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Na,K-ATPase in Dog Red Cells

IMMUNOLOGICAL IDENTIFICATION AND MATURATION-ASSOCIATED DEGRADATION BY THE PROTEOLYTIC SYSTEM*

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The Na,K-ATPase of red cells from high K^+ and low K^+ dogs was studied immunologically by using antibodies raised against dog kidney enzyme. Anti-α subunit IgGs, which also recognized $\alpha(+)$ from brain enzyme, identified the larger subunit of erthrocyte Na,K-ATPase as a homogeneous polypeptide with M_r = 96,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting. In addition, erythrocyte Na,K-ATPase, purified by immunoaffinity chromatography on a monoclonal antibody-coupled column, showed the identity of its polypeptide composition to that of the renal enzyme. Furthermore, it was shown that reticulocyte lysates from high K^+ and low K^+ dogs substantially degraded ^{125}I-Bolton-Hunter reagent-labeled Na,K-ATPase. This degradation of the enzyme protein was significantly enhanced by the addition of ATP and Mg^{2+}. These results indicate that dog reticulocytes possess some mechanism for protein breakdown involving an ATP-dependent proteolytic system, resulting in the dramatic breakdown of Na,K-ATPase activity during dog reticulocyte maturation into erythrocytes (Maede, Y., and Inaba, M. (1985) *J. Biol. Chem.* 260, 3337–3343).

Na,K-ATPase is an essential membrane-bound enzyme, playing a key role in transporting sodium out of and potassium into cells (for review, see Ref. 1). Most mammalian cells possess the enzyme to maintain intracellular high potassium, low sodium concentrations, which are necessary to carry out their physiological functions. Previous studies have consistently shown that the enzyme is composed of two noncovalently attached subunits, an α subunit (M_r = 90,000–110,000) possessing catalytic activity, and a glycosylated β subunit (M_r = 40,000–60,000) of unknown function. The primary structures of these polypeptides in some preparations have recently been established by deduction from their cDNA sequences (2–4). In addition, biosynthesis of the enzyme protein has been reported by some investigators (5–9).

Erythrocytes from various vertebrates have often been used as subjects for ion transport studies (10–15) since Post *et al.* (16) demonstrated the presence of Na,K-ATPase in human erythrocytes. However, studies of the molecular structure of this enzyme in erythrocytes have lagged behind structural studies conducted on specialized tissues much richer in the enzyme (1, 17, 18). To our knowledge, disregarding the many arguments on the function and kinetics of the enzyme, only the cross-reactivity of the antibodies raised against kidney Na,K-ATPase with human erythrocytes (19–21) has supported the molecular similarity of the erythrocyte enzyme to the renal one. Moreover, very little is known about the regulation of the enzymatic activity in erythrocytes or in other tissues and cells.

Recently, we have demonstrated that dogs are genetically divided into two distinct types with regard to the Na,K-ATPase activity of their erythrocytes, the HK (high activity) and LK (no activity) types (22, 23). We have also found that reticulocytes, immature red cells, from both types of dogs possess Na,K-ATPase activity and reported on some properties of the decrease in enzyme activity during reticulocyte maturation into erythrocytes (24). However, questions have been newly raised as to (i) whether the Na,K-ATPase of dog erythrocytes is identical to the enzyme from other sources, (ii) whether the enzyme proteins from HK erythrocytes, HK reticulocytes, and LK reticulocytes are homogeneous molecules or not, and (iii) whether the decrease of enzymatic activity during reticulocyte maturation is caused by alterations of the enzyme protein itself. It is of general biochemical and biological significance to elucidate these questions, since information about the structure and regulation of the Na,K-ATPase in two such typical types of erythrocytes may be relevant for the Na,K-ATPase in a variety of cells from many species.

The present study was done to examine the molecular identity of the erythrocyte Na,K-ATPase with Na,K-ATPase from other tissues and cells. The proteolysis of this enzyme during reticulocyte maturation into erythrocytes is also discussed.

EXPERIMENTAL PROCEDURES

Materials—^{125}I-Labeled Bolton-Hunter reagent (4400 Ci/mmol) was obtained from New England Nuclear. Apparatus and reagents for SDS-PAGE[1] and immunoblotting, including goat anti-rabbit IgG horseradish peroxidase conjugate, were purchased from Bio-Rad. Protein A-Sepharose CL-4B, CNBr-activated Sepharose 4B, and Percoll were obtained from Pharmacia P-L Biochemicals. The sources of other chemicals were as follows. PMSF and pepstatin were from Boehringer Mannheim, Durapore GVHP membrane from Millipore, RPMI 1640 medium from GIBCO, Freund's complete adjuvant from Difco, SDS from BDH Pharmaceuticals, and $C_{12}E_8$ from Nikko Chemicals (Tokyo). All other chemicals used were purchased from Wako Pure Chemical Industries (Osaka, Japan).

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; $C_{12}E_8$, octaethylene glycol dodecyl ether; MoAb, monoclonal antibody.

Mouse myeloma cells (line Sp2/0-Ag14) were kindly provided by Dr. Hiroshi Kida (Hokkaido University). K562 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Flow Laboratories).

Preparation of Cell Membranes from Erythrocytes and K562 Cells—Heparinized blood was filtered through an α-cellulose/microcrystalline cellulose column to remove leukocytes and platelets. Filtered cells were washed three times with 170 mM NaCl, 5 mM Tris/Cl, pH 7.4. Washed cells were lysed with 40 volumes of lysing solution (5 mM Tris/Cl, pH 7.8, 1 mM EDTA, 0.2 mM PMSF) and centrifuged for 15 min at 18,000 × *g*. Membranes were washed twice with the same solution, then once with 250 mM sucrose, 0.2 mM PMSF, and 1 mM EDTA/Tris, pH 7.4. The resultant erythrocyte membranes were suspended in the same sucrose solution and stored at −80 °C until use. Patterns of Coomassie Brilliant Blue-stained proteins from HK and LK dog erythrocyte membranes prepared as described above were shown in Fig. 1*A*.

K562 cells were collected and washed. Cells were homogenized in 20 volumes of lysing solution using a glass-Teflon homogenizer and centrifuged at 1,000 × *g* for 5 min. Supernatants were centrifuged at 85,000 × *g* for 30 min. The resultant pellet was suspended in sucrose solution and stored at −80 °C.

Preparation of Brain Microsomes—Dog and pig brain microsomes were prepared as described by Sweadner (25).

Preparation of Dog Kidney Na,K-ATPase and Purification of the α Subunit—Na,K-ATPase was prepared from dog kidney according to the method of Jørgensen (26). The specific activity of the preparations was about 1200–2000 μmol/mg of protein/h. The α subunit was purified by gel filtration chromatography on a Bio-Gel A-1.5m column (1.6 × 90-cm) in the presence of 0.1% SDS as described previously (27). Fractions containing the α subunit were concentrated on a Millipore immersible CX-30 membrane. The complete purification of the α subunit was achieved by loading the concentrated fractions on a column of TSK-GEL G3000SW (Toyo Soda, Tokyo, Japan) through a high-performance liquid chromatography system from Waters Associates. The column was equilibrated and eluted with 0.1 M Na_2SO_4, 0.1 M sodium phosphate, pH 7.0, and 0.1% SDS at a flow rate of 0.3 ml/min. Purified α subunit was dialyzed against 150 mM NaCl, 10 mM sodium phosphate, pH 7.4, concentrated to 1 mg protein/ml, and used as an antigen.

Preparation of Anti-α Subunit IgGs—The α subunit (1 mg) was emulsified with an equal volume of Freund's complete adjuvant and injected into a New Zealand White rabbit twice with an interval of 3 weeks. 4 weeks later, a booster injection (0.5 mg) was given intravenously, and the antiserum was obtained 4 days after the final injection. Anti-α IgGs were purified by affinity chromatography on Protein A-Sepharose CL-4B column. Anti-α IgGs, eluted with 1 M acetic acid, were dialyzed against 150 mM NaCl, 10 mM Tris/Cl, pH 7.4, for 2 days with several changes of buffer and stored at −80 °C.

Production of Monoclonal Antibody Secreting Hybridomas—Two BALB/c mice were immunized with 20 μg of membrane-bound Na,K-ATPase from dog kidney emulsified with Freund's complete adjuvant. Mice were boosted 3 weeks later with a similar enzyme preparation by intravenous injection. After 3 days, spleen cells were fused with Sp2/0-Ag14 cells (28), and hybrid cells were grown and cloned in soft agar to ensure stable antibody production (29). Hybridomas were screened for production of antibodies against membrane-bound enzyme (30) using a Bio-Rad clone-selecting enzyme-linked immunosorbent assay kit.

Hybridomas 24/1 and 202/4 used in this study were among 10 cloned hybrids derived by the procedures outlined above. These cells produced IgGs which recognized the ^{125}I-Bolton-Hunter reagent-labeled Na,K-ATPase solubilized with $C_{12}E_8$ and precipitated it with Protein A-Sepharose beads as shown in Fig. 1*B*.

Preparation of Immunoaffinity Beads—Hybridomas 24/1 and 202/4 were grown in peritoneal cavities of pristane-primed BALB/c mice. Monoclonal antibodies were purified from ascitic fluids by ammonium sulfate precipitation followed by affinity chromatography on a Protein A-Sepharose column. Purified monoclonal IgG was covalently coupled to CNBr-activated Sepharose 4B as recommended by the manufacturer. Approximately 3–5 mg of antibodies were coupled to 1 ml of the packed beads.

Immunoaffinity Purification of the Na,K-ATPase from HK Dog Erythrocytes—HK dog erythrocyte membranes were depleted of spectrin according to the method of Cohen and Foley (31) with minor modifications. Ghost membranes were suspended in 0.1 mM EDTA, 0.1 mM EGTA, and 0.5 mM PMSF (pH 8.0, adjusted with Tris), incubated at 37 °C for 30 min, and centrifuged at 165,000 × *g* for 30 min. Pelleted vesicles were washed once using the same procedure and resuspended in 50 mM Tris/Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3, 0.2 mM PMSF, and 10% glycerol (TNG buffer). Membranes were solubilized by the addition of an equal volume of $C_{12}E_8$ in TNG buffer to yield a final concentration of 7.5 mg $C_{12}E_8$/2.5 mg protein/ml and centrifuged at 208,000 × *g* for 30 min. Supernatant was applied to an immunoaffinity column (MoAb 24/1- or 202/4-coupled Sepharose 4B (1 × 2-cm)) equilibrated with TNG buffer containing 0.5 mg $C_{12}E_8$/ml at a flow rate of 2 ml/min. Each column was washed with 20 volumes of the same buffer and 5 volumes of buffer containing 1 M NaCl, followed by 5 volumes of TNG buffer again. The bound proteins then were eluted with 50 mM diethylamine/Cl, pH 11.5, containing 0.5 mM PMSF and 0.5 mg of $C_{12}E_8$, followed by neutralization with 1 M Tris/Cl, pH 7.5.

Experimental Reticulocytosis in Dogs and Separation of Reticulocytes—Experimental reticulocytosis was induced in HK and LK dogs as previously described (24). Reticulocytes were separated from whole blood using the Percoll discontinuous gradient centrifugation technique (24).

Reticulocyte Culture—Dog reticulocytes separated as outlined above were cultured as previously described (24). At the indicated times, cells were collected and washed. The membranes then were prepared as mentioned above.

Preparation of Reticulocyte Lysates—Reticulocytes obtained from anemic dogs were washed with 10 mM Tris/Cl, pH 7.4, 150 mM NaCl. Lysates were prepared as described by Tanaka *et al.* (32).

^{125}I-Labeling of the Na,K-ATPase from Dog Kidney—Membrane-bound Na,K-ATPase purified from dog kidney was washed and suspended in 50 mM sodium phosphate buffer, pH 8.0. 10 μl of enzyme solution (20 μg) were reacted with 500 μCi of ^{125}I-Bolton-Hunter reagent (33) at 0 °C for 15 min. After that, 500 μl of 50 mM phosphate buffer, pH 7.8, containing 0.2 M glycine was added and allowed to stand for 5 min. 500 μl of 50 mM phosphate buffer, pH 7.5, containing 1% bovine serum albumin then was added and centrifuged for 10 min at 15,000 × *g* in a microcentrifuge apparatus. Membranes were washed three times in the same manner, then washed two times with 50 mM phosphate buffer without bovine serum albumin. The resultant ^{125}I-labeled enzymes were suspended and homogenized in 250 mM sucrose, 1 mM EDTA/Tris, pH 7.4. Thus, ^{125}I-labeled Na,K-ATPase (3.3 × 10^6 cpm/μg protein) was obtained. This preparation was stored at −80 °C and was used within 7 days after labeling.

Proteolysis of ^{125}I-Labeled Na,K-ATPase by Dog Reticulocyte Lysates—^{125}I-Labeled Na,K-ATPase (1.5 × 10^5 cpm) was dissolved in 15 μl of 50 mM Tris/Cl, pH 7.8. When present, ATP and $MgCl_2$ were included in the reaction at concentrations of 2 and 5 mM, respectively. Incubations were started by the addition of 5 μl of reticulocyte lysates and were performed at 37 °C. At the indicated time, an equal volume of × 2 concentrated SDS-PAGE sample buffer (34) containing 4 mM PMSF, 4 μg/ml pepstatin, and 1 mM EDTA was added and allowed to stand for 30 min. After electrophoresis, gels were fixed, dried, and exposed to Kodak X-Omat S film with a Cronex intensifying screen at −80 °C for 1–3 days.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed as described by Laemmli (34) using 8% acrylamide gel. Samples were electrophoresed without heat treatment, except that marker proteins (Bio-Rad) were boiled for 2 min before electrophoresis. Gels were stained with Coomassie Brilliant Blue or with silver according to the manufacturer's directions (Bio-Rad). For immunoblotting, gels were equilibrated with a blotting buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 30 min, and proteins were transferred from gels onto Durapore GVHP membranes at 60 V (200–600 mA) for 12–14 h in a cold room. Immunological detection of the α subunit was carried out using a Bio-Rad Immuno-blot™ assay system. The primary antibody was rabbit anti-α IgGs prepared as mentioned above, and the secondary antibody was horseradish peroxidase conjugated antibody to rabbit IgG. All washing solutions contained 0.05% Tween 20.

Assay of Protein Concentrations—Protein concentration was measured by the method of Bradford (35) with a Bio-Rad protein assay kit using bovine serum albumin as the standard.

RESULTS

Immunoblot Detection of the α Subunit of Na,K-ATPase in HK Dog Erythrocytes—We previously reported that HK dog erythrocyte membranes showed high Na,K-ATPase activity and [^{3}H]ouabain binding capacity (22–24). It has been con-

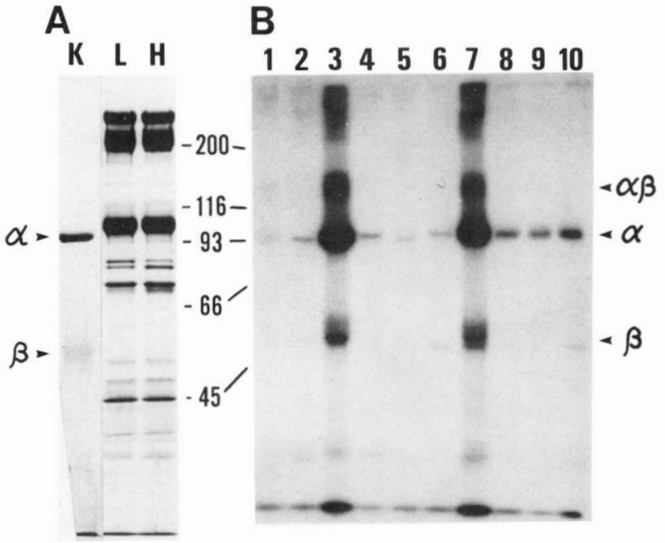

FIG. 1. **SDS-PAGE analysis of dog erythrocyte membranes and Na,K-ATPase from dog kidney (*A*) and ^{125}I-labeled proteins precipitated by monoclonal antibodies raised against kidney holoenzyme (*B*).** *A*, erythrocyte membrane proteins and kidney enzyme were prepared as described under "Experimental Procedures" and analyzed by SDS-PAGE with Coomassie staining. *Lanes H* and *L*, 30 μg of erythrocyte membrane from HK (*H*) and LK (*L*) dogs. No significant difference is observed in the polypeptide composition of HK and LK dog cell membranes except the a:b ratio of *band* 4.1.[2] *Lane K*, 6.6 μg of purified Na,K-ATPase from dog kidney. Migration positions of molecular weight standards are indicated at right ($M_r \times 10^{-3}$). *B*, immunoglobulins secreted by 10 clones of hybridomas were reacted with Protein A beads. Washed beads were incubated for 2 h with ^{125}I-Bolton-Hunter reagent-labeled Na,K-ATPase solubilized in TNG buffer containing 5 mg/ml of $C_{12}E_8$. Beads were washed five times with the same buffer, two times with buffer additionally containing 0.5 M NaCl, then with 5 mg/ml $C_{12}E_8$ in 20 mM Tris/Cl, pH 7.5. Proteins bound to the beads were loaded on SDS-PAGE and autoradiographed as described under "Experimental Procedures." *Lanes 3* and *7*, samples immunoprecipitated with MoAb 24/1 and 202/4, respectively.

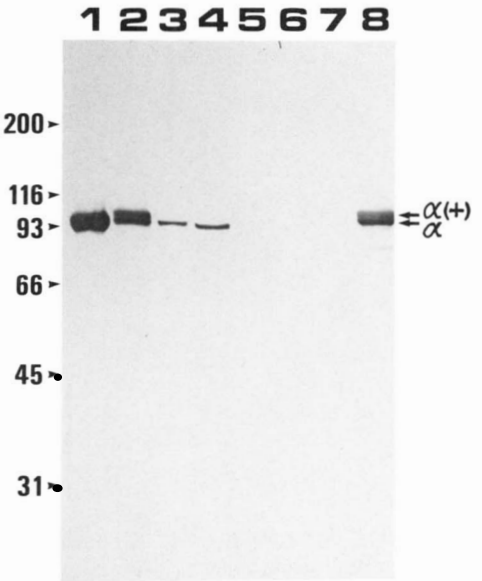

FIG. 2. **Identification of α subunit with immunoblotting using anti-α IgGs.** Immunoblotting was carried out to identify the α subunit of Na,K-ATPase on SDS-PAGE as described under "Experimental Procedures." *Lane 1*, 3.3 μg of kidney Na,K-ATPase; *lanes 2* and *8*, 25 μg of brain microsomes from dog; *lanes 3* and *4*, 20 and 30 μg of HK dog erythrocyte membranes; *lanes 5*, *6*, and *7*, 30, 50, and 20 μg of LK cell membranes, respectively. Molecular weight markers were myosin (M_r = 200,000), β-galactosidase (M_r = 116,250), phosphoryase B (M_r = 92,500), bovine serum albumin (M_r = 66,200), ovalbumin (M_r = 45,000), and carbonic anhydrase (M_r = 31,000). Migration positions of α and α(+) subunits are also indicated.

cluded that HK dog erythrocytes contain Na,K-ATPase, which was long thought to be lacking in normal dog (LK dog) red cells (10, 36). However, it has been difficult to reveal direct evidence for the presence of the enzyme protein, since it is only a very minor, latent component of erythrocyte membrane proteins (Fig. 1*A*). From our previous studies (24), the number of enzyme copies was calculated to be approximately 1500 ouabain binding sites per HK erythrocyte. Thus, we first attempted to detect the α subunit of the enzyme in HK erythrocyte membranes subjected to SDS-PAGE by immunoblotting with anti-α subunit IgGs. From such immunoblots in HK erythrocyte membranes, a polypeptide was immunospecifically visualized that co-migrated on SDS-PAGE with dog kidney and dog brain α subunit (Fig. 2). No bands were observed in LK erythrocyte membranes, and increasing the amounts of proteins subjected to the gel had no effect on this observation. Fig. 2 shows that the anti-α IgGs used here recognized α(+), which is a variant polypeptide of α in brain tissues (25). Such bands co-migrating with α(+) were not detected in HK cell membranes. Based on the mobility on SDS-PAGE and specific immunorecognition with anti-α IgGs, we concluded that this membrane protein from HK erythrocytes was the α subunit of the Na,K-ATPase and not α(+) as designated. Moreover, in LK cell membranes, the absence of bands recognized by anti-α antibodies was in accordance with the lack of enzymatic activity and ouabain binding sites in the preparations (22, 24).

The M_r of the α subunit from HK erythrocytes was calculated to be 96,000 on SDS-PAGE, and thus was equal to the renal α subunit. As shown on SDS-PAGE in Fig. 1*A*, it seems that the α subunit of erythrocytes moves slightly faster than the 100,000-Da polypeptide designated as *band 3*, which is a major component of erythrocyte membrane proteins (37) mainly consisting of anion transporter (38).

Cross-reactivity of Anti-α IgGs with Membrane Proteins from Various Mammalian Erythrocytes and from Erythroid Precursor Cells—Fig. 3 shows the immunoblots of α subunits from various mammalian cellular membranes visualized with anti-α IgGs. In erythrocyte membranes from three species, HK dog, cow, and pig, the α subunit was visualized as a single band with high intensity. Faint bands were observed in horse and human erythrocytes. Electrophoretic mobilities of these polypeptides were homologous to each other and corresponded well to the α subunits of renal and brain enzymes. The smiling of bands observed in *lanes 1–5* is attributed to the species difference in the amount of *band 3* in membrane proteins. No bands were seen in red cell proteins from cat. These observations are in good agreement with the characteristics of the compositions of sodium and potassium in these mammalian erythrocytes (36), which might reflect the activity of Na-K pump. For example, cats, as well as LK dogs, are well known to possess erythrocytes which lack the Na,K-ATPase activity (10). Although the amount of α subunit in human erythrocyte membranes was much smaller than expected from our previous results (22), a clear homologous band was observed in a membrane preparation of K562 erythroid progenitor cells derived from human erythroleukemia (39–41). In all preparations of erythrocytes and erythroleukemic cell membranes, no bands of polypeptide co-migrating with α(+) were visualized by anti-α IgGs. Thus, it is suggested that the larger

[2] Y. Maede and M. Inaba, unpublished data.

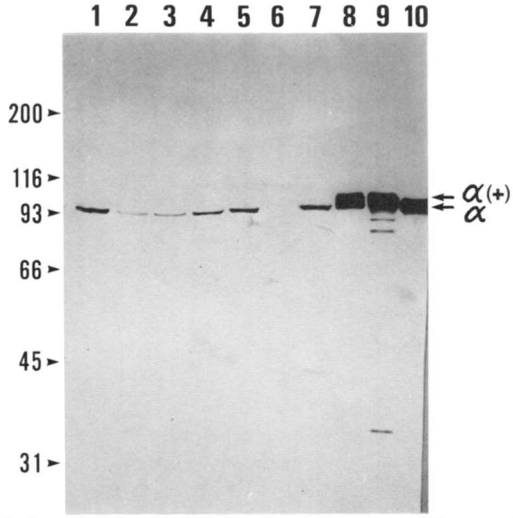

FIG. 3. **Immunological detection of α subunit in membranes from various mammalian erythrocytes and K562 cells.** Various membrane preparations subjected to SDS-PAGE were transferred onto Durapore membranes and visualized by anti-α IgGs as described under "Experimental Procedures." *Lane 1*, 30 μg of erythrocyte membranes from HK dog; *lanes 2–6* contained 50 μg of erythrocyte membranes from human, horse, cow, pig, and cat, respectively; *lane 7*, 40 μg of K562 cell membranes; *lanes 8* and *9*, 30 μg of brain microsomes from pig and dog, respectively; and *lane 10*, 1.2 μg of dog renal Na,K-ATPase. Molecular weights of standards are shown × 10^{-3}, and α and α(+) subunits of the enzyme are also indicated.

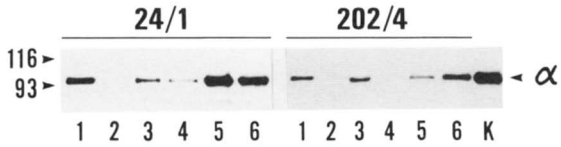

FIG. 4. **Immunological detection of the α subunit of MoAb 24/1- or 202/4-coupled Sepharose column fractions.** Spectrin-depleted erythrocyte membranes from HK dogs were solubilized and loaded on MoAb 24/1 (*left*)- or 202/4 (*right*)- coupled CNBr-activated Sepharose 4B columns (1 × 2-cm). Each column was washed, and proteins bound to the MoAb were eluted with 50 mM diethylamine/Cl, pH 11.5. Fractions were subjected to SDS-PAGE and immunoblotting using anti-α IgGs. For each column, *lane 1* shows the peak fraction of proteins passed through the column; *lane 2*, the fraction of the washing step; *lane 3*, the peak fraction of the 1 M NaCl elution; *lane 4*, the washing step again; and *lanes 5* and *6*, fractions of the pH 11.5 elutions. As a control, an immunoblot of 1 μg of kidney Na,K-ATPase α subunit is shown (*K*).

subunit of the Na,K-ATPase in erythropoietic cells is very similar, or identical, to the renal α subunit, regardless of species difference.

Immunoaffinity Purification of the Na,K-ATPase from HK Dog Erythrocyte Membranes—Next, we tried to purify the Na,K-ATPase from HK dog erythrocyte membranes to obtain further evidence for the equivalence of the erythrocyte enzyme to the renal enzyme. We previously utilized some conventional techniques on $C_{12}E_8$-solubilized membrane proteins (42), but no procedures led us to a satisfactory resolution. Thus, we tried to produce monoclonal antibodies which could recognize the solubilized enzyme and attempted to purify the enzyme using the immunoaffinity binding to antibodies (Fig. 1*B*).

Fig. 4 shows the process of immunoaffinity purification of HK dog erythrocyte Na,K-ATPase using MoAb 24/1- or 202/4-coupled Sepharose beads. The α subunit in each fraction was detected by immunoblots as shown in Fig. 4, since the protein concentrations were very low without fractions passed through the column. Fig. 5 shows the silver stained gel (*lanes 4* and *5*) after SDS-PAGE of proteins bound to a MoAb 24/1-coupled Sepharose column and eluted with 50 mM diethylamine/Cl, pH 11.5 (corresponding to *lanes 5* and *6* (*24/1*) in Fig. 4). Fractions eluted with alkaline buffer from MoAb 202/4-coupled bead columns contained many other polypeptides nonspecifically bound to the column (Fig. 5, *lane 6* (*202/4*)). For the antigens eluted from the MoAb 24/1 column, three predominant polypeptides were observed. A sharp band (M_r = 96,000) and a broad band (approximately M_r = 53,000) corresponded to the purified α and β subunits of renal Na,K-ATPase (*lane 2*), respectively. We estimated a part of the latter 53,000-Da protein as IgG heavy chain and the lower 30,000-Da polypeptide as IgG light chain by the analysis of purified MoAb 24/1 (IgG) on SDS-PAGE. Moreover, both the 96,000- and 53,000-Da polypeptides were observed in the proteins eluted from MoAb 202/4-coupled bead columns. The larger polypeptide was estimated by immunoblots to be the α subunit, as shown in Fig. 4. Based on copurification with the α subunit and its resemblance in molecular weight on SDS-PAGE, we determined that the 53,000-Da polypeptide was the β subunit of Na,K-ATPase in HK dog erythrocyte membranes. These results indicated that the erythrocyte Na,K-ATPase was composed of two heterogeneous polypeptides, the α and β subunits, which, judging from the molecular weights and molecular structure, appeared to be very similar to the subunits of kidney enzymes.

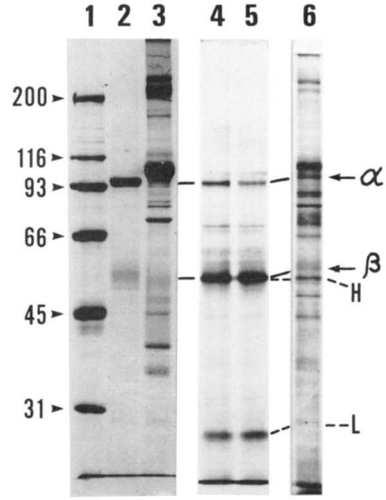

FIG. 5. **SDS-PAGE analysis of MoAb 24/1- or 202/4-coupled Sepharose column fractions.** Immunoaffinity purification was carried out as described under "Experimental Procedures" and the legend for Fig. 4. Proteins eluted with pH 11.5 buffer were analyzed by SDS-PAGE and stained with silver. *Lane 1*, molecular weight standards are the same as in Figs. 2 and 3; *lane 2*, 6.6 μg of kidney enzyme; *lane 3*, 20 μg of spectrin-depleted ghost proteins from HK dog. *Lanes 1–3* are shown with Coomassie staining. *Lanes 4* and *5*, the fractions of pH 11.5 elution from MoAb 24/1-column; *lane 6*, the fraction of pH 11.5 elution from MoAb 202/4-column, with silver staining. The heavy (*H*) and light (*L*) chains of MoAb 24/1 and 202/4 (IgG) are indicated.

Detection of the α Subunit in HK and LK Dog Reticulocytes and Their Degradation during Maturation into Erythrocytes—From the results described above and from our previous studies (24), it was suggested that the reticulocytes from LK and HK dogs might have Na,K-ATPase identical to that of HK erythrocytes and of kidney. Furthermore, the rapid decrease in enzymatic activity during cellular maturation into erythrocytes (24) seemed to be derived from the depression of the enzyme protein itself. Therefore, reticulocytes were separated from whole blood of HK and LK dogs made anemic, and the alteration of the enzyme protein during incubation was estimated.

Reticulocytes of HK and LK dogs were separated by the method described previously (24) at the second day after successive bleeding for 3 days (300 ml of blood/day). Membranes were prepared and subjected to immunoblotting after SDS-PAGE. Fig. 6*A* shows the immunoblots of proteins immunorecognized and visualized by anti-α IgGs. Proteins comigrated on SDS-PAGE with an apparent M_r of 96,000, and reactions with anti-α antibodies were observed in both reticulocytes and erythrocytes from HK dogs and in reticulocytes from LK dogs. No polypeptide bands were detected in any membrane preparation of LK mature erythrocytes again (see Fig. 2). These results provide direct evidence that the immature erythrocytes, *i.e.* reticulocytes of LK dogs, possess Na,K-ATPase protein.

Using the same methods, we also analyzed the alteration of α subunit during reticulocyte maturation *in vitro*. Reticulocyte membranes from LK dogs exhibited a significant decrease in immunorecognition by anti-α antibodies in proportion to cellular aging *in vitro* (Fig. 6*B*). At 8 h after the onset of incubation, the immunoreactivity of the α subunit of LK cells was already decreased. Faint bands of α subunit were observed after 24 and 48 h in culture, and the complete loss of the α subunit was achieved on immunoblots at 120 h. The half-life of the degradation of the LK reticulocyte α subunit seemed to be from 12 to 24 h. In contrast, the α subunit of HK reticulocytes revealed no marked decrease in immunoreactivity with antibodies throughout incubation. It was difficult to calculate the rate of this regression process, since the procedure was based solely on SDS-PAGE and immunoblotting. Therefore, we could not determine the precise relationship between the regression in immunorecognition by anti-α IgGs and the decrease of enzymatic activity. However, the profiles of the regression of α subunit immunoreactivity in LK reticulocytes were in good agreement with those of changes in [^{3}H] ouabain binding capacity accompanying cellular maturation (see Ref. 24 and Fig. 7). Thus, it was suggested that the α subunit of dog reticulocytes was rapidly degraded during its maturation by some mechanism involving changes of antigenic determinants, such as proteolysis.

Breakdown of Na,K-ATPase by Dog Reticulocyte Lysates—Since, as indicated above, it was suggested that the α subunit of reticulocyte Na,K-ATPase was degraded by proteolytic systems during cellular maturation, we examined whether dog reticulocytes possessed such abilities or not.

Renal Na,K-ATPase preparations labeled with ^{125}I-Bolton-Hunter reagent were incubated with reticulocyte lysate from HK or LK dogs prepared as reported by Tanaka *et al.* (32). Reactions were quenched at the indicated times by the addition of SDS-PAGE sample buffer containing a set of protease inhibitors and followed by SDS-PAGE and autoradiography (Fig. 7). Both α and β subunits were substantially and rapidly reduced by incubation with reticulocyte lysates from HK and LK dogs. There was a corresponding increase (at 15 min), followed by a reduction, in 70,000–75,000-Da polypeptides, especially in reactions by LK lysates. These polypeptides (M_r = 70,000–75,000) seem to be proteolytic fragments of the α subunit, since anti-α IgGs specifically recognized 69,000- and 72,000-Da polypeptides and other proteins (M_r = 66,000, 64,000, 56,000, 45,000, and 44,000) when kidney enzymes were treated in the same manner and analyzed by SDS-PAGE and immunoblotting (data not shown). The radioactivity remaining in the α subunits was quantified by slicing the gel and counting γ-emissions (Fig. 8). When ATP was absent from the reactions, for the initial 15 min of incubation, LK reticulocyte lysate caused a 50% decrease of radioactivity in the α subunit, whereas the regression by the lysate from HK retic-

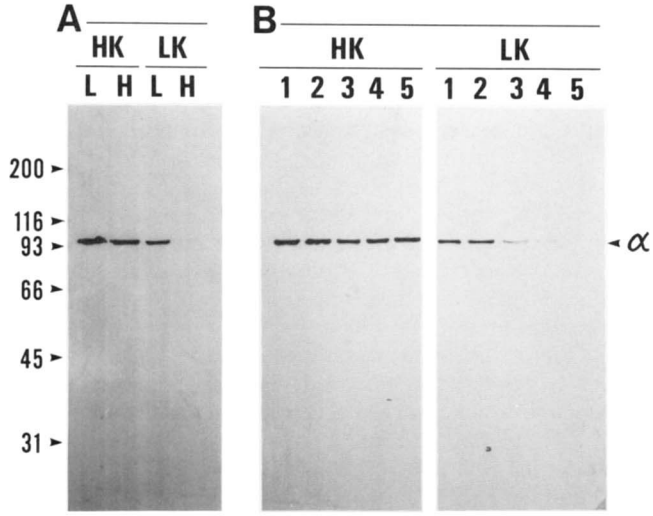

FIG. 6. **Decrease in immunological reactivity of the α subunit in reticulocytes from HK and LK dogs during cellular maturation *in vivo* (*A*) and *in vitro* (*B*).** *A*, red cells from anemic dogs were separated into low (*L*, 1.070 < *d* < 1.090 g/ml) and high (*H*, *d* > 1.090 g/ml) density fractions and the cell membranes of each fraction were prepared. Membranes were subjected to SDS-PAGE followed by immunoblotting. The reticulocyte contents of each fraction were 73% (*L*), 2% (*H*) and 77% (*L*), and 5% (*H*) for HK and LK dogs, respectively, after staining with new methylene blue. *B*, reticulocytes separated from HK (60%) and LK (78%) dogs were incubated for 0, 8, 24, 48, and 120 h (*lanes 1–5*) as described under "Experimental Procedures." At the indicated times, cells were harvested to prepare membranes. After 100 h in culture, only a few reticulocytes (4–6%) were observed. No significant changes in the total cell counts were observed during the culture (see Ref. 24). Molecular weight standards are shown × 10^{-3}.

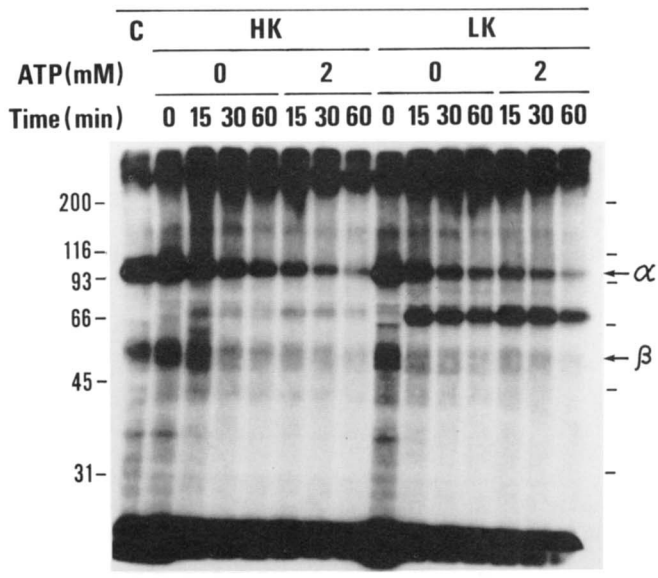

FIG. 7. **Proteolysis of ^{125}I-Bolton-Hunter reagent-labeled Na,K-ATPase by reticulocyte lysates from HK and LK dogs and the effect of ATP on protein breakdown.** The ^{125}I-Bolton-Hunter reagent-labeled Na,K-ATPase from dog kidney was incubated at 37 °C with reticulocyte lysates from HK and LK dogs. When present, ATP and $MgCl_2$ were included in the reaction at concentrations of 2 mM and 5 mM, respectively. For detailed procedures, see "Experimental Procedures." *Lane C* contains ^{125}I-labeled enzyme (1 × 10^5 cpm) incubated for 60 min at 37 °C in the presence of 2 mM PMSF. The purities of reticulocytes used for the preparation of lysates were 65 and 59% for HK and LK dogs, respectively.

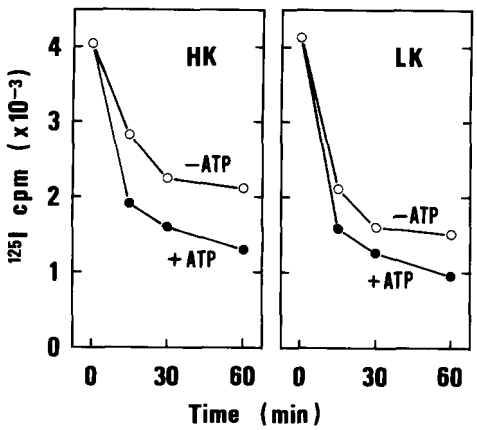

FIG. 8. **Degradation of the α subunit by reticulocyte lysates from HK and LK dogs.** Incubations were carried out as described under the legend for Fig. 7 in the presence (●) or absence (○) of 2 mM ATP. After exposure to x-ray film, the gels corresponding to the α subunit region were cut off and counted for retained radioactivities in a γ-counter. Data are mean values of two experiments using two different lysate preparations for both types of cells. The purities of reticulocytes used were 65 and 73% for HK, and 59 and 77% for LK dogs. Leukocyte contamination was not significant (lower than 0.3% in all cases).

ulocytes was no more than 30% of the initial amount. After 60 min of incubation, the remaining radioactivity in the α subunit was reduced to 51 and 43% by lysates from HK and LK reticulocytes, respectively. The degradative effects of reticulocyte lysates from HK and LK dogs were significantly increased in the presence of ATP and Mg^{2+}, as shown in Fig. 8. These results demonstrate that dog reticulocytes contain some proteolytic systems for Na,K-ATPase proteins similar to those for some soluble proteins reported in reticulocytes from other mammals (32, 43–48). In addition, they indicate that the proteolytic activity for Na,K-ATPase protein in HK reticulocytes might be lower than in LK immature cells, resulting in the high activity of the enzyme in mature HK red cells.

DISCUSSION

Although some studies on stoichiometry and kinetics of cation transport by the Na,K-ATPase have been carried out in erythrocytes or ghosts prepared from them (12, 13, 16), the molecular characteristics of the Na,K-ATPase in erythrocyte membranes are as yet unclear. Only a few investigators have reported that antibodies raised against purified kidney holoenzyme blocked the ouabain-sensitive fluxes of Na^+ and K^+ in human red cells and suggested that the antigenic determinants of the enzyme are the same regardless of their origin (19–21). In the present study, immunoblotting analysis clearly demonstrated that erythrocytes from HK dogs possess a catalytic subunit of the Na,K-ATPase which is identical in molecular mass to the α subunit of kidney enzyme, which was calculated to be 96,000 in our electrophoretic system (Fig. 2). Furthermore, we purified enzyme proteins from HK erythrocyte membranes using immunoaffinity chromatography on MoAb-coupled beads, and showed that the erythrocyte enzyme is composed of two heterogeneous subunits, the α and β subunits, as in all preparations reported until now (1, 17). Purified β subunit showed a broad molecular weight distribution (approximately M_r = 53,000) on SDS-PAGE, as is the case with kidney enzyme. These results agreed with our previous observation that similar elution profiles were obtained on gel permeation chromatography of [^{3}H]ouabain-labeled particles solubilized from HK erythrocyte membranes and dog kidney enzymes with an apparent molecular mass of 470,000–490,000, assuming $\alpha_2\beta_2$ formation (42). Based on the specific immunorecognition of erythrocyte enzyme by mono- (MoAb 24/1 and 202/4) and polyclonal (anti-α IgGs) antibodies raised against renal enzyme proteins, and the coincidence in molecular weight of each subunit, it is suggested that the enzyme proteins from HK erythrocytes and kidney are products derived from the same genes.

As shown in Fig. 3, similarities in the molecular weight and antigenic determinants of the larger subunit of Na,K-ATPase are also investigated for various mammalian erythrocytes. The larger subunits of erythrocyte enzyme in dog, pig, horse, cow, and human are all recognized by anti-α IgGs as 96,000-Da polypeptide, and equal to the kidney and brain α subunit, but not to $\alpha(+)$, a heterogeneous protein, in brain tissues (25). A similar result was obtained for K562 cell membranes (Fig. 3, *lane 7*). K562 cells were derived from a chronic myelogeneous leukemia patient (39) and recently suggested to be multipotential leukemia cells with potential for erythroid differentiation (40, 41, 51–53). Therefore, we may refute the possibility that precursors of mammalian erythrocytes might contain $\alpha(+)$ polypeptide. Thus, it appears that only the α subunit with M_r = 96,000 is present in myelogeneous cells as a catalytic subunit of the Na,K-ATPase.

The present study also demonstrates that the decrease of Na,K-ATPase activity accompanying dog reticulocyte maturation described in our previous study (24) is caused by the breakdown of enzyme protein (Fig. 6). Based on the reduction of the immunorecognition of the α subunit by anti-α IgGs (Fig. 6) and the proteolytic activities in dog reticulocyte lysates on ^{125}I-Bolton-Hunter reagent-labeled enzyme (Figs. 7 and 8), we can conclude that the Na,K-ATPase of reticulocyte membranes is degraded by a proteolytic system in these immature cells.

Recently, Weigensberg and Blostein (54) reported that the loss of Na^+-dependent glycine transport activity and [^{3}H] ouabain binding sites during reticulocyte maturation in sheep was retarded by ATP depletion, suggesting that an ATP-dependent proteolytic system (32, 43–50) may modulate the number of ouabain binding sites, the Na,K-ATPase. This nonlysosomal ATP-dependent degradation of proteins in reticulocytes appears to be responsible for the selective elimination of many normal proteins during reticulocyte maturation into erythrocytes (49, 50), as well as for the selective degradation of abnormal protein (43, 47). In addition, two distinct roles for ATP in this proteolytic process, one requiring, and one independent of, ubiquitin, were reported by Tanaka *et al.* (48). Our results demonstrate that dog reticulocytes contain such an ATP-dependent proteolytic system for the degradation of the membrane-bound Na,K-ATPase, indicating a modulation of this enzyme and the related cellular functions in dog reticulocytes by energy-requiring proteolysis, as suggested in sheep cells (54). However, this does not appear to hold true for maturation-associated changes of the Na,K-ATPase in all functions; in fact, more than half of the breakdown of the enzyme protein did not require the addition of ATP (Fig. 8). The extent to which degradation of the enzyme is energy dependent remains to be determined.

Furthermore, as shown in Figs. 7 and 8, reticulocyte lysates from HK dogs showed proteolytic activity lower than that from LK dogs in the breakdown of ^{125}I-labeled α subunit, especially when a sufficient amount of ATP (2 mM) was not included in the system. In HK dog erythrocytes, some of the glycolytic enzymes, such as glucose-6-phosphate dehydrogenase, hexokinase, glyceraldehyde-3-phosphate dehydrogenase, and pyruvate kinase, showed higher activities than those in

LK cells. Some of this might be due to the presence of isozymes characteristic to reticulocytes.[3] From these findings, it is suggested that the proteolytic system for the Na,K-ATPase in dog reticulocytes may be related to the degradation of some other cytosolic and membranous enzymes and that lower activity of this proteolytic system in HK reticulocytes may be one of the reasons why the HK erythrocytes remain a characteristic of immature cells (23, 24, 55). In addition, since cation fluxes due to the function of Na,K-ATPase have been implicated as signals in the cellular regulation of growth and differentiation (56–60), informatin as to whether the Na,K-ATPase is regulated by a proteolytic system as reported in the present work, and if so, how, is crucial for the understanding of cell maturation and differentiation. Recently, Waxman *et al.* (61) and Rieder *et al.* (62) have shown that some erythroleukemic cells contain an ATP-dependent system for protein breakdown, indicating the presence of such systems in normal erythroid cells. It will thus be of importance in the future to investigate the mechanism of the proteolytic system in dog erythropoietic cells and its relationship to Na,K-ATPase.

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