affinity purified polyclonal antibodies was used to assess the presence of intermolecular disulfide bonds. Under either reducing or nonreducing conditions, immunoreactive bands at ≈65 kDa were observed for rabbit transverse tubule membranes and chicken gizzard membranes with the polyclonal antibodies (Figure 7), indicative of monomeric ecto-ATPase, and consistent with previous results (Stout & Kirley, 1994a; Stout et al., 1995b; Treuheit et al., 1992). To demonstrate more clearly the oligomerization state of rabbit ecto-ATPase, digitonin-solubilized rabbit t-tubule membranes were depleted of IgG by protein A Sepharose, and the depleted sample was analyzed by

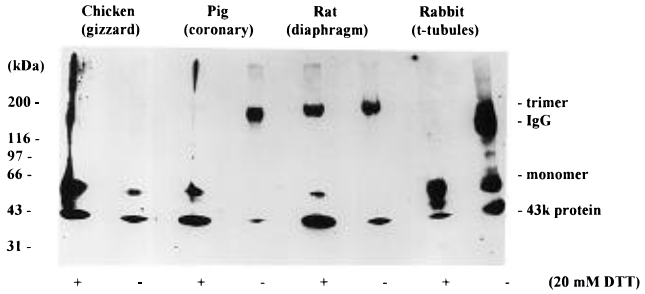


FIGURE 7: Analysis of intermolecular disulfide bonds between ecto-ATPase monomers from different species. Membranes from several species were prepared in reducing (containing 20 mM DTT) or nonreducing sample buffer at 1.0 mg/mL protein concentration and boiled 5 min, and 10 $\mu$g per lane was resolved by SDS−PAGE and Western blotted with an anti-ecto-ATPase polyclonal antibody. Molecular weight markers are on the left, and labels for the immunoreactive proteins are on the right; monomer and trimer refer to oligomers of the ecto-ATPase. "IgG" indicates immunoglobulin present in the rabbit t-tubule membrane preparation which directly reacts with the anti-rabbit secondary antibody used to detect the primary antibody. The species and tissues are indicated at the top, and the presence (+) or absence (−) of 20 mM DTT in the SDS−PAGE sample buffer is indicated at the bottom of each lane.

SDS−PAGE/Western analysis with the polyclonal antibodies. Either with or without reduction, a 66 kDa protein was recognized by the polyclonals on Western blots (results not shown). This indicates that the rabbit ecto-ATPase is not intermolecularly disulfide linked, consistent with earlier purification data (Treuheit et al., 1992).

Immunoreactive bands at ≈200 kDa (homooligomeric ecto-ATPase) were observed for rat diaphragm and pig coronary artery under nonreducing conditions (Figure 7). After reduction, these immunoreactive bands at ≈200 kDa shift to ≈65 kDa (Figure 7), the molecular size of monomeric ecto-ATPase. It should be noted that although this shift due to reduction is complete in the pig coronary sample, the rat diaphragm sample is poorly reduced under identical conditions, indicating that the rat enzyme is refractory to reduction and suggesting a difference in structure. Also, the results were qualitatively the same when the samples were boiled or heated at 60 °C prior to electrophoresis either in the presence or absence of 8 M urea (not shown), indicating that the 200 kDa band is indeed held together by disulfide bonds and is not due to aggregation caused by heating the sample under unusual conditions.

The identity of the 43 kDa protein cross-reacting with the affinity purified polyclonal antibodies, but not the Mabs (see Figure 7), was investigated. The 43 kDa protein could be separated from the ecto-ATPase activity by ion-exchange [no 43 kDa protein detected in the pig coronary ion exchange fractions (see Figure 6B and unpublished results using chicken gizzard ecto-ATPase)]. The 43 kDa protein was detected in almost every tissue and species examined. Experiments designed to examine the possibility that it could be a proteolytic fragment of the ecto-ATPase were negative, in agreement with the consistent finding that the ecto-ATPase is very resistant to proteolysis (Kirley, 1988; Saborido et al., 1991). It is unlikely that this immunoreactivity was due to an impurity in the antigen used to generate the polyclonal antisera, since the antigen was size selected after immunoaffinity purification, and the antisera was affinity purified using size selected, immunoaffinity pure 66 kDa chicken gizzard ecto-ATPase. The molecular weight, widespread distribu-

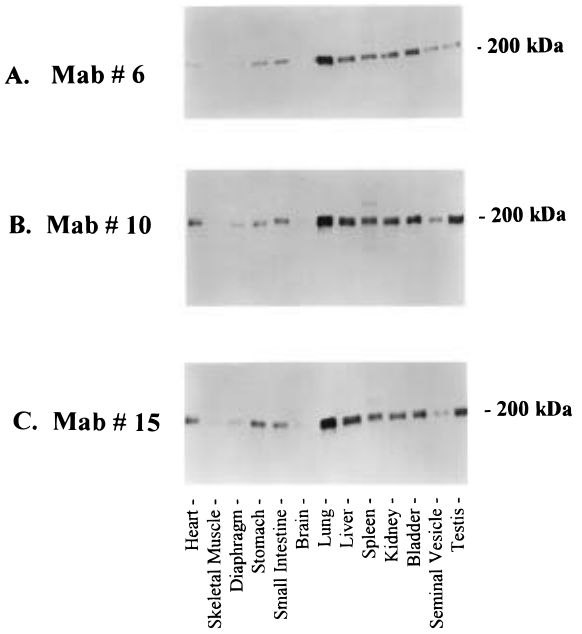


FIGURE 8: Distribution of the ecto-ATPase in rat tissues. Membranes from various rat tissues were prepared in nonreducing sample buffer, and 10 $\mu$g of protein per lane was resolved by SDS−PAGE and analyzed by Western blot using three different anti-ecto-ATPase monoclonal antibodies. Rat tissues are indicated at the bottom of each lane. Monoclonal antibodies used for each blot are indicated on the left, and the corresponding molecular weight markers are on the right.

tion, and presence of a blocked N-terminus (data not shown) led to the hypothesis that the 43 kDa immunoreactive protein might be actin. This hypothesis was tested and found to be correct by Western blot reactivity of purified actin with the affinity purified anti-ecto-ATPase polyclonal antibodies (data not shown). In addition, the hypothesis was also confirmed by partially purifying the 43 kDa immunoreactive protein from chicken gizzard and demonstrating that it reacts with an actin-specific commercial antibody (not shown). We do not know why immunostaining of this band (actin) by the polyclonal antibodies is more pronounced upon reduction, although we speculate that this may be because the antigen used to generate the polyclonal antibody (66 kDa gizzard ecto-ATPase) was reduced prior to gel purification.

To confirm that the 200 kDa band represents an oligomer of the ecto-ATPase and to gain insight into the possible function(s) of mammalian ecto-ATPase, the Western blot distribution in rat tissues was determined using three different Mabs (#6, #10, and #15) that each recognize specifically the monomeric 66 kDa chicken gizzard ecto-ATPase on Western blots (Stout et al., 1995b) (Figure 8, only the molecular mass range around 200 kDa is shown, since there were no other bands detected). Immunoreactive bands at ≈200 kDa were observed in all tissues screened; the relative intensities were very similar for the three different Mabs (Figure 8).

## DISCUSSION

The study of cell membrane ecto-ATPases is still in its infancy compared to the progress made on the more well known classes of ion motive ATPases (E-, P-, and V-type ATPases). There exists a considerable amount of contradictory and controversial data and conclusions in the literature concerning the structure, physiological function, and enzymology of the ecto-ATPases. In our work and throughout this study, the term "ecto-ATPase" refers to those enzymes that hydrolyze extracellular nucleoside triphosphates but do not hydrolyze nucleoside diphosphates to an appreciable extent (less than 5% of the hydrolysis rate observed with ATP). This is a distinct class of enzymes from the ecto-adenosine diphosphohydrolases (ecto-ATPDases, or apyrases), which hydrolyze ADP and other nucleoside diphosphates at rates similar to that of ATP. One of the best characterized examples of a vertebrate ecto-ATPDase is the 85 kDa glycoprotein isolated from chicken oviduct (Strobel & Rosenberg, 1993). This enzyme has been immunolocalized to the apical membranes of the oxyntico-peptic cells of the chicken stomach, a localization suggesting that the ecto-ATPDase is involved in secretion (Stout et al., 1995a).

The ecto-ATPase which our laboratory has purified to homogeneity from both rabbit skeletal muscle t-tubules (Treuheit et al., 1992) and chicken gizzard smooth muscle (Stout & Kirley, 1994a) is a very high specific activity enzyme which does not hydrolyze ADP. Much of the literature on the enzymology of this class of ATPase is derived from studies on skeletal muscle t-tubule membranes from several species. Although the rabbit enzyme follows classical Michaelis−Menton enzyme kinetics (Hidalgo et al., 1983), the chicken and rat enzymes do not, with the rat enzyme exhibiting nonlinear hydrolysis time courses and substrate (ATP)-induced inactivation (Beeler et al., 1983) and the chicken enzyme exhibiting negative cooperativity and substrate inhibition, both of which are abolished by preincubation (and stimulation of ATPase activity) by the lectin Con A (Moulton et al., 1986). Addition of Con A shifts the $K_m$ for ATP of rat t-tubule ecto-ATPase to higher substrate concentrations (Beeler et al., 1983). The reason for such varied enzymology is unclear, especially since it now appears that the molecular mass of the ecto-ATPase monomer of all species examined is very similar (≈66 kDa). Ecto-ATPase from all species is very susceptible to inactivation by most detergents [especially Triton X-100 (Hidalgo et al., 1983; Moulton et al., 1986)], and very resistant to inactivation by most chemical modification reagents and proteases.

Here, we propose that the native, active ecto-ATPase from all species is homotrimeric in quaternary structure and that the differences in enzymology between species can be explained by differences in the strengths of monomer−monomer interactions within the active complex. Intermolecular disulfide bonds are one important mechanism whereby the oligomer would be stabilized, but they are not the only, or even the most important, factor determining the stability and enzyme activity [note the differences between the rabbit and chicken enzymes, both of which contain no intermolecular disulfide bonds (see Table 1)]. The cross-linking data reported here demonstrate that the ecto-ATPase activity is modulated by intermolecular cross-linking (see Figures 2−5). The effect is opposite for the chicken vs the rabbit; stimulation of the chicken (Figures 2 and 3) vs inhibition of the rabbit enzyme [Figure 5 and Kirley (1991)]. We postulate that the reason for the opposite effect of cross-linking of the two species is due to a difference in the strength of the intermolecular (noncovalent) interactions between 66 kDa ecto-ATPase monomers. The pure rabbit enzyme has a much higher specific activity than that of the chicken, approximately 400 000 $\mu$mol mg$^{-1}$ h$^{-1}$ (Treuheit et al., 1992) compared to about 5000 $\mu$mol mg$^{-1}$ h$^{-1}$ [without

Table 1: Antibody Reactivity and Disulfide Linkages of Ecto-ATPases from Different Species[a]

| species | intermolecular disulfides | lectin and cross-linker effects | polyclonal antibody (reduced) | polyclonal antibody (non-red.) | Mab #6 (non-red.) | Mab #10 (non-red.) | Mab #15 (non-red.) |
|---|---|---|---|---|---|---|---|
| chicken | no | stimulates | (+) ≈66 k | (+) ≈66 k | (+) ≈66 k | (+) ≈66 k | (+) ≈66 k |
| rabbit | no | inhibits (solubilized) | (+) ≈66 k | (+) ≈66 k | (−) | (−) | (−) |
| rat | yes (trimer) | prevents ATP and detergent inhibition[b] | (+) ≈66 k | (+) ≈200k | (+) ≈200k | (+) ≈200k | (+) ≈200k |
| mouse[c] | yes (trimer) | ? | (+) ≈66 k | (+) ≈200k | (+) ≈200k | (+) ≈200k | (+) ≈200k |
| pig | yes (trimer) | ? | (+) ≈66 k | (+) ≈200k | (+) ≈200k | (+) ≈200k | (+) ≈200k |

[a] (+) Positive response to Western analysis at the given molecular size. (−) Negative (no band detected) response to Western analysis. [b] Lectin and cross-linker (glutaraldehyde) effects on rat ecto-ATPase taken from Beeler et al. (Beeler et al., 1983). [c] Results not shown.

Con A stimulation (Stout & Kirley, 1994a)]. We postulate that the rabbit enzyme is maximally active because of optimized monomer−monomer interactions: it has a more stable and enzymatically active quaternary structure than the chicken enzyme. Thus, cross-linking could only interfere and perturb this already optimized homooligomeric structure for the rabbit enzyme, whereas cross-linking would stimulate the chicken enzyme by enhancing the monomer−monomer interactions and thereby mimicking, however poorly, a more active quaternary conformation of the enzyme. Consistent with this hypothesis is the observation that even after maximal stimulation of the chicken ecto-ATPase by Con A [19-fold (Stout & Kirley, 1994a)] or Mabs [13-fold (Stout et al., 1995b)], the specific activity of the chicken enzyme is still about 4−5 times lower than that of the rabbit t-tubule enzyme (Treuheit et al., 1992). This theory would also explain why the Mabs and lectins stimulate the chicken enzyme but not the rabbit enzyme: both the antibody and the lectin are multivalent, effectively cross-linking ecto-ATPase monomers. This is consistent with the findings that succinylated Con A is much less stimulatory for the chicken enzyme (Stout & Kirley, 1994a) and that monovalent Fab fragments generated by papain cleavage from Mab # 6 (Stout et al., 1995b) only marginally stimulated activity (1.6-fold versus 9.5-fold for the intact antibody under identical conditions). Interestingly, in rat skeletal muscle, Beeler et al. (1983) found that treatment with glutaraldehyde stabilized the ecto-ATPase against detergent inactivation and prevented ATP-dependent inactivation of ATPase activity. However, probes were not available at that time to distinguish if the effects were due to inter- and/or intramolecular cross-linking of the ecto-ATPase. We have not examined rat ecto-ATPase enzymology; however, it appears as if the data and hypotheses set forth in this work may not explain all of the enzymatic properties of the rat ecto-ATPase as reported in earlier studies (Beeler et al., 1983).

Although we did not demonstrate directly the formation of intermolecular rabbit ecto-ATPase cross-linked species, it is interesting to note that a 200 kDa cross-linked species was previously observed in our laboratory (and the 66 kDa band disappeared) after cross-linking partially purified rabbit t-tubule ecto-ATPase with DTSSP [see Kirley (1991), Figure 2, lane 13]. It now seems likely that the native, active rabbit t-tubule ecto-ATPase (like the chicken gizzard enzyme) is a homotrimer of noncovalently associated monomers. In light of our current findings, it appears likely that the 200 kDa band observed in that earlier work was a homotrimer of the 66 kDa rabbit ecto-ATPase. Also, it appears as if the assignment of the active, digitonin-solubilized, rabbit t-tubule ecto-ATPase as a homodimer made in our previous publication (Treuheit et al., 1992) should be revised to a homotrimer in light of our present sizing experiments and disulfide bond determinations. The molecular size determined in that earlier work was 168 kDa (in the presence of 0.2 M NaCl), a value interpreted as a dimer of 67 kDa ecto-ATPase monomers. This value is actually in between that expected for a dimer and a trimer, and, in light of our current experiment demonstrating that the rabbit ecto-ATPase activity eluted earlier on a gel filtration column than did the IgG in the same sample, it seems reasonable to reclassify the active, solubilized rabbit ecto-ATPase as a homotrimer.

The detection of an immunoreactive band from several species at ≈200 kDa after nonreducing SDS−PAGE was at first surprising, since there was no evidence for intermolecular disulfide bonds existing in either the purified rabbit t-tubule (Treuheit et al., 1992) or chicken gizzard (Stout & Kirley, 1994a) ecto-ATPases. However, the hypothesis that the 200 kDa band is a trimer of the ecto-ATPase is strengthened by the observation that three Mabs which recognize different epitopes on the ecto-ATPase [based on data from their original characterization (Stout et al., 1995b)] recognize a 200 kDa band from a variety of rat tissues (Figure 8). Rat lung was found to contain the highest levels of the ecto-ATPase, and, in general, tissues known to be enriched in smooth muscle were enriched in ecto-ATPase. Ironically, skeletal muscle, the tissue from which the t-tubule membranes used for the first purification of the ecto-ATPase (Treuheit et al., 1992) is derived, is apparently a poor source for this enzyme, since ecto-ATPase immunoreactivity is low in both hind limb and diaphragm skeletal muscle (Figure 8).

Thus, it now seems clear that the 200 kDa band, observed in pig, rat, and mouse tissues, is an intermolecularly disulfide-bonded homotrimer of the ≈66 kDa ecto-ATPase. The apparent size of the digitonin-solubilized, active ecto-ATPase is the same for all mammalian species, as determined by HPSEC elution times (≈200 kDa, data not shown), so it appears that the active form of the ecto-ATPase is a homotrimer in all mammalian tissues/species. Why the homotrimer is intermolecularly disulfide-linked in some species but not others (see a summary in Table 1) is not clear, but it is hypothesized that the strength of the monomer-monomer interactions (both covalent and noncovalent interactions) is a key determinant of the rate of ATP hydrolysis by the enzyme. We postulate that at least part of the reason for the enzymatic differences observed among ecto-ATPases derived from different species could be accounted for by differences in the strength of monomer interactions in the active trimer. Not only is this hypothesis consistent with the chemical, antibody, and lectin "cross-linking" modulation of ATPase activity described above, but it may also explain modulation of activity of the ecto-ATPases by a variety of other agents. Thus, the class of hydrophobic, polysulfonated compounds found to be inhibitors of the purified chicken

ecto-ATPase, such as suramin, Coomassie blue, and the triazine-based reactive textile dyes such as Cibacron blue (Stout & Kirley, 1995) could easily inhibit by interfering with noncovalent monomer−monomer interactions, since it has been demonstrated that suramin induces deoligomerization of the tumor necrosis factor α native trimer (Alzani et al., 1995). Also consistent with this theory is the inactivation of the ecto-ATPases by a wide variety of biological detergents. Detergents, with the exception of digitonin, saponin, and lysolecithin, inactivate ecto-ATPases, probably resulting from the disruption of the native oligomeric structure necessary for expression of high ATPase specific activity. In addition, the finding that cholesterol oxidase treatment of brain ecto-ATPase inhibits activity (Wood et al., 1995) is also consistent with this hypothesis, since cholesterol oxidation increases membrane fluidity with a concomitant increase in protein mobility, possibly weakening ecto-ATPase monomer interactions within the membrane. Conversely, high levels of cholesterol would impose order on the phospholipid membrane by decreasing fluidity, which could subsequently stabilize the oligomerization state of the ecto-ATPase enzyme. It is relevant here that the rabbit t-tubule membranes are very high in cholesterol content (Hidalgo et al., 1983; Rosemblatt et al., 1981) and that the cholesterol co-purifies with the ecto-ATPase (Treuheit et al., 1992) and may be important for the high specific activity and stability of this enzyme. Modulation of ecto-ATPase activity by fatty acids and related compounds (Kang et al., 1991) may also be mediated in this manner. Taken as a whole, these data and observations offer insight into the mechanism of many modulators of ecto-ATPase activity and support a unifying hypothesis that modulation of enzyme activity by a variety of inhibitors and activators is mediated via a common pathway, regulation of the interactions between ≈66 kDa ecto-ATPase monomers in a homotrimeric quaternary structure.

It is interesting to speculate that the ecto-ATPase might also be regulated *in vivo* by alterations in ecto-ATPase monomer interactions induced by contact with other biomolecules. Several laboratories have reported that ecto-ATPases from various sources copurify and/or coimmunoprecipitate with cell adhesion molecules (Cunningham et al., 1993; Dzhandzhugazyan & Bock, 1993; Lin et al., 1991). The regulation mechanism as described above could be invoked by the interaction of adhesion molecules with their macromolecular receptors. Thus, the ATPase activity (and function) of the ecto-ATPase could be switched on and off by contact with an extracellular target, resulting in either initiation or termination of an adhesive response. Related to this speculation, extracellular ATP and ADP have been known for a long time to modulate cell aggregation events (Knight et al., 1966), including platelet aggregation, and the ecto-ATPase would control the concentrations of the nucleotide triggers. This mechanism could also be invoked during the rapid termination of a purinergic response to extracellular ATP by turning on the ATPase activity of the ecto-ATPase by a change in its oligomerization state mediated via interaction with a purinergic receptor, ATP itself, or some associated protein.

We originally used cleavable (by reduction) cross-linking agents, expecting to cross-link the ecto-ATPase to some protein other than itself and to identify the "associated" protein after cleaving the cross-links. The possibility of closely associated proteins was examined since others have reported copurification and coimmunoprecipitations with adhesion molecules (Cunningham et al., 1993; Dzhandzhugazyan & Bock, 1993; Lin et al., 1991), and this laboratory reported that antibodies which recognized LEP100 and integrin proteins on Western blots were capable of immunoprecipitating chicken gizzard ecto-ATPase activity (Stout et al., 1995b). However, we found no evidence of the 66 kDa ecto-ATPase being chemically cross-linked to anything but itself. It is possible that a different choice of cross-linkers would yield different results, but the results reported here suggest, along with previous purification data (Stout et al., 1994, 1995b; Stout & Kirley, 1994a; Treuheit et al., 1992), that the ecto-ATPase does not exist in a tight association with an adhesion molecule, or any other protein. However, it is always possible that these results are species-dependent, or that there may be subpopulations of ecto-ATPases or isoforms of the ecto-ATPase which are in close association with specific proteins. The observation that anti-ecto-ATPase polyclonal antibodies, which were affinity purified using immunopure 66 kDa chicken gizzard ecto-ATPase, cross-react with actin is interesting. This suggests that there are similar epitopes on both proteins, possibly implying some structural or functional relatedness between the two proteins. The significance of this observation may only become evident when the ecto-ATPase is cloned and sequenced or when the physiological significance of the ecto-ATPases is elucidated.

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