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Purification and Partial Characterization of the (H^+,K^+)-Transporting Adenosinetriphosphatase from Fundic Mucosa†

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Received December 22, 1986; Revised Manuscript Received March 17, 1987

ABSTRACT: The microsomal (H^+,K^+)-ATPase systems from dog and pig fundic mucosa were purified to homogeneity and partially characterized. The method involves sodium dodecyl sulfate (SDS) (0.033% w/v) extraction of the microsomal non-ATPase proteins under appropriate conditions followed by sucrose density gradient centrifugation. Two distinct membrane bands of low (buoyant density = 1.08 g/mL) and high (buoyant density = 1.114 g/mL) densities having distinct enzymatic and chemical composition were harvested. The low-density membrane was highly enriched in Mg^{2+}- or Ca^{2+}-stimulated ATPase and 5′-nucleotidase activities but totally devoid of (H^+,K^+)-ATPase and K^+-*p*-nitrophenylphosphatase activities. The latter two activities were found exclusively in the high-density membrane. SDS–polyacrylamide gel electrophoresis revealed the high-density membranes to consist primarily of a major 100-kilodalton (kDa) protein and a minor 85-kDa glycoprotein, the former being the catalytic subunit of the (H^+,K^+)-ATPase. The amino acid composition of the pure dog (H^+,K^+)-ATPase revealed close similarities with that from pig. The N-terminal amino acid was identified to be lysine as the sole residue. Similar to the high-density membrane-associated pure (H^+,K^+)-ATPase, the low-density membranes containing high Mg^{2+}-ATPase activity also contained a 100-kDa peptide and a 85-kDa glycopeptide in addition to numerous low molecular weight peptides. Also, similar to the pure (H^+,K^+)-ATPase, the Mg^{2+}-ATPase-rich fraction produced an E∼P unstable to hydroxylamine and partially (about 25%) sensitive to K^+ but having a slow turnover. The levels of E∼P produced by the pure (H^+,K^+)-ATPase- and Mg^{2+}-ATPase-rich fractions were 1400 and 178 pmol/mg of protein, respectively. The possibility of the low-density membrane-associated Mg^{2+}-ATPase to be a modified form of the (H^+,K^+)-ATPase has been discussed.

The ouabain-insensitive, membrane-bound (H^+,K^+)-ATPase activity, depending on Mg^{2+} as the sole divalent cation, transports protons across the secretory membrane of the parietal cells in the fundic mucosae (Forte et al., 1976; Ray & Fromm, 1981; Faller et al., 1982). Although much progress has been made in recent years regarding the mechanism of action and regulation of the enzyme system (Forte et al., 1976; Ray & Fromm, 1981; Faller et al., 1982), the precise mechanisms at the molecular level are unknown. One of the approaches for obtaining such molecular insight would be to purify the enzyme to homogeneity in a highly active form and perform appropriate experiments. For instance, studies on the

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0006-2960/87/0426-4264$01.50/0

molar stoichiometry of the (H^+,K^+)-ATPase and the pure endogenous activator (Bandopadhya & Ray, 1986) and the precise mode of activator regulation of the (H^+,K^+)-ATPase could only be studied by using the pure (H^+,K^+)-ATPase preparation as has recently been done (Bandopadhya et al., 1987).

Recently, Peters et al. (1982) reported the purification of the gastric (H^+,K^+)-ATPase protein following sodium dodecyl sulfate (SDS) solubilization and column chromatography. Although a homogeneous 100-kilodalton (kDa) band, presumably the catalytic subunit of the (H^+,K^+)-ATPase, was purified, the absence of enzyme activity as well as the lack of any data showing reconstitution of the activity made such preparation of limited use. Contrary to the reports of Peters et al. (1982), Saccomani et al. (1977) reported the purification of the (H^+,K^+)-ATPase in an active form using free-flow electrophoresis. However, the reported (Saccomani et al., 1977; Rabon et al., 1986) K^+-stimulated activity of the purified enzyme was only 2-fold higher than the total microsomes (specific activity about 60 μmol mg^{-1} h^{-1}). Subsequently, Schrijen et al. (1983), using zonal electrophoresis, reported purification of the (H^+,K^+)-ATPase to a similar degree of (H^+,K^+)-ATPase specific activity as reported by Sachs et al. (1977). Both the procedures mentioned above used rather expensive equipment not available in most laboratories.

The purpose of the present investigation was to purify the gastric (H^+,K^+)-ATPase to a homogeneous and highly active form using a simpler approach which may be used in any modern biochemical laboratory. In addition, we wanted to find out if the Mg^{2+}-dependent (basal) activity is associated with the "pure" (H^+,K^+)-ATPase and if so to what extent. The data demonstrate that appropriate modification of the Jorgensen procedure (Jorgensen, 1974) for the purification of (Na^+,K^+)-ATPase could be conveniently used for the purification of the (H^+,K^+)-ATPase in a highly active and homogeneous form as demonstrated by SDS–polyacrylamide gel electrophoresis (PAGE) and a single N-terminal lysine residue. The preparation was nearly free from Mg^+-dependent ATPase. Almost all of the Mg^{2+}-ATPase appeared in a lighter density (band 1) region and was completely separated from the high-density (band 2) membrane fraction containing the (H^+,K^+)-ATPase and K^+-*p*-nitrophenylphosphatase (pNPPase) activities. SDS–PAGE revealed that while the low-density band was quite heterogeneous, the characteristics 100-kDa protein and 85 kDa glycoprotein bands were present in both low- and high-density bands. In addition to characterization of the bands in terms of enzymatic activities and ^{32}P-labeled intermeidate studies, analytical data for the lipid, protein, and glycoprotein contents have also been provided. The data have been discussed in relation to the (Na^+,K^+)-ATPase and Ca^{2+}-ATPase since both enzymes are known to share (Schuurmans & Bonting, 1981; Ray & Nandi, 1986) many features.

Materials and Methods

Isolation of Plasma Membranes. Purified pig and dog gastric microsomal vesicles highly enriched in (H^+,K^+)-ATPase and K^+-pNPPase activities were isolated by the method of Ray (1978) with minor modification as described in detail elsewhere (Ray et al., 1982, 1983). No ouabain-sensitive (Na^+,K^+)-ATPase is detected in membranes prepared this way. The preparation is also free from the mitochondrial marker enzymes such as succinic dehydrogenase and HCO_3^--stimulated ATPase. Also, lack of glucose 6-phosphatase, RNA, and DNA suggested negligible contamination by rough endoplasmic reticulum (ER) and nuclear materials. The microsomes are derived primarily from the apical and tubulovesicular membranes of the parietal cells (Forte et al., 1976; Ray & Fromm, 1981; Faller et al., 1982). Isolated microsomes appear in the form of tightly sealed vesicles with the ATP hydrolytic site facing the vesicle exterior and the K^+ effector site for the ATPase facing the interior of the vesicles (Forte et al., 1976; Ray & Fromm, 1981; Faller et al., 1982).

Assay of Enzymes. The ATPase and pNPPase activities were assayed as previously described (Ray & Nandi, 1985, 1986). The incubation mixture for the ATPase contained, in a total volume of 1 mL, 50 μmol of piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) (pH 6.8), 1 μmol of $MgCl_2$, 2 μmol of tris(hydroxymethyl)aminomethane (Tris)–ATP, and 2–5 μg of membrane protein in the presence and absence of 20 μmol of KCl. After 10-min incubation at 37 °C, the reactions were started with ATP and incubated for 15 min. P_i was assayed according to Sanui (1974).

For pNPPase activity, the incubation mixture contained, in a total volume of 1 mL, 50 μmol of Tris buffer (pH 7.5), 2 μmol of $MgCl_2$, 5 μmol of *p*-nitrophenyl (pNPP), phosphate, and 2–10 μg of membrane protein with an without 25 μmol of KCl. After an incubation period of 10 min, the reactions were started by addition of pNPP and continued for 15 min at 37 °C. The reactions were stopped with 1 mL of 1.5 N NaOH. Following a brief centrifugation, the supernatant was read at 410 nm.

The Mg^{2+}- or Ca^{2+}-stimulated ATPase activity was assayed according to the procedure described recently (Nandi & Ray, 1984). Briefly, 1 mL of reaction mixture consisting of 50 μmol of Pipes (pH 6.8), 0.2 μmol of *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), 1 μmol of Ca^{2+} or Mg^{2+}, and 10 μg of membrane protein was preincubated for 10 min at 37 °C. Following incubation, Tris-ATP (1 μmol) was added to start the reaction, and incubation was continued for an additional 20 min. The reactions were terminated by 1 mL of 12% ice-cold trichloroacetic acid, and P_i was assayed.

The 5′-nucleotidase was assayed as previously described (Ray, 1970). The incubation condition was the same as for the Ca^{2+}- or Mg^{2+}-ATPase described above except that 5 mM AMP was used as substrate in the absence of Ca^{2+} and the incubation time was 30–60 min.

Assay of ^{32}P-Labeled Intermediates. The ^{32}P-labeled intermediates were assayed following the procedures described recently (Ray & Forte, 1976; Ray & Nandi, 1983; Nandi & Ray, 1987). Briefly, the incubation mixture contained, in a total volume of 0.2 mL, 50 mM Pipes–Tris buffer (pH 7.0), 0.125 mM [γ-^{32}P]ATP (about 8×10^6 cpm), 0.5 mM Mg^{2+}, and 50 μg of membrane protein with and without 20 mM K^+. The reactions were stopped after 15–30 s at room temperature with 0.2 mL of 35% ice-cold perchloric acid. The ^{32}P-labeled membranes were collected by Millipore (0.45 μm) filtration followed by three 5-mL washings with 5% perchloric acid (PCA) containing carrier ATP and P_i. The filter paper was washed twice with 5-mL portions of ethanol, dried, and then counted in 10 mL of Aquasol.

Assay of Lipids. The lipids were extracted from the membranes following a modification of the method of Bligh and Dyer (1959). The procedure for extraction, identification, and quantitation of each of the lipids is described in detail elsewhere (Nandi et al., 1983; Nandi & Ray, 1985).

Assay of Glycoproteins and Glycolipids. The membranes (5 mg) used for the analysis of glycoproteins and glycolipids were first pelleted by ultracentrifugation and resuspended in 80 mL of sucrose-free medium consisting of 2 mM Pipes (pH

6.8) containing 0.2 mM ethylenediaminetetraacetic acid (EDTA). The suspension was kept at 0–4 °C overnight for complete lysis of vesicles and consequent release of trapped sucrose. The membranes were harvested at 150000*g* for 90 min, and the pellet was washed once more in the sucrose-free medium by using the procedure described above. The final membrane pellet free from contaminating sucrose was suspended in 2 mL of sucrose-free medium, and aliquots were used for the measurement of total sugar content by the orcinol method (Svennerholm, 1956). The membranes were then extracted according to Bligh and Dyer (1959), and the totally delipidated membranes following repeated (3 times) extraction were assayed for sugar (Svennerholm, 1956) to quantify the protein-bound sugar (glycoprotein). The lipid extract was evaporated to dryness under N_2 and dissolved in a known volume of chloroform, and aliquots were assayed for sugar. The sugar content of the lipid extract from low- and high-density membranes was found to be exactly to the phosphatidylinositol content when assayed following two-dimensional separation of the total lipid extract.

SDS–PAGE and Electron Microscopy. SDS–PAGE was run following the procedure of Fairbanks et al. (1971) and as described earlier (Nandi & Ray, 1985). The membranes were prepared for electron microscopy as detailed elsewhere (Ray et al., 1983).

Purification of Gastric Microsomal (H^+,K^+)-ATPase Activity. Two methods were used for the purification of the (H^+,K^+)-ATPase. Initially, a continuous sucrose density gradient method was developed, and the buoyant densities of the various membrane subfractions were determined accurately. On the basis of such information, an appropriate step gradient method was developed for purification of the (H^+,K^+)-ATPase in bulk quantity.

Purified dog gastric microsomes (8 mg) made leaky by 4 times freezing and thawing (Ray & Nandi, 1986) were extracted with SDS (0.033% w/v) in a total volume of 4 mL containing 2 mM ATP, 0.5 mM dithiothreitol, 250 mM sucrose, and 50 mM Tris–glycine buffer (pH 9.0) by sonication (Heat System Ultrasonics, Inc., Model W-225R; output control set at 10) for 6 min at 21 °C followed by an incubation period of 30 min. The membrane suspension was then diluted with 4 mL of ice-cold 0.2 mM Pipes, 250 mM sucrose, and 0.2 mM EDTA buffer (pH 6.8) and layered over a linear sucrose gradient (20–40%). After 18 h of centrifugation at 25000 rpm using an SW27 rotor, 24 fractions of 1.5 mL each were collected with an LKB fraction collector. Proteins were assayed by the procedure of Lowry et al. (1951) after precipitation of 0.5 mL of each fraction with 7% trichloroacetic acid. Each fraction from the sucrose gradient was checked for density, protein content, and Mg^{2+}- or Ca^{2+}-ATPase, 5′-nucleotidase, K^+-pNPPase, and (H^+,K^+)-ATPase activities. The buoyant densities of the two membrane bands having distinct enzyme activities (Figure 1) were found to be 1.08 and 1.114 g/mL, respectively, the lighter band containing nearly all the Mg^{2+}-ATPase activity and the heavier one all of the (H^+,K^+)-ATPase and K^+-pNPPase activities.

For bulk purification of the (H^+,K^+)-ATPase, freshly prepared gastric microsomes (50–60 mg) were suspended in a medium (pH 9.0) identical with the one stated above at a concentration of 2 mg/mL. The suspension was frozen and thawed 3 times in an ethanol–dry ice mixture to make the vesicles leaky. Subsequently, ATP (2 mM), dithiothreitol (0.5 mM), and SDS (0.033% w/v) were added, and the mixture was sonicated in a manner identical with that stated above. The SDS incubation was then stopped by adding an equal volume of ice-cold Pipes buffer (pH 6.8) as mentioned above. The SDS-treated membranes were then layered over a single-step sucrose gradient made up of 17 mL of 32% sucrose and centrifuged for 18 h at 25000 rpm in an SW-27 rotor (Beckman). The light membrane appears at the interface of 32% sucrose, and the heavy membrane appears in the form of pellet.

Assay of Proteins. Protein was assayed by the method of Lowry et al. (1951). In some cases when there was SDS in the sample, a simple modification in the sample preparation was adopted. Since SDS interferes with the Lowry protein assay, it was essential to remove the SDS before the assay. To 0.2 mL of the sample, 1.0 mL of ice-cold 5% trichloroacetic acid was added, and the mixture was allowed to stay overnight at 0–4 °C. The suspension was then centrifuged at 10000*g* for 15 min. The pellet was dissolved in 0.05 mL of 6 N NaOH and assayed for protein by the Lowry procedure (Lowry et al., 1951).

Determination of the Amino Acid Composition and the N-Terminal Amino Acid. The amino acid composition of the pure dog gastric (H^+,K^+)-ATPase was determined as follows. The pure (H^+,K^+)-ATPase (about 5 mg) in 50 mM Tris–Pipes buffer, 0.2 mM dithiothreitol (DTT), and 0.2 mM ethylenediaminetetraacetic acid (EDTA), pH 6.8, was dialyzed exhaustively against glass-distilled water and lyophilized. The dry protein was dissolved in 6 N HCl and hydrolyzed in the presence of a trace (10 μL in 1 mL) amount of phenol at 105 °C for 24, 48, and 72 h in an N_2 atmosphere. The hydrolyzed protein was evaporated to dryness and the amino acid composition determined by a Beckman System 6300 high-performance analyzer. The cysteine residue in the AF was confirmed by oxidation to cysteic acid and amino acid analysis.

The N-terminal amino acid was determined by dansylation method (Grey, 1972). The salt-free lyophilized protein was dansylated in 0.5 M $NaHCO_3$ buffer (pH 9.0) containing 1% SDS at 37 °C for 4 h. The labeled protein was first dialyzed and subsequently hydrolyzed in 6 N HCL at 110 °C for 16 h. The (Dansyl)-N-terminal amino acid was identified by thin-layer chromatography (TLC) in three different solvent systems such as (a) benzene/acetic acid/pyridine (16:1:4 v/v), (b) chloroform/benzyl alcohol/acetic acid (70:30:3 v/v), and 1-butanol/pyridine/acetic acid/water (30:20:6:24 v/v). Lysine was found to be the only N-terminal amino acid residue for the pure dog gastric (H^+,K^+)-ATPase.

RESULTS

Effects of Detergents on the Enrichment of Gastric Microsomal (H^+,K^+)-ATPase Activity. Various detergents such as 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), deoxycholate (DOC), and SDS were tested at various concentrations for their ability to extract the non-ATPase proteins from the microsomal membranes. Detergent extraction was conducted for various times (up to 30 min) at different pHs (6.8, 7.5, 8.0, and 9.0) and temperatures (0, 21, and 37 °C), to assess the optimal conditions. Following detergent treatment, the membranes were harvested by high-speed centrifugation. Both the untreated control and the detergent-treated membranes were then assayed for protein and (H^+,K^+)-ATPase activity.

It was observed that CHAPS was not a suitable detergent even at high concentratrion (10 mM) and did not cause any appreciable enrichment of the (H^+,K^+)-ATPase under all conditions tested. DOC, on the other hand, was found to be

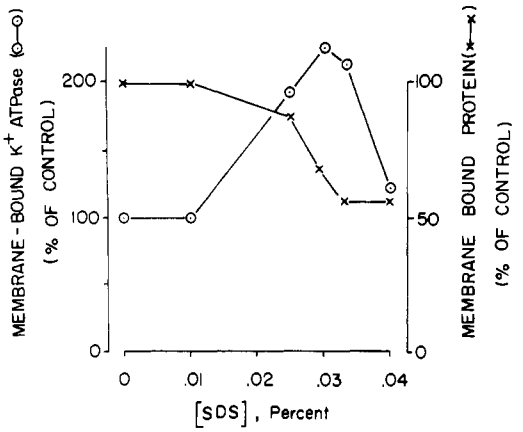

FIGURE 1: Effects of treatment of gastric microsomes with different concentrations of SDS on the relationship between the extraction of membrane proteins and the activity of membrane-bound (H^+,K^+)-ATPase. Dog gastric microsomes (1 mg/mL) in 0.25 M sucrose, 0.5 mM EDTA, and 2 mM Pipes buffer (pH 6.8) were made leaky by 4 times freezing and thawing. The leaky membranes were pelleted by ultracentrifugation (150000*g*, 60 min) and resuspended in 1 mL of a medium consisting of 2 mM ATP, 0.5 mM DTT, 250 mM sucrose, the indicated concentration of SDS, and 50 mM Tris–glycine buffer (pH 9.0). The suspension was sonicated (Heat System Ultrasonics Inc., Model W-225R; output control set at 10) for 6 min at 21 °C and subsequently incubated at the same temperature for 30 min. Following the treatment, the membranes were pelleted at 4 °C using 15000*g* for 60 min. The pellets were resuspended in 1 mL of the Pipes buffer mentioned above. Both the supernatant and the pellet were assayed for protein and ATPase activity. The supernatant did not have any detectable ATPase activity. Details of the assay are given under Materials and Methods.

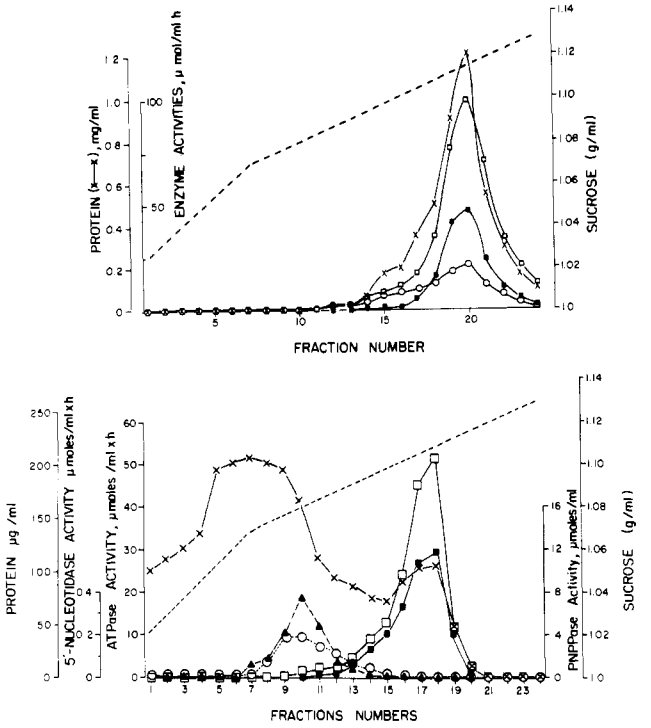

FIGURE 2: Fractionation of the SDS (0.033%)-treated dog gastric microsomes on a linear sucrose density gradient. Details of the fractionation procedure are given under Materials and Methods. The upper graph shows the density gradient profile of the control membranes (without treatment with SDS) and the lower following SDS (0.033% w/v) treatment. Both the control and SDS-treated membranes were run on the same linear sucrose gradients under identical conditions. Note (lower graph) that the (H^+,K^+)-ATPase and the K^+-pNPPase activities associated with the heavy microsomes (buoyant density = 1.114 g/mL) are completely separated from the Mg^{2+}-ATPase and 5′-nucleotidase activities associated with the low-density (buoyant density = 1.08 g/mL) membranes. A large part of the total membrane proteins which did not have any detectable enzymic activities remained in a soluble form and did not penetrate the gradient. The notations are as follows: sucrose gradient (---); protein (×); (H^+,K^+)-ATPase (□); K^+-pNPPase (■); Mg^{2+}-ATPase (○); and 5′-nucleotidase (▲). The profile is a typical of four separate runs, one of which was from pig gastric microsomes. Both dog and pig membranes give identical patterns.

effective at 2.5 mM but ineffective at 0.5 and 1.0 mM concentrations. Treatment with DOC (2.5 mM) at pH 9.0, with sonication for 6 min at 21 °C, extracted about 30% of the microsomal non-ATPase proteins accompanied by significant enrichment of the (H^+,K^+)-ATPase activity. However, SDS proved to be a far superior detergent in terms of both membrane protein extraction and (H^+,K^+)-ATPase enrichment. Very low concentrations (0.033% w/v) of SDS under appropriate conditions were found to be effective in purifying the (H^+,K^+)-ATPase in a highly active and nearly homogeneous pure form (see below).

Conditions for Optimal SDS Effects in Purifying the Gastric (H^+,K^+)-ATPase. Both ATP and DTT protected the enzyme from inactivation during SDS treatment and subsequent purification and were used routinely. Effects of different concentrations of SDS on solubilization of microsomal proteins and (H^+,K^+)-ATPase activity (Figure 1) demonstrated that 0.033% SDS was highly effective. Even a slightly higher SDS concentration (0.04%) inhibited the ATPase. It is noteworthy that while 0.02% SDS solubilized only about 20% of the microsomal protein, the (H^+,K^+)-ATPase activity was stimulated almost 2-fold, suggesting an activation by a low concentration of SDS per se. A similar effect was also observed with 2.5 mM DOC. Since recent studies (Ray et al., 1983; Ray & Nandi, 1986) demonstrated that nearly 100% of the gastric microsomal vesciles are of inside-out orientation, the demonstrated activation of the (H^+,K^+)-ATPase by low concentrations of detergents was not due to availability of substrate but appears to be due either to a direct effect on the ATPase molecule or to changes in permeability of the remaining intact vesicles to K^+ or both.

Purification of the Gastric Microsomal (H^+,K^+)-ATPase. Figure 2 (bottom) shows the data on the purification of the SDS-solubilized gastric (H^+,K^+)-ATPase on a continuous sucrose gradient. The (H^+,K^+)-ATPase and K^+-pNPPase activities appear in a heavy membrane fraction (buoyant density, 1.114 g/mL) which is clearly separated from the Mg^{2+}-ATPase and 5′-nucleotidase activities associated with a light membrane fraction (buoyant density, 1.08 g/mL). The control microsomes (without SDS treatment), on the other hand, when run in a similar gradient under identical conditions, do not show any separation of the (H^+,K^+)-ATPase and associated Mg^{2+}-ATPase activities and appear in a region of higher density (buoyant density, 1.118 g/mL). SDS–PAGE profiles of the various sucrose gradient fractions are shown in Figure 4A,B for the proteins and glycoproteins, respectively.

On the basis of knowledge about the density of the low- and high-density membranes, a one-step sucrose gradient was developed for the purification of the (H^+,K^+)-ATPase in bulk quantities. The high-density membranes harvested as a pellet from the single-step gradient were washed twice by resuspension in about 30 mL of sucrose–EDTA–Pipes buffer (pH 6.8) and centrifugation. SDS–PAGE of the washed high-density membrane is shown in Figure 4. When the pooled fractions (fractions 15–19) from the continuous sucrose gradient (Figure 3A) were washed as described above and run on SDS–PAGE, significant portions of the low molecular

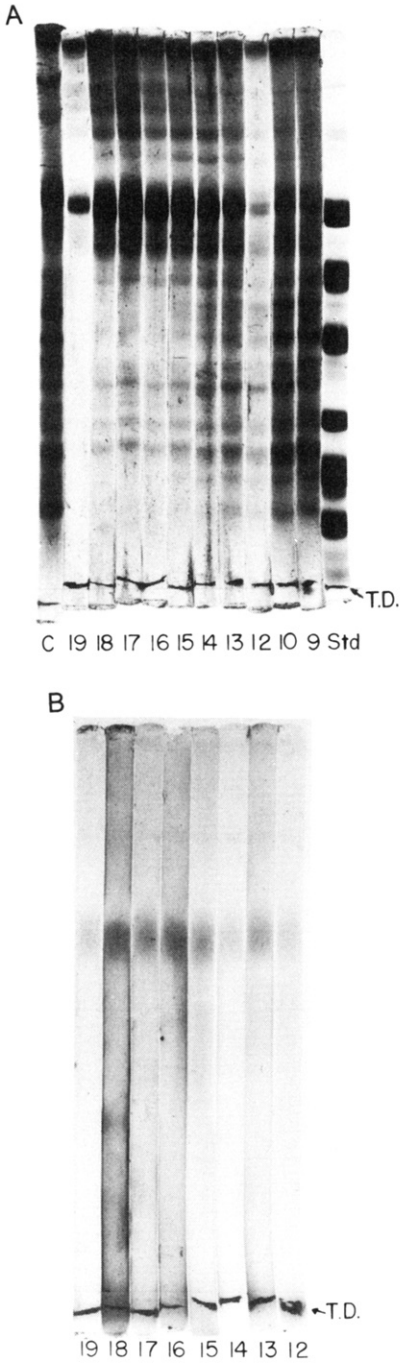

FIGURE 3: SDS–PAGE patterns of the various fractions from the continuous sucrose gradient. The gels werre stained for protein (A) and carbohydrates (B), respectively. The numbers at the bottom of the gels correspond to the fraction numbers shown in Figure 2. C stands for control membrane and Std for molecular weight standards, respectively. The molecular weight standards ($\times 10^{-3}$) from the top were 94, 68, 43, 30, 21, and 14.3, respectively.

weight peptides (Figure 3A) disappeared (not shown). This result suggests that some of these peptides are loosely adsorbed from the soluble fraction during the gradient run. SDS–PAGE shows that the high-density subfraction consists primarily of a major 100-kDa peptide and a glycopeptide (about 85 kDa); the former peptide has been demonstrated to be the catalytic subunit of the (H^+,K^+)-ATPase. The low-density band, on the other hand, contained numerous other peptides in addition to 100- and 85-kDa bands similar to the high-density membranes (Figure 4).

Chemical and Enzymatic Characterization of the Low- and High-Density Membranes. Table I gives data on the specific activities of the (H^+,K^+)-ATPase associated with the starting gastric microsomes and the high-density membranes from several dog and pig fundic mucosae. The basal activities (Mg^{2+}-dependent ATPase) for all preparations are also shown. The basal activities were eliminated from most of the (H^+,K^+)-ATPase prepared by the continuous sucrose gradient method. However, in the step gradient method, a significant number of batches show a small but measurable basal activity. The purified (H^+,K^+)-ATPase activity is enriched 4–8-fold over that in gastric microsomes from either species giving high specific activities. SDS-PAGE of the purified (H^+,K^+)-ATPase from either species also shows, an identical peptide profile. It may be noted in this connection that even though the pu-

Table I: (H^+,K^+)-ATPase Activities Associated with the High-Density Subfraction and the Corresponding Native Microsomes from Dog and Pig Fundic Mucosa[a]

	(H^+,K^+)-ATPase activity (μmol mg^{-1} h^{-1})			
	microsome		high-density fraction	
species	$-K^+$	$+K^+$	$-K^+$	$+K^+$
(A) Continuous Sucrose Gradient				
dog	20.2	51.3	0	353.5
dog	20.5	95.5	0	621.6
dog	20.8	52.5	0	350.2
pig	10.1	62.4	0.5	320.4
(B) Single Step Gradient				
dog	20.5	80.3	0.5	410.3
pig	9.2	63.5	1.5	201.5
pig	10.0	44.8	0	160.2
pig	11.5	55.3	0.8	197.8
pig	9.5	40.2	0	180.9

[a] Each value was derived from a separate individual preparation from the species as indicated. The yield of the pure (H^+,K^+)-ATPase activity (heavy band) was 10–15% of the original starting material (gastric microsomes) in various preparations from both species. The extent of purification thus achieved in terms of specific activity of the pure enzyme was (4–8)-fold in various preparations.

Table II: Chemical Composition of Dog Gastric Microsomes and the Low- and High-Density Subfractions

mg/mg of membrane protein	microsome subfractions		
	microsome	low density	high density
total lipid	0.985 ± 0.045	2.084 ± 0.084	1.47 ± 0.087
phospholipid (total)	0.752 ± 0.014	1.638 ± 0.054	1.048 ± 0.079
cholesterol	0.166 ± 0.001	0.52 ± 0.044	0.341 ± 0.042
cholesterol (mol)/phospholipid (mol)	0.43	0.61	0.63
total carbohydrate	0.172 ± 0.007	0.241 ± 0.002	0.155 ± 0.019
protein–carbohydrate	0.059 ± 0.002	0.067 ± 0.003	0.060 ± 0.003
lipid–carbohydrate[a]	0.062 ± 0.005	0.174 ● 0.017	0.095 ± 0.016
glycolipid[a]	0.053 ± 0.005	0	0
nonprotein constituent (0/0 of total)[b] (%)	53	70	62

[a] The carbohydrates associated with the lipid fraction (chloroform–methanol extract) were found to be due entirely to the phosphatidylinositol. The glycolipid content was calculated by subtracting the carbohydrate content of protein and lipid from the total carbohydrate. [b] The nonprotein constituents include all of the lipids and carbohydrates of the microsomal fractions. The "total" means the total of all constituents including protein.

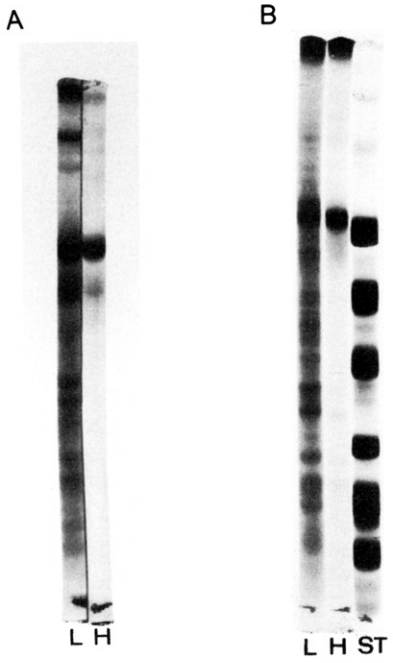

FIGURE 4: SDS–PAGE of the low- and high-density membranes prepared by the step gradient methods, L and H, low- and high-density membranes, respectively. Panel A is from dog, and panel B is from pig gastric membranes. It is noteworth that the pure (H^+,K^+)-ATPase preparations from two different species, such as pig and dog, are nearly indistinguishable. The molecular weight standards (ST) were the same as given in Figure 3. Details of the procedure for preparing the gastric membrane fractions by step gradient are given under Materials and Methods.

rified (H^+,K^+)-ATPase preparation of Saccomani et al. (1977) showed a major 100-kDa peptide on SDS-PAGE, the reported specific activity was considerably lower than that obtained in the present study. Since these authors reported the valinomycin–K^+-stimulated activity to be nearly the same as those of the leaky membranes containing the pure (H^+,K^+)-ATPase, the limited availability of either K^+ or ATP as the cause of such low activity may be ruled out. It is, however, likely that some firmly bound potent inhibitors of the (H^+,K^+)-ATPase such as heavy metals, may have been removed in the SDS procedure but retained in the Saccomani et al. (1977) preparation, generating lower activity.

Chemical compositions of the native gastric microsomes and the high- and low-density membrane subfractions enriched in (H^+,K^+)-ATPase and Mg^{2+}-ATPase, respectively, are shown in Table II. Consistent with their buoyant densities, the total lipid content (milligrams per milligram of membrane protein) was highest in the low-density membranes and lowest in the native membranes; the high-density membranes had an intermediate value. Similar to the total lipids, the total carbohydrate, cholesterol, and phospholipid values (milligrams per milligram of membrane protein) also hold an inverse relationship with the buoyant densities of the respective membrane fractions. The protein-bound carbohydrate, on the other hand, was found to be distributed equally in all three membranes. Also, glycolipid, which was present in a significant amount in the native microsome, was totally absent in the subfractions. However, no phospholpids could be detected in the SDS extract, implying specific solubilization of glycolipids during such treatment. The cholesterol to phospholipid molar ratios of the low- and high-density membranes were nearly identical and considerably higher than that of the microsomes.

The N-terminal amino acid of the pure dog gastric

Table III: Amino Acid Composition of the Pure (H^+,K^+)-ATPase from Dog Gastric Mucosa[a]

	% of total amino acids			
amino acid	dog (H^+,K^+)-ATPase (present method)	dog AF (Ray et al., 1987)	pig (H^+,K^+)-ATPase (Peters et al., 1981)	pig (H^+,K^+)-ATPase (Sachs et al., 1980)
nonpolar				
Ala	8.85	6.92	8.4	8.3
Val	7.88	6.62	6.0	5.4
Leu	7.79	6.92	10.0	9.3
Ile	4.41	6.32	5.2	4.5
Pro	4.59	5.72	5.0	6.4
Met	0.9	0	2.8	1.9
Phe	3.73	4.21	4.5	4.7
Trp	nd	2.41	1.7	nk
polar (uncharged)				
Gly	8.9	10.84	7.6	7.5
Ser	9.38	12.05	6.7	8.4
Thr	7.4	7.53	6.2	6.3
Cys	0.41	0.003	0.8	1.6
Tyr	2.28	3.61	2.7	3.3
negatively charged				
Asp	9.16	11.14	9.6	9.6
Glu	13.66	10.24	10.9	10.8
positively charged				
Lys	5.08	2.1	5.6	5.7
Arg	4.21	2.1	5.0	4.9
His	1.35	0.009	1.6	1.9

[a] nd stands for not determined; nk stands for not known. The values for valine, leucine, and isoleucine were determined from the samples hydrolyzed for 72 h. The serine and threonine values were obtained by extrapolation to 0 h of the data from 12-, 24-, 48-, and 72-h-hydrolyzed samples. The details are given under Materials and Methods. The table also shows the amino acid composition of the pure dog activator protein (AF) and the pure pig (H^+,K^+)-ATPase reported earlier for comparative purposes.

(H^+,K^+)-ATPase was identified to be lysine, demonstrating the homogeneity of the preparation. The amino acid composition of the pure (H^+,K^+)-ATPase preparation from dog fundic mucosa is shown in Table III. The amino acid compositions of the pure pig (H^+,K^+)-ATPase reported by Peters et al. (1981) and Sachs et al. (1980) are also shown in the table for comparison. In addition, the amino acid composition of the pure dog AF which appreciably stimulates (3–5-fold) the pure (H^+,K^+)-ATPase (Bandopadhya et al., 1987) is presented for the sake of comparison in the amino acid makeup of the possible intracellular regulator (AF) and the regulated (H^+,K^+)-ATPase molecules. The data (Table III) demonstrate close similarities in the amino acid profiles of the (H^+,K^+)-ATPase from the two different species. The contents of acidic amino acids such as glutamic and aspartic acids and the basic amino acids like lysine, arginine, and histidine are nearly identical in the (H^+,K^+)-ATPase from dog and pig. The contents of polar (uncharged) and nonpolar amino acids were also very similar. Comparison of the pure AF with the (H^+,K^+)-ATPase shows that despite having the level of nonpolar amino acids in the AF as high as that of the (H^+,K^+)-ATPase, the AF is a soluble protein. Other major differences observed were as follows: The amount of basic amino acids is about 60% lower in the AF compared to the (H^+,K^+)-ATPase, making the former a much more acidic protein than the latter. Such differences in charge distribution may have important implications in the interaction between the two molecules.

Figure 5 shows the characteristics of the ^{32}P-labeled intermediates produced by the low- and high-density fractions.

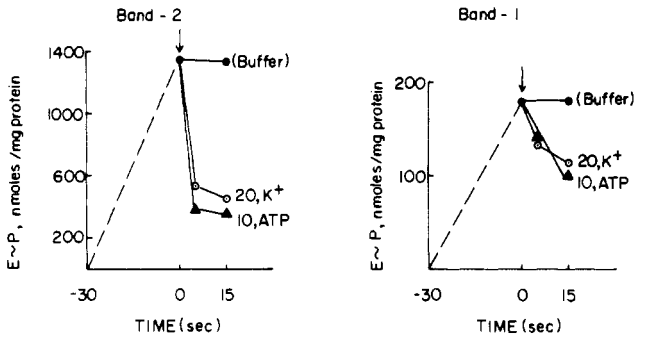

FIGURE 5: ^{32}P-Labeled intermediates produced by the low- and high-density membranes. Band 1 indicates the low- and band 2 the high-density membrane fraction from dog gastric microsomes, respectively. The details for E~P measurement are given under Materials and Methods. Data are representative of four separate studies.

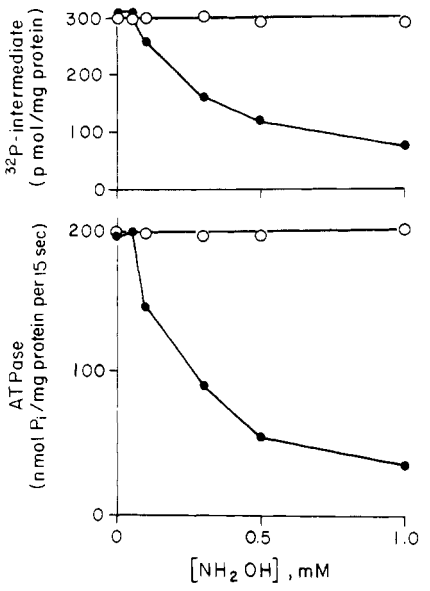

FIGURE 6: Effects of various concentrations of hydroxylamine on E~P formation and ATP hydrolysis by the low-density membranes from dog fundic mucosa. The indicated concentrations of NH_2OH (adjusted to pH 7.0) were incorporated into 200 μL of the incubation mixture (pH 7.0) containing 0.125 mM [γ-^{32}P]ATP, 0.5 mM $MgCl_2$, and 100 μg of membrane protein as detailed under Materials and Methods. The reactions were started with the addition of gastric microsomes at 21 °C and terminated following 60-s incubation with an equal volume of ice-cold 35% PCA. The E~P and P_i were assayed as described under Materials and Methods and elsewhere (Nandi & Ray, 1987).

The high-density membranes produce about 1400 pmol/mg of membrane protein of steady-state E~P highly sensitive to K^+. This is consistent with the E~P being an intermediate of the (H^+,K^+)-ATPase (Ray & Forte, 1976; Ray & Nandi, 1983). Contrary to the pure (H^+,K^+)-ATPase, the steady-state level of E~P produced (about 550 pmol/mg) by the untreated microsomes (Ray & Forte, 1976; Ray & Nandi, 1983) is appreciably lower. The low-density membranes, on the other hand, produce a low steady-state level of E~P which is relatively insensitive to K^+. Similar to the E~P of the (H^+,K^+)-ATPase (Ray & Forte, 1976), the E~P produced by the low-density membranes is sensitive to hydroxylaminolysis (Figure 6), suggesting the acyl phosphate nature of the intermediate.

Effects of vanadate on the inhibition of the (H^+,K^+)-ATPase and Mg^{2+}-ATPase activities associated with the high- and low-density membranes, respectively, are shown in Figure 7.

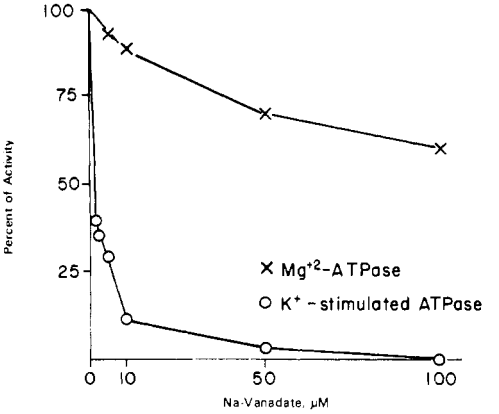

FIGURE 7: Vanadate inhibition of the Mg^{2+}-ATPase and (H^+,K^+)-ATPase activities associated with the respective low- and high-density membrane fractions. The membranes were preincubated for 10 min at 37 °C with the designated concentrations of vanadate in the ATPase assay medium as detailed under Materials and Methods. The control activities (in the absence of vanadate) have been plotted as 100%.

The data clearly demonstrated that, up to 10 μM vanadate, only slight (about 10%) inhibition of the Mg^{2+}-ATPase is observed while the (H^+,K^+)-ATPase activity is nearly obliterated.

Discussion

The present method of purification of the gastric (H^+,K^+)-ATPase is similar to that of Jorgensen (1974) for the purification of (Na^+,K^+)-ATPase from kidney cortex in that both methods use low concentrations of SDS and take advantage of ATP protection of the enzyme during SDS treatments. Our data reveal that, under appropriate conditions, a sublytic concentration (about 1 mM) of SDS, which is below the critical micelle concentration of 8.2 mM (Vendittis et al., 1981), is capable of causing some noteworthy changes in gastric microsomes described as follows: A large portion (about 40%) of the gastric microsomal proteins are solubilized without causing significant solubilization of the associated phospholipids and cholesterol (see Results). Treatment with SDS (0.033%) also causes alteration of the gastric microsomal vesicles (buoyant density 1.118 g/mL), generating two membrane subfractions of buoyant densities 1.114 (high density) and 1.08 (low density) g/mL, respectively, with distinct enzymatic and lipid profiles. The high-density fraction contained all the (H^+,K^+)-ATPase and K^+-pNPPase showing high specific activities while the low-density fraction contained the Mg^{2+}-ATPase and 5′-nucleotidase activities. SDS–PAGE reveals that the high-density fraction is composed primarily of a single 100-kDa peptide characterized to be the catalytic subunit of the (H^+,K^+)-ATPase. A glycoprotein band (about 85 kDa) is revealed upon periodic acid–Schiff (PAS) staining and appears to be copurified during (H^+,K^+)-ATPase purification. Our data demonstrating lysine to be the sole residue at the N-terminal end of the pure (H^+,K^+)-ATPase complex may suggest that the glycoprotein also has an N-terminal lysine. Alternatively, the possibility of the N-terminal residue of the glycoprotein being inaccessible to dansylation due to some masking effect of the associated carbohydrate moieties cannot be ruled out.

The amino acid composition (Table III) revealed that, with the exception of cysteine and methionine, the dog (H^+,K^+)-ATPase has an amino acid makeup closely similar to that of the pig preparation reported by others (Peters et al., 1981; Sachs et al., 1980). It is noteworthy in this connection that

the amino acid composition also compares very well with that of rat gastric (H^+,K^+)-ATPase (Schull & Lingrel, 1986) gathered recently by using the cDNA cloning technique. Major differences observed between the sequences of rat (H^+,K^+)-ATPase and the present study with dog are in the N-terminal residue and in the cysteine and methionine content. Thus, contrary to dog, the N-terminal residue is reported to be methionine in the rat. Also, the content of cysteine and methionine in rat gastric (H^+,K^+)-ATPase is 2.4% and 2.8%, respectively, compared to 0.4% and 0.8% for the dog. The cysteine and methionine values of the rat gastric (H^+,K^+)-ATPase, however, are closer to those reported for pig (Sachs et al., 1980; Peters et al., 1981).

Copurification of the 85-kDa glycoprotein might suggest this to be an integral part of the gastric (H^+,K^+)-ATPase system. Similar glycoproteins of 55 and 53 kDa have been demonstrated to copurify with the (Na^+,K^+)-ATPase and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase, respectively. For the Ca^{2+}-ATPase, the 53-kDa glycoprotein has recently been strongly suggested to regulate the coupling between ATP hydrolysis and Ca^{2+} transport (Leonards & Kutchai, 1985). It was noted (Leonards & Kutchai, 1985) that the degree of coupling obtained in reconstituted SR vesicles could be altered by varying the KCl concentration during cholate solubilization and the extent of 53-kDa peptide associated with the catalytic subunit in the reconstituted vesicles showed strong correlation with the coupling of transport but not the Ca^{2+}-ATPase activity. In view of the striking analogies among the three ion-transporting ATPase systems (Jorgensen, 1974; Ray & Nandi, 1986; Schuurmans & Bonting, 1981), it is likely that the glycoproteins associated with the (Na^+,K^+)-ATPase and (H^+,K^+)-ATPase systems may also be involved in similar coupling indicated as above. These aspects need to be carefully investigated.

The PAS-stained 85-kDa band appears as a diffused and weakly stained region following staining of the SDS–PAGE (5.6% acrylamide) by Coomassie blue. The presence of a similar diffused zone in the SDS–PAGE of the purified pig (H^+,K^+)-ATPase (Sacomani et al., 1977) suggests a similar glycopeptide (85 kDa) to be present also in pig. The presence of an 85-kDa glycopeptide (data not shown) in the purified pig and rabbit (H^+,K^+)-ATPase prepared by the present method would support such a notion.

As mentioned earlier, the 100-kDa peptide characteristics of the high-density membrane-associated pure (H^+,K^+)-ATPase are also present in the low-density membrane devoid of (H^+,K^+)-ATPase but highly enriched in Mg^{2+}-ATPase activity. This observation raised the question about the relationship of the 100-kDa peptide with that of the Mg^{2+}-ATPase activity. Previous autoradiographic studies (Forte et al., 1976) using [γ-^{32}P]ATP-labeled gradient purified gastric microsomes having both Mg^{2+}-ATPase and (H^+,K^+)-ATPase activities demonstrated the generation of a hydroxylamine-labile and K^+-sensitive E$\sim$P associated almost exclusively with the 100-kDa band. The present study reveals that the light membrane subfraction also produces an E$\sim$P (about 170 pmol/mg of protein) partially sensitive to K^+ and unstable to hydroxylamine. Such a low level of E$\sim$P (on a milligram of protein basis) appears to be due to the presence of numerous other proteins besides the 100-kDa peptide in this fraction. The observed vanadate sensitivity of the Mg^{2+}-ATPase, although small compared to the (H^+,K^+)-ATPase reaction, also implicates the involvement of an E$\sim$P in the Mg^{2+}-ATPase reaction. Hence, it appears likely that the E$\sim$P associated with the low-density membranes belongs to the 100-kDa peptide and is probably related to the Mg^{2+}-ATPase activity. Our recent data (Banerjee et al., 1987) demonstrating the generation of K^+-stimulated ATPase and pNPPase activities with high vanadate sensitivity in the low-density membrane subfraction in the presence of the pure endogenous activator (Bandopadhyay & Ray, 1986) will be totally consistent with such an idea.

The present procedure for the purification of the (H^+,K^+)-ATPase could be valuable in the future investigation on the molecular aspects of gastric acid secretion. Recent studies (Bandopadhyay et al., 1987) on the mechanism and regulation of the pure gastric (H^+,K^+)-ATPase system by the pure endogenous activator clearly emphasize the usefulness of the present preparation.

ACKNOWLEDGMENTS

We thank Allegra Giordano for expert secretarial help, Peggy Monday for typing the manuscript, Mark V. Wright for technical assistance, and Dr. David Fromm, chairman of our department, for encouragement and support. We also thank Dr. Debasis Bhattacharyyay for his help in the amino acid analysis.

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Examination of the Na^+-Induced Conformational Change of the Intestinal Brush Border Sodium/Glucose Symporter Using Fluorescent Probes†

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Received June 23, 1986; Revised Manuscript Received March 16, 1987

ABSTRACT: The Na^+-induced change in conformation of the intestinal brush border glucose carrier has been examined by three procedures. In the first, we have measured the effect of Na^+ on the binding of fluorescein isothiocyanate (FITC) to the glucose site; 100 mM Na increased the specific [blocked by D-glucose, *p*-(chloromercuri)benzenesulfonic acid, and *N*-acetylimidazole] FITC binding to a 75-kilodalton polypeptide 3-fold. In the second series, we have examined the effect of Na^+ on the susceptibility of the fluorescently labeled glucose site [pyrene isothiocyanate (PYTC) labeled] to a hydrophilic quencher (Tl^+); 100 mM NaCl increased the fraction of PYTC sites available to Tl^+ from 32% to 92% and decreased the apparent quenching constant from 94 to 44 M^{-1}. Finally, in the third series, we probed the distribution of tryptophan residues 15-30 Å from the glucose site using a "distant reporter group method", where tryptophan was used an an energy donor to anthracene isothiocyanate bound to the glucose site. Tryptophan quench reagents (I^-, Cs^+, and acrylamide) were then employed to probe the accessibility of the glucose site tryptophans in the presence and absence of sodium. In the absence of Na^+, there were two major classes of glucose tryptophans—exterior surface residues and residues buried in the hydrophobic protein matrix. Na^+ caused a redistribution of the donor tryptophans such that a higher percentage were accessible to I^- (51% vs. 25%) and fewer were accessible to Cs^+ (13% vs. 25%) and acrylamide (27% vs. 57%). These results indicate that during the Na^+-induced conformational change, there is a redistribution of tryptophans at the surface of the protein. These short- and long-range conformational changes induced by Na^+ are consistent with the Na^+-induced increase in accessibility of the glucose site to D-glucose, phlorizin, and FITC.

The Na^+ and glucose binding sites of the intestinal brush border Na^+/glucose cotransporter are located on a 75000-dalton polypeptide (Peerce & Wright, 1984a,b, 1985). Furthermore, Na^+ binding to the carrier produces a conformational change at the glucose site which results in an increase in the affinity for glucose binding (Kaunitz & Wright, 1984; Peerce & Wright, 1984a,b). The conformational change is also observed as a Na^+-dependent quench of fluorescein isothiocyanate (FITC)[1] bound to the glucose site on the carrier.

In the present study, we have used two new isothiocyanate derivatives, 1-pyrene isothiocyanate (PYTC) and 2-anthracene isothiocyanate (AITC), to probe the structure of the glucose site in its two conformations. With PYTC, Tl^+ quenching was used to determine the fraction of pyrenes that are exposed to the external aqueous environment in the presence and absence of Na^+. AITC, on the other hand, was used to monitor the solvent exposure of tryptophan residues close to the glucose site. AITC is a good energy acceptor up to 30 Å away from donor tryptophans. Tryptophan quench reagents (Eftink & Ghiron, 1981) were then employed to examine the solvent exposure and charge surrounding these neighborhood residues in the two conformations of the glucose site.

† This work was supported by NIH Grants DK 34807 and DK 19567.

[1] Abbreviations: PITC, phenyl isothiocyanate; FITC, fluorescein isothiocyanate; PYTC, pyrene isothiocyanate; AITC, anthracene isothiocyanate; Trp_G, tryptophan residues energy-donating to the glucose site; TFE, 2,2,2-trifluoroethanol; kDa, kilodalton(s); pCMBS, *p*-(chloromercuri)benzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

0006-2960/87/0426-4272$01.50/0