Rat Hepatic (Na^+,K^+)-ATPase: α-Subunit Isolation by Immunoaffinity Chromatography and Structural Analysis by Peptide Mapping†

J. J. Hubert, D. B. Schenk,‡ H. Skelly, and H. L. Leffert*

Department of Medicine, Division of Pharmacology, University of California, San Diego, La Jolla, California 92093

Received January 29, 1986; Revised Manuscript Received March 20, 1986

ABSTRACT: The catalytic α-subunit of rat hepatic (Na^+,K^+)-ATPase (EC 3.6.1.3) has been isolated by immunoaffinity chromatography from microsomes solubilized in *n*-dodecyl octaethylene glycol monoether. The procedure employs an anticatalytic mouse monoclonal antibody ("9-A5") covalently linked to Sepharose 4B that specifically blocks phosphorylation of the sodium pump's α-subunit from [γ-^{32}P]ATP [Schenk, D. B., Hubert, J. J., & Leffert, H. L. (1984) *J. Biol. Chem. 259*, 14941–14951]. The hepatic subunit is virtually identical with purified rat, dog, and human renal α-subunits as judged by its apparent molecular weight after polyacrylamide gel electrophoresis in sodium dodecyl sulfate (M_r 92K) and its two-dimensional tryptic and chymotryptic peptide maps on cellulose-coated thin-layer plates. In contrast, the structures of authentic renal β-subunits from the three species differ significantly from each other as judged by their peptide maps; no detectable homologies are seen between their chymotryptic maps and those of putative hepatic "β"-subunits (M_r 50K and 55K) eluted from 9-A5–Sepharose. Additional studies of ouabain-sensitive $^{86}Rb^+$ uptake in primary cultures of adult rat hepatocytes reveal inhibition curves with single inflection points (ID_{50} = 0.1 mM ouabain) in the absence or presence of pump-stimulating peptides like insulin, glucagon, and epidermal growth factor. These findings indicate that rat hepatocytes express only one of two known structurally conserved forms of catalytic subunit (the renallike α form) and, if at all, structurally divergent forms of the sodium pump's β-subunit. In addition, immunoaffinity chromatography with 9-A5–Sepharose facilitates the isolation of (Na^+,K^+)-ATPases from nonrenal tissues with low levels of sodium pumps.

Mammalian (Na^+,K^+)-ATPase (the "sodium pump", EC 3.6.1.3) is a plasma membrane spanning protein composed of catalytic (M_r 92–110K) "α"-subunits and glycosylated (M_r 50–56K) "β"-subunits whose postulated stoichiometry is 1:1 (Kyte, 1981). Biochemical evidence suggests that two types of α-subunit exist—a renal α and a neuronal "α^+" form—which differ in their primary sequences (Sweadner, 1979; Sweadner & Gilkeson, 1985). These differences have not yet been defined though the primary sequence of an α-subunit was deduced recently from the sequence of its cloned cDNA (Shull et al., 1985). No functions or primary sequences have been assigned yet to β-subunits, and there is evidence that certain cells may lack them (Fambrough & Bayne, 1983).

The pump actively transports three Na^+ ions out of and two K^+ ions into the intracellular space during a turnover cycle (Goldin, 1977). Ion gradients generated by this process ($[Na^+]_{out} > [Na^+]_{in}$ and $[K^+]_{in} > [K^+]_{out}$) regulate many cellular functions (Jörgensen, 1982) and have been implicated, as well, in the control of differentiation and proliferation (Kaplan, 1978; Leffert & Koch, 1979, 1985). For example, when quiescent cells are exposed to mitogens, the rates of K^+ influxes mediated by the sodium pump are stimulated (Averdunk & Lauf, 1975). Elevated rates of pump activity occur twice during the prereplicative interval of the animal cell "cycle": rapidly, within seconds, following increases in rates of plasma membrane Na^+ influxes (Koch & Leffert, 1979a,b) and, more gradually, within 6–12 h, as reflected by elevated levels of membrane (Na^+,K^+)-ATPase activity (Schenk et al., 1984). An interesting but unresolved problem concerns the issue of whether the second late prereplicative "phase" of pump activation, which occurs without an increase in the number of active membrane-bound sodium pumps, is caused by physicochemical changes in the plasma membrane and/or the structure of the sodium pump itself (Schenk et al., 1984).

While testing the hypothesis that pump-mediated ion fluxes are necessary to initiate hepatocyte DNA synthesis, we constructed a panel of mouse monoclonal antibodies directed against the rat renal α-subunit (Schenk & Leffert, 1983). Since little was known of the molecular structure of hepatic (Na^+,K^+)-ATPase and because the enzyme's abundance in rat liver is less than 1% of the renal level (Schenk et al., 1984), we used one of the extensively characterized anticatalytic antibodies ("9-A5") to isolate the hepatic pump. Here we show that hepatic microsomes solubilized in *n*-dodecyl octaethylene glycol monoether ($C_{12}E_8$)[1] retain (Na^+,K^+)-ATPase activity, a prerequisite for isolating catalytic subunits by immunoaffinity chromatography with 9-A5. Comparative studies of two-dimensional tryptic and chymotryptic peptide maps and of $^{86}Rb^+$ uptake in vitro (with primary hepatocyte cultures) indicate that hepatocytes contain only the renallike α form of catalytic subunit and, if at all, a β-subunit without detectable homologies to authentic renal β-subunits from rat, dog, or human sources. These findings exclude a role for α^+-subunits in hepatic function. They also illustrate the utility of immunoaffinity chromatography for the isolation of (Na^+,K^+)-ATPases from tissues containing low levels of these membrane proteins.

† This work was supported by the U.S. Public Health Service (AM28215, AM 28392, and GM 07752) and the American Liver Foundation.

* Correspondence should be addressed to this author.

‡ Present address: California Biotechnology, Inc., Mountain View, CA 94043.

[1] Abbreviations: $C_{12}E_8$, *n*-dodecyl octaethylene glycol monoether; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STA, silicotungstic acid; NP-40, Nonidet P40; EGF, epidermal growth factor; ID_{50}, drug concentration that inhibits response by 50%; kDa, kilodalton(s).

0006-2960/86/0425-4156$01.50/0

Experimental Procedures

Preparation of Renal (Na^+,K^+)-ATPase. (Na^+,K^+)-ATPases were isolated from outer renal medullas of male Sprague-Dawley or Fischer rats (300–400 g) or from human kidney medullas (25 g) obtained at autopsy by standard procedures (Jörgensen, 1974; Braughler & Corder, 1977). Following SDS titrations, microsomal protein concentrations were adjusted (Lowry et al., 1951) to 1.4 mg·mL^{-1}. Five milliliters of these solutions was loaded onto discontinuous gradients [5 mL of 37%, 8 mL of 28.3%, and 7 mL of 15% (w/v) sucrose] in polyallomer tubes and centrifuged by using a Beckman fixed-angle Ti-60 rotor (Jorgensen, 1974). Pure enzyme from dog kidney was generously supplied by J. Kyte (Kyte, 1971). Specific activities of fresh enzyme preparations were 15–24 μmol of ATP hydrolyzed·mg^{-1}·min^{-1} (Schenk et al., 1984) as determined by a standard (Na^+,K^+)-ATPase assay (Schwartz et al., 1969).

Preparation of Rat Hepatic Microsomes. Microsomes were prepared from fresh Fischer or Sprague-Dawley rat livers (25–30 g total) by standard procedures through step 10 (Neville, 1968). Following liver perfusion with 0.15 M NaCl at 21 °C, all manipulations were performed at 4 °C unless noted.

Tissue extracts (2 mL of step 10 material) were layered onto discontinuous gradients [11 mL of 44%, 10 mL of 42.3%, and 2 mL of 10% (w/v) sucrose, adjusted to pH 7.5 with 0.01 M imidazole and 0.01 N HCl] in a final volume of 26.3 mL, using 25.4 mm × 92 mm polyallomer tubes (Beckman Corp., Irvine, CA). The tubes were centrifuged at 90000*g* for 2 h in a Beckman L2-65B ultracentrifuge using the Ti-60 rotor. Microsomes banded at the 10–42.3% sucrose interface and were removed by aspiration with a Pasteur pipet. Protein concentrations in 5–10-μL aliquots were determined by adding 1 mL of STA[2] in 10 mm × 75 mm borosilicate glass tubes (White et al., 1972; Martin & Doty, 1949). The suspensions were mixed to denature proteins and centrifuged at 5000*g* for 10 min at 21 °C. The resulting supernatants were removed by aspiration, and the protein contents of the pellets were estimated (Lowry et al., 1951) by using bovine serum albumin as a standard. (Na^+,K^+)-ATPase activity in microsomal fractions ranged from 0.1 to 0.3 μmol of ATP hydrolyzed·mg^{-1}·min^{-1} as determined after storage at −20 °C for periods of ≤6 months.

Solubilization of Rat Hepatic Microsomal Proteins. Fresh solutions of $C_{12}E_8$ (Calbiochem/Behring, La Jolla, CA)[3] were used to solubilize microsomes at a $C_{12}E_8$:protein ratio of 1:4 (w/w), respectively. For routine preparations, suspensions containing 0.25 mg of $C_{12}E_8$·mL^{-1}, 1 mg of microsomal protein·mL^{-1}, 0.1 M KCl, 10% (v/v) glycerol, and 0.01 M imidazole (adjusted to pH 7.0 with 0.01 N HCl) were incubated for 5 min at 21 °C. $C_{12}E_8$ was added last, slowly titrating it into the rapidly stirred suspension, in order to avoid high "localized" concentrations of $C_{12}E_8$ which were found to inactivate hepatic (Na^+,K^+)-ATPase. Resulting solutions were centrifuged in 1.5-mL conical polypropylene Eppendorf tubes using an Eppendorf Model 5415 microfuge at 10000*g* for 5 min at 4 °C. The supernatants were removed and assayed for (Na^+,K^+)-ATPase activity.

Immunoaffinity Chromatography. Purified 9-A5 (Schenk & Leffert, 1983) was covalently bonded to CNBr-activated (March et al., 1974) Sepharose 4B (Pharmacia, Uppsala, Sweden). The beads were swollen in and washed with a 100-fold volume excess of 0.001 N HCl on a scintered glass filter at 21 °C (Goding, 1980). The resulting loosely packed beads (2 mL) were mixed together with 12 mg of 9-A5 in 2 mL of "coupling buffer" (0.5 M NaCl containing 0.2 M $NaHCO_3$, adjusted to pH 8.2 with 0.2 N NaOH). The mixture was agitated gently on a platform rocker for 6 h at 21 °C (Calton, 1984). The coupling efficiency of 9-A5 to CNBr-activated Sepharose 4B beads was 90%, as determined by monitoring 9-A5 concentrations (A_{280nm} = 1.34 mg of 9-A5·mL^{-1}) before and after incubation with the beads. Nonbonded 9-A5 was removed by filtration, and 2 mL of coupling buffer containing 0.2 M glycine was added to the 9-A5–Sepharose beads. This mixture was agitated for 2 h at 21 °C to block unreacted Sepharose sites. Unreacted material was removed by washing the beads 5 times, alternating with 50 mL each of a solution of 0.5 M NaCl containing 0.1 M sodium bicarbonate (adjusted to pH 8.2 with 0.1 N sodium hydroxide) or 0.1 M sodium acetate (adjusted to pH 4.0 with 0.1 N acetic acid). The resulting immunoaffinity adsorbant was washed with 50 mL of phosphate-buffered saline (0.15 M NaCl containing 0.01 M Na_2HPO_4 and 0.01 M NaH_2PO_4, adjusted to pH 7.5 with 0.01 M NaH_2PO_4) and stored in this solution at 4 °C.

For chromatography, the immunoaffinity adsorbant (1 mL) was mixed with 5 mL of "wash buffer" [0.25 mg of $C_{12}E_8$·mL^{-1}, 0.1 M KCl, 10% (v/v) glycerol, and 0.01 M imidazole, adjusted to pH 7.5 with 0.01 N HCl]. After 30 min at 21 °C, the buffer was removed by filtration. The beads were added to 5 mL of solubilized hepatic microsomes containing 1 mg of protein·mL^{-1} (as above). The mixture was incubated for 16 h at 4 °C with constant, gentle agitation to ensure equilibrium binding of specific proteins to the beads (Schenk et al., 1984). The resulting immunoadsorbant was transferred into a 10-mL plastic column (1.3 cm × 8.5 cm). The beads were allowed to settle, and the microsome solution was removed. The column was then rinsed with 50 mL of wash buffer.

The column was eluted with 1-mL aliquots of "elution buffer" (3 M KSCN, 0.5 M NaCl, and 0.5 M imidazole, adjusted to pH 7.5 with 0.05 N HCl). Fractions (1 mL) were collected and dialyzed separately against 4 L of 0.05% (w/v) SDS for 16 h at 4 °C using 1-cm diameter Spectra/Por dialysis membranes (molecular weight cutoff ≥8K).

Dialyzed fractions (100-μL aliquots) were analyzed by SDS–PAGE (Laemmli, 1970), and this first set of gels (7.5%, w/v) was stained with silver nitrate (Morrisey, 1981). Fractions containing high concentrations of α-subunits as judged by visual inspection of stained gels were pooled (fractions 2 and 3), concentrated to 50 μL in a Schleicher & Schuell collodion bag (molecular weight cutoff ≥25K), and subjected again to SDS–PAGE. The second set of gels provided the source of hepatic proteins for peptide mapping. These gels were stained with Coomassie Brilliant Blue R250 and scanned under white light with a Beckman DU-8 densitometer to determine the relative proportions of proteins.

To estimate α-subunit recoveries from hepatic microsomes, a second identical sample of solubilized microsomal proteins was purified by affinity chromatography and eluted in parallel with the above sample. After the concentration step, fractions

[2] When microsomal fractions are incubated with STA, protein values were ≃30% higher than the values obtained when identical samples were incubated with trichloroacetic acid. The differences apparently were due to increased solubility of microsomal proteins in the reagents used in the protein assay after STA precipitation (Martin & Doty, 1949).

[3] We found that $C_{12}E_8$ is unstable because (Na^+,K^+)-ATPase did not show measurable enzymatic activity after solubilization with $C_{12}E_8$ solutions stored for ≥3 weeks at 21 °C, or with $C_{12}E_8$ solutions prepared from solid $C_{12}E_8$ reagent stored for >1 year at −20 °C.

2 and 3 obtained in the second sample were assayed as above for total proteins.

Peptide Mapping. Two-dimensional peptide mapping was performed by a modified standard procedure (Kennel, 1976; Elder et al., 1977; Hubert & O'Brien, 1983). Proteins were eluted from 1 mm^2 gel slices (gel thickness = 1 mm) excised from Coomassie Blue stained gel bands ($\simeq$1–2 mm high). The proteins were labeled with 200–300 μCi of $Na^{125}I$ (ICN Radiochemicals, Irvine, CA) and digested with proteases (see legends to Figures 3A and 4). The resulting peptides were recovered and spotted onto 10 cm × 10 cm cellulose precoated thin-layer glass plates without fluorescent indicator (EM Reagents, Darmstadt, FDR). Peptides were separated by electrophoresis at 21 °C in the first dimension (1000 V for 10–15 min) and by ascending partition chromatography in the second dimension in a solvent system of butanol, pyridine, water, and acetic acid (65:50:40:10 v/v, respectively). The radiolabeled peptides were visualized on the plates as peptide maps by autoradiography using Kodak XRP-1 film at −70 °C; Dupont Hi-Plus intensifying screens were used to enhance detection of labeled peptides.

$^{86}Rb^+$ Uptake Studies. Primary adult rat hepatocyte cultures were established as described previously (Koch & Leffert, 1979b). Ion uptake assays were performed with quiescent 12-day-old cells (8×10^5 cells·$dish^{-1}$). The cultures were shifted into fresh serum-free media (1 mL per 35-mm dish) containing 1 μCi of ^{86}RbCl·mL^{-1} (New England Nuclear, Boston, MA) with or without 40 ng each of insulin, glucagon, and EGF (Collaborative Research, Waltham, MA). Half of the cultures in each of the groups also received varying doses of ouabain (10^{-8}–10^{-3} M). After 40 min at 37 °C, the dishes were washed and cell extracts harvested as described previously (Koch & Leffert, 1979a; Leffert & Koch, 1982) for measurements of radioactivity and proteins. A parallel set of dishes was used to monitor growth reinitiation competence (Koch & Leffert, 1979a; see legend to Figure 6).

Results

Solubilization of Hepatic (Na^+,K^+)-ATPase. In order to solubilize hepatic (Na^+,K^+)-ATPase prior to immunoaffinity chromatography, we first employed a standard procedure for isolating the enzyme from tissues containing high levels of this protein. For example, after SDS titration and centrifugation, renal microsomes from rat, dog, and human sources yielded virtually pure (Na^+,K^+)-ATPases as judged by SDS–PAGE (Figure 1). Molecular weight values for α-subunits under these conditions were 92K (all three species); molecular weight values for β-subunits were 53K (rat) and 56K (dog and human). However, similar attempts to solubilize (Na^+,K^+)-ATPase from rat hepatic microsomes were unsuccessful due to nearly quantitative losses of enzyme activity under these conditions.

For these reasons, we approached the problem by titrating hepatic microsomes in the nonionic detergent $C_{12}E_8$. Figure 2 shows this titration curve; optimal levels of soluble (Na^+,K^+)-ATPase activity were obtained at 0.25 mg of $C_{12}E_8$·(mg of microsomal proteins)$^{-1}$. This detergent:protein ratio (1:4 w/w) was 2.6-fold less than the optimal ratio used to solubilize the renal enzyme (Craig, 1982). The reasons for these tissue-specific differences are unknown.

Immunoaffinity Chromatography. Hepatic microsomes solubilized in $C_{12}E_8$ showed a complex protein profile after SDS–PAGE (Figure 3A, lane 1). In contrast, microsomal fractions that were bound to and then eluted from a 9-A5–Sepharose affinity column (and analyzed similarly) showed a prominent band of silver nitrate stained protein with a molecular weight of $\simeq$92K (Figure 3B, lanes 2–6). This band was not observed when starting material was incubated either

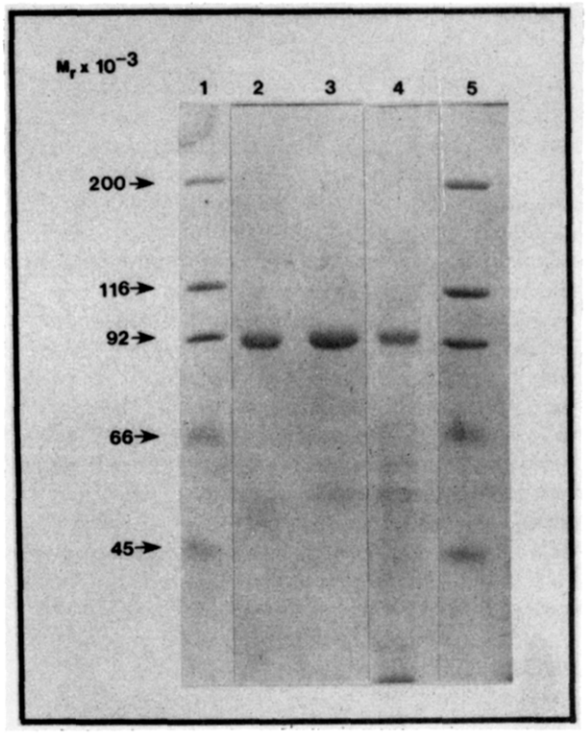

FIGURE 1: Gel electrophoretic analyses of purified rat, dog, and human renal (Na^+,K^+)-ATPase. Microsomes were prepared from renal tissues and solubilized with SDS. Solubilized microsomes were adjusted to 4.2% (v/v) sucrose, and 5 mL was layered onto discontinuous sucrose gradients as described under Experimental Procedures. The tubes were centrifuged at 140000*g* for 90 min at 4 °C. Under these conditions, (Na^+,K^+)-ATPase activity banded at the 28.3%–37% interface. Material at this interface was aspirated with a Pasteur pipet and assayed for protein content. An aliquot of each fraction was solubilized in a final volume of 100 μL of loading buffer under reducing conditions (Schenk & Leffert, 1983) and analyzed by SDS–PAGE on a 7.5% gel according to Laemmli (1970). Lanes 1 and 5, commercial molecular weight standards (Bio-Rad Corp., Richmond, CA) of myosin (M_r 200K), β-galactosidase (116K), phosphorylase *b* (92K), bovine serum albumin (66K), and ovalbumin (45K); lane 2, rat (Na^+,K^+)-ATPase (20 μg); lane 3, dog (Na^+,K^+)-ATPase (20 μg); lane 4, human (Na^+,K^+)-ATPase (100 μg).

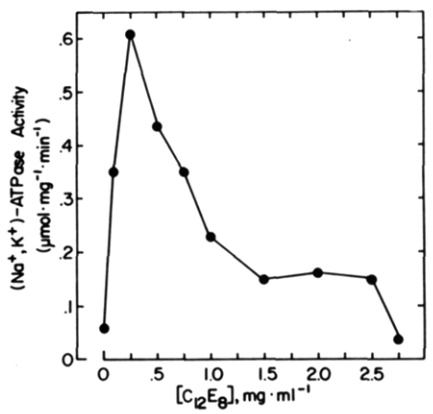

FIGURE 2: (Na^+,K^+)-ATPase activity in rat hepatic microsomes treated with $C_{12}E_8$. Rat hepatic microsomes were incubated for 5 min at 21 °C in a buffer containing (final concentrations) 1 mg of protein·mL^{-1}, 0.1–2.75 mg of $C_{12}E_8$·mL^{-1}, 0.1 M KCl, 10% (v/v) glycerol, and 0.01 M imidazole (adjusted to pH 7.0 with 0.01 N HCl). The suspensions were centrifuged at 10000*g* for 10 min at 4 °C, and the resulting supernatants were assayed immediately for enzyme activity. Solubilized (Na^+,K^+)-ATPase activity is expressed as the difference between the amounts of ATP hydrolyzed (micromoles of ATP hydrolyzed per milligram of protein per minute) in the absence and presence of 1 mM ouabain.

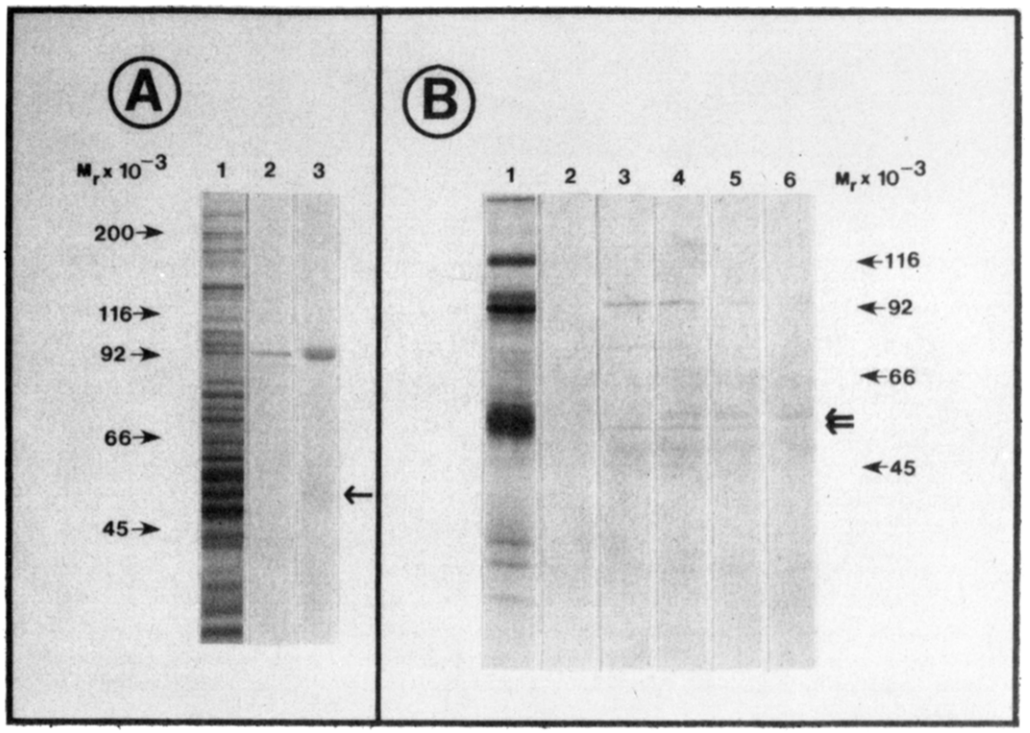

FIGURE 3: Gel electrophoretic analyses of rat hepatic microsomal proteins before and after 9-A5 immunoaffinity chromatography. (A) Coomassie Blue stained gel. (B) Silver nitrate stained gel. Hepatic microsomes were suspended in 5 mL of a buffer containing (final concentrations) 0.25 mg of $C_{12}E_8 \cdot mL^{-1}$, 1 mg of microsomal protein·mL^{-1}, 0.1 M KCl, 10% (v/v) glycerol, and 0.01 M imidazole (adjusted to pH 7.0 with 0.01 N HCl). The suspension was incubated for 5 min at 21 °C and centrifuged at 10000*g* for 5 min at 4 °C. The resulting supernatant was mixed with 1 mL of prewashed 9-A5–Sepharose immunoadsorbant beads. This mixture was agitated on a platform rocker for 16 h at 4 °C, transferred to a 10-mL column, and, after the microsome solution was allowed to drain, washed again with 50 mL of a buffer containing 0.25 mg of $C_{12}E_8 \cdot mL^{-1}$, 0.1 M KCl, 10% (v/v) glycerol, and 0.01 M imidazole (adjusted to pH 7.5 with 0.01 N HCl). The column was eluted, and the fractions were collected and analyzed by SDS–PAGE as described under Experimental Procedures. Proteins analyzed were as follows: rat hepatic microsomes (10 μg, panel A, lane 1); pooled and concentrated column fractions 2 and 3 (2 μg, panel A, lane 2; see below); purified renal (Na^+,K^+)-ATPase [7 μg, panel A, lane 3 (arrow denotes M_r ~53K), and panel B, lane 1]; and individual 9-A5 affinity column fractions 1–5 (panel B, lanes 2–6, respectively). Arrows (panel B, lane 6) denote molecular weight values of 50K and 55K. The virtual differences in comparative mobilities of authentic α- and β-subunits of renal (Na^+,K^+)-ATPase are due to shrinkage artifacts incurred by gels prepared for Coomassie staining in comparison to gels stained with silver nitrate. Molecular weight values were obtained from known protein standards (45–200 kDa) that were run in parallel lanes on each gel (data not shown; see Figure 1).

with a glycine-blocked Sepharose 4B column or with a column of Sepharose 4B covalently bonded to "S-63", a purified myeloma immunoglobulin (Hood et al., 1977).

Gel analyses of pooled and concentrated fractions (fractions 2 and 3) showed an identical 92-kDa band after Coomassie Blue staining (Figure 3A, lane 2) that comigrated with pure authentic renal α-subunits [Figure 3A, lane 3 (Coomassie stained), or Figure 3B, lane 1 (silver nitrate stained)]. When lane 2 (Figure 3A) was scanned with a densitometer, >85% of the stained proteins were confined to the 92-kDa band; the faster moving 88–90-kDa "contaminant" was not further studied since its appearance in Coomassie-stained gels was variable.

Under these conditions, 92-kDa bands were isolated by immunoaffinity chromatography only from hepatic microsomes containing (Na^+,K^+)-ATPase activity. Solubilized microsomes (Figure 2) retained ≃80% and <1% of the starting (Na^+,K^+)-ATPase activity at 4 °C for 6 and 24 h, respectively. Microsomes solubilized with 0.2% (w/v) Triton X-100 or 0.2% NP-40 did not retain enzymatic activity, and 92-kDa bands could not be recovered from these preparations after 9-A5–Sepharose chromatography (data not shown). Silver nitrate stained gels (Figure 3B) showed several additional proteins with molecular weight values ≃60–80K or <48K (lanes 3–6). It was not possible to selectively elute these proteins by washing the column with either Triton X-100 or NP-40 before elution with KSCN, since 92-kDa proteins also were eluted nonspecifically under these conditions.

Two-Dimensional Peptide Mapping. (A) α-Subunits. Tryptic (Figure 4A) and chymotryptic (Figure 4B) peptide maps of the 92-kDa band isolated by immunoaffinity chromatography (Figure 3A, lane 2) were virtually identical with tryptic and chymotryptic maps of the authentic rat renal α-subunit (panels E and F, respectively, of Figure 4). Mixtures of hepatic and renal tryptic and chymotryptic peptides also were indistinguishable in their mapping patterns (panels C and D, respectively, of Figure 4) when compared to maps derived from samples treated with a single proteolytic enzyme. When peptide maps were prepared from crude fractions of microsomal proteins (Figure 3A, lane 1) eluted from 92-kDa regions corresponding to the molecular weight of renal α-subunits, the resulting patterns were unlike those obtained with the isolated 92-kDa hepatic protein (data not shown). These results indicated that immunoaffinity chromatography significantly enriched protein fractions for (Na^+,K^+)-ATPase α-subunits and that α-subunits from rat liver and kidney were structurally identical. The results also provided evidence against artifactual isolation by immunoaffinity chromatography of a subpopulation of rat hepatic α-subunits.

Although rat renal α-subunits were used as standards in peptide mapping for the positive identification of rat hepatic 92-kDa proteins, a similar conclusion could have been made from renal α-subunit maps of dog (Figure 4G,H) and human proteins (Figure 4I,J). Greater than 95% homology was seen among the three sources when comparisons were made among their chymotryptic maps (Figure 4B,F,H,J). Near identity

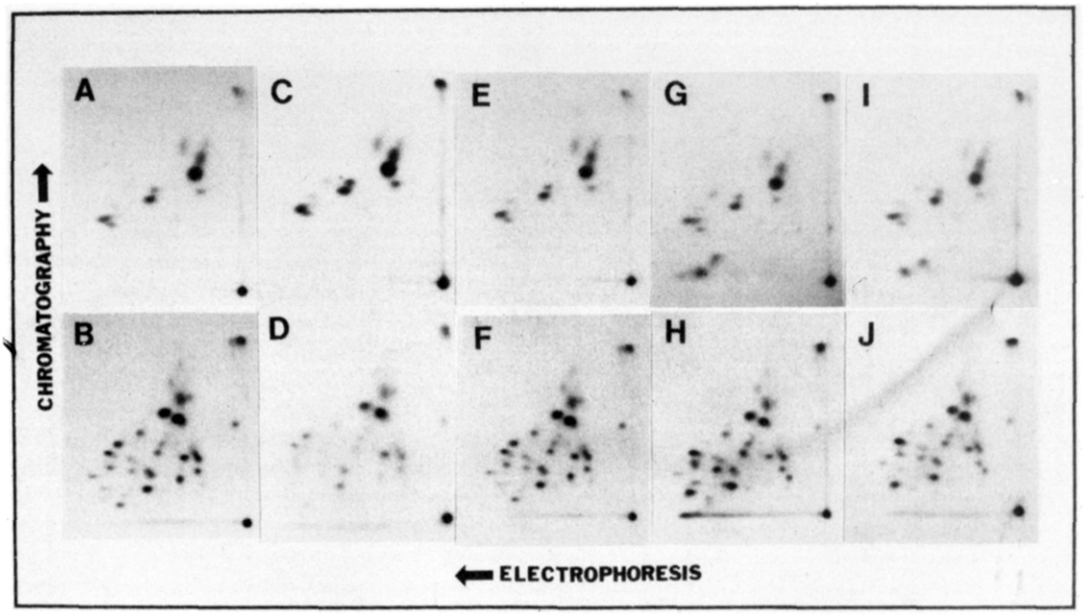

FIGURE 4: Two-dimensional peptide maps of ^{125}I-labeled polypeptides from (Na^+,K^+)-ATPase α-subunits. Proteins eluted from slices of Coomassie Blue stained bands on SDS gels were labeled with $Na^{125}I$ and digested with 100 μL of a 0.1 mg·mL^{-1} solution of either trypsin or chymotrypsin (type XIII, 12 000 units·mg^{-1}, or type II, 45 units·mg^{-1}, respectively; Sigma, St. Louis, MO). The ^{125}I-labeled peptides were recovered, and 250 000 cpm from each enzyme digest were spotted onto cellulose plates. Peptides were separated in the first dimension by electrophoresis from right (anode) to left (cathode) and in the second dimension by ascending partition chromatography (from bottom to top) under alkaline conditions for 60–90 min. Peptides were visualized as described under Experimental Procedures. Tryptic peptides are shown from rat liver [panel A (proteins obtained from 92-kDa band, Figure 3A, lane 2)], rat kidney [panel E (proteins obtained from 92-kDa band, Figure 3A, lane 3)], rat liver and rat kidney mixtures (panel C), dog kidney [panel G (proteins obtained from 92-kDa band, Figure 1, lane 3)], and human kidney [panel I (proteins obtained from 92-kDa band, Figure 1, lane 4)]. Chymotryptic peptides are shown from rat liver [panel B (as above)], rat kidney [panel F (as above)], rat liver and rat kidney mixtures (panel D), dog kidney [panel H (as above)], and human kidney [panel J (as above)].

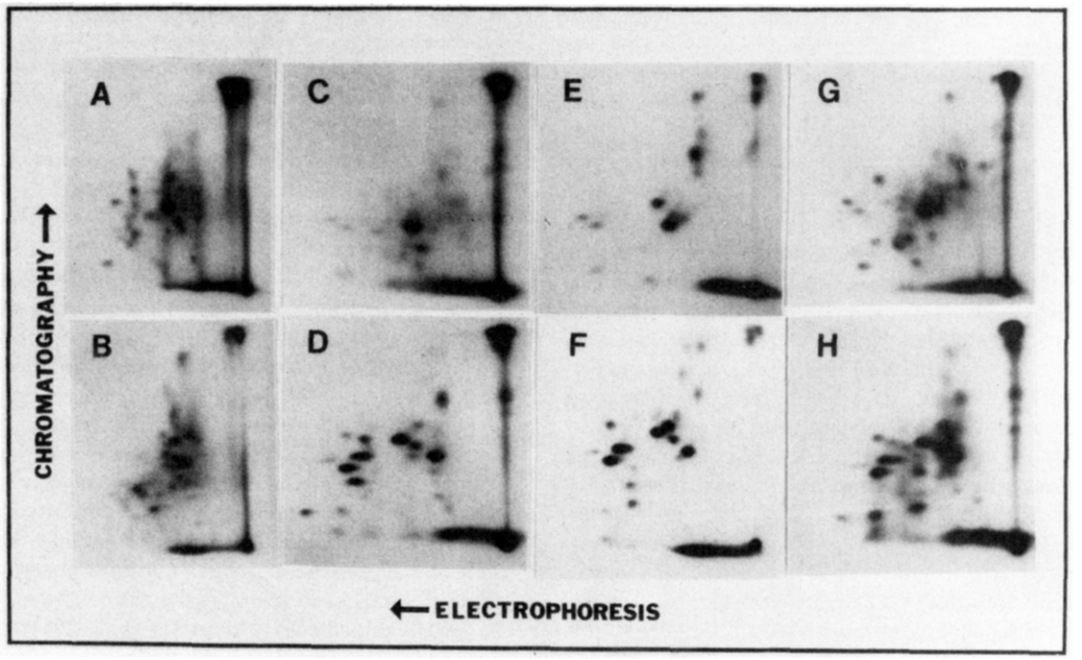

FIGURE 5: Two-dimensional peptide maps of ^{125}I-labeled polypeptides from (Na^+,K^+)-ATPase β-subunits. Tryptic and chymotryptic maps of authentic β-subunits were prepared as described under Experimental Procedures and in the legend to Figure 4. Tryptic peptides are shown from rat kidney [panel C (proteins obtained from 53-kDa band, Figure 3A, lane 3)], dog kidney [panel E (proteins obtained from 56-kDa band, Figure 1, lane 3)], and human kidney [panel G (proteins obtained from 56-kDa band, Figure 1, lane 4)]. Chymotryptic peptides are shown for rat kidney [panel D (as above)], dog kidney [panel F (as above)], and human kidney [panel H (as above)]. Chymotryptic maps of putative hepatic β-subunits are shown in panels A (M_r ~55K band) and B (M_r ~50K band); the proteins were obtained from the 50–55-kDa region of the gel shown in Figure 3A, lane 2.

was observed between dog (Figure 4G) and human forms (Figure 4I), which differed from rat subunits (Figure 4E) by only two to three basic peptides when their tryptic maps were compared.

(*B*) *β-Subunits.* Figure 5 shows peptide maps of authentic renal (Na^+,K^+)-ATPase β-subunits from rat (tryptic and chymotryptic maps, panels C and D, respectively; see arrow at $M_r \simeq 53$K region in Figure 3A, lane 3), dog (panels E and F), and human sources (panels G and H). In contrast to structural homologies among α-subunits, less than 20% homology among β-subunits was observed, although all were derived from renal tissues. Microheterogeneity could not account

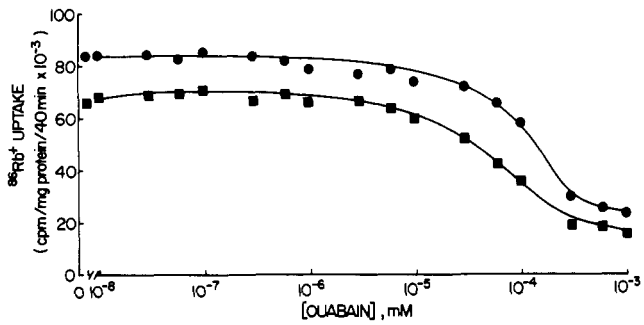

FIGURE 6: Ouabain-sensitive uptake of $^{86}Rb^+$ in primary cultures of adult rat hepatocytes. Culture conditions and $^{86}Rb^+$ uptake assays were performed as described under Experimental Procedures. Radioactive thymidine uptake rates, which confirm growth-reinitiation competence, were as follows: unchanged "conditioned" medium, 11 300 cpm of [^{3}H]dT·(10^6 cells)$^{-1}$·(24 h)$^{-1}$ [see Koch & Leffert (1979a)]; fresh medium, 17 500 cpm of [^{3}H]dT·(10^6 cells)$^{-1}$·(24 h)$^{-1}$ (■); fresh medium plus insulin, glucagon, and EGF, 77 800 cpm of [^{3}H]dT·(10^6 cells)$^{-1}$·(24 h)$^{-1}$ (●).

for these differences since upper or lower regions of each of the diffusely (rat and dog) or more "tightly" (human) staining β-subunit bands (see Figure 1) generated identical chymotryptic maps (data not shown).

Chymotryptic maps of putative rat hepatic β-subunits of M_r 55K/50K are shown in panels A and B, respectively of Figure 5. These proteins were detected by visual inspection of (and obtained from) Coomassie-stained gels shown in lane 2 of Figure 3A. [Because of their faint staining compared to the two silver nitrate stained protein bands (Figure 3B, lanes 3–6; see arrows), the proteins were not readily apparent in the photograph of the gel (Figure 3A, lane 2), and because of their low levels, tryptic maps were not prepared.] Not only were the "β-subunit" chymotryptic maps different from each other (55- vs. 50-kDa proteins) but also they were distinct from all of the authentic renal β-subunit maps analyzed.

In several additional experiments with proteins of $M_r \simeq 45$K, 47K, 63K, 68K, and 77K (see Figure 3B, lanes 3–5) that were radioiodinated and eluted from Coomassie-stained gels (Figure 3A, lane 2), no detectable homologies were found between their tryptic and chymotryptic peptide maps and the peptide maps of authentic α- or β-subunits (data not shown). Therefore, these unidentified proteins were probably not proteolytic fragments of the sodium pump that might have been generated artifactually before or during the immunoaffinity adsorption step. Instead, they could represent either nonspecifically adsorbed proteins and/or degradation products of the 9-A5 immunoadsorbant. More work with larger amounts of starting material (or with purified renal membranes) might help to clarify this interpretation.

Effects of Peptide Hormones on Ouabain-Sensitive $^{86}Rb^+$ Uptake in Cultured Hepatocytes. Figure 6 shows the effects of polypeptide growth factors on ouabain-sensitive uptake of $^{86}Rb^+$ (a K^+ analogue) in quiescent cultures of adult cells. The rate of isotope uptake was linear during the time interval of incubation that was used in these experiments (Leffert & Koch, 1982). A mixture of insulin, glucagon, and EGF stimulated ouabain-sensitive $^{86}Rb^+$ uptake 25% ($p < 0.01$). A single inflection point ($ID_{50} \simeq 0.1$ mM ouabain) was observed for both stimulated and unstimulated rates of $^{86}Rb^+$ uptake. No additional inflection points were seen at concentrations of ouabain which ranged between 10^{-8} and 10^{-4} M.

DISCUSSION

Structural and functional studies of rat liver (Na^+,K^+)-ATPase reveal that hepatocytes contain only one of two known polypeptide forms of the pump's catalytic subunit, the so-called α or renallike form. This conclusion was obtained by isolating detergent-solubilized hepatic catalytic subunits by specific immunoaffinity chromatography and by comparing the molecular weight values (92K) of these subunits and their two-dimensional tryptic and chymotryptic peptide maps with those of conventionally purified authentic renal α-subunits. Functional evidence supporting this conclusion was obtained from findings that cultured hepatocytes display only one inflection point in their dose–response curve for ouabain-sensitive $^{86}Rb^+$ uptake (ID_{50} = 0.1 mM ouabain), before or after exposure to known pump-stimulating mitogenic peptides like insulin, glucagon, and EGF (Fehlmann & Freychet, 1981; Leffert & Koch, 1982). These and other (Schenk et al., 1984) observations suggest that hormonal stimulation of hepatic (Na^+,K^+)-ATPase activity in vitro (and in vivo) during the late prereplicative phase of liver regeneration involves the activation of α-subunits without the emergence of α^+-subunits. These findings are in contrast to the activation of (Na^+,K^+)-ATPase activity by insulin in isolated adipocytes (Lytton et al., 1985). Therefore, it would appear that if two different genes code for α- and α^+-subunits (Sweadner & Gilkeson, 1985), of if alternative mRNA processing occurs, then hepatocytes—unlike central nervous system neurons (Sweadner, 1979) and insulin-treated adipocytes (Lytton et al., 1985)—selectively inhibit α^+ gene expression in the presence or absence of pump-stimulating factors. The molecular mechanisms responsible for this selectivity are unknown.

The application of monoclonal antibody 9-A5 for immunoaffinity chromatography illustrates the utility of this highly specific sodium pump ligand (Leffert et al., 1985)—whose binding site was shown recently to be localized to the "energy transduction" region of the pump's catalytic subunit (Farley et al., 1986)—for the isolation of this type of membrane-bound enzyme from low abundance sources like the liver. According to an experimental determination of 238 000 (Na^+,K^+)-ATPases per hepatocyte (Schenk et al., 1984), we estimate that from 5-mg proteins of starting microsomes, 56 μg of pump proteins would represent theoretical 100% yield. This value is based upon experimental yields of $\simeq$0.5 mg of microsomal proteins per gram of rat liver ($\simeq 1 \times 10^8$ hepatocytes) and the assumption that all sodium pumps with molecular weights $\simeq$145K (Kyte, 1981; Craig, 1982) remain catalytically active during the isolation procedure. Under the actual experimental conditions used, however, $\simeq 2$ μg of "pump"-related proteins (3.6% yield) was recovered from the pooled 9-A5 affinity column fractions (Figure 3B). We attribute this reduced yield to the ligand's inability to bind to denatured enzymes, that is, to bind to solubilized enzymes whose α-subunit activities in $C_{12}E_8$ detergent are lost beyond 6 h.

This explanation seems a more plausible one than the fortuitous isolation or the loss of a "subpopulation" of hepatic sodium pumps. In fact, the available evidence suggests that rat hepatic (Na^+,K^+)-ATPases represent an homogeneous population of epitopes recognized by 9-A5. This conclusion is based upon (a) linear curves and equivalent B_{max} values generated by Scatchard analyses of the hepatic pump in hepatic plasma membrane (or whole tissue homogenate) fractions with either ^{125}I-9-A5 or ^{35}S-9-A5 (Schenk et al., 1984), (b) the turnover number of hepatic (Na^+,K^+)-ATPase, calculated directly from studies of ^{125}I-9-A5 binding to hepatic microsomal proteins, which is 150 ATP molecules hydrolyzed per pump per second (Schenk et al., 1984), a value in excellent agreement with the one calculated for pure renal (Na^+,K^+)-ATPase (Jorgensen, 1982), and (c) the identity of

two-dimensional tryptic and chymotryptic peptide maps of authentic rat renal α-subunits with the 92-kDa hepatic membrane protein that binds specifically to 9-A5–Sepharose. This explanation is supported further by the failure to unmask, following hormonal stimulation of pump activity, a system of "high-affinity" ouabain-sensitive $^{86}Rb^+$ uptake in primary cultures of adult rat hepatocytes and by the failure to detect α^+-subunits in hepatic membranes after immunoblotting with α^+-specific polyclonal antisera.[4]

Comparative studies of two-dimensional tryptic and chymotryptic rat, dog, and human peptide maps reveal significant structural diversity among authentic renal β-subunits in comparison to α-subunits. Since renal and hepatic membranes do not contain α^+-subunits,[4] the minor differences that we observed between the rat and the dog and human catalytic subunit forms probably arise from single gene polymorphisms and not from mixed subunit populations (α and α^+) coded for by two distinct but evolutionarily related genes. This issue seems less equivocal with regard to β-subunits, whose primary sequences appear to diverge so much so (<20% homology as judged from peptide maps) that it would seem more likely that they are coded for by a family of unrelated or, at best, distantly related genes. Structural conservation and genetic relatedness among 92-kDa subunits of (Na^+,K^+)-ATPase (with respect to species and tissues) would be expected for the catalytic subunits of a crucial cellular enzyme that is distributed widely in nature. The reasons for the marked diversity in structural forms of the enzyme's β-subunit are unknown.

Structural diversity of β-subunits precludes a rigorous identification of a hepatic (Na^+,K^+)-ATPase β-subunit since no authentic "standard" is available for comparative study. Consequently, different interpretations of the results shown in Figure 5 are possible. On the one hand, it is conceivable that the 55- and 50-kDa proteins recovered after immunoaffinity chromatography (whose peptide maps are given in panels A and B, respectively, of Figure 5)—or one or more of the other "contaminants" in silver nitrate stained gels shown in Figure 3B—are, in fact, bonafide hepatic β-subunits. Verification of this conclusion must await appropriate reconstitution experiments in natural or synthetic lipid membranes using separately purified α-subunits and, if isolatable, β-like components. On the other hand, β-subunits might have been present in bound column fractions but at levels below detection by silver staining. This seems unlikely because when the 9-A5 immunoadsorbant is used to bind purified ^{125}I-labeled renal (Na^+,K^+)-ATPase solubilized in $C_{12}E_8$, and the specifically bound material analyzed by SDS–PAGE and autoradiography, ^{125}I-labeled bands with molecular weights of 92K and 53K are observed [and both bands comigrate with authentic unlabeled renal α- and β-subunits, respectively; see Schenk (1984)]. Alternatively, it might be argued that authentic hepatic β-subunits simply do not copurify on 9-A5–Sepharose and that these subunits dissociate from native holoenzyme–9-A5 complexes on the Sepharose matrix during the $C_{12}E_8$ washing step. However, this too seems unlikely since no evidence has been found of intact 50–55-kDa proteins among such wash fractions analyzed by SDS–PAGE (J. J. Hubert et al., unpublished results).

Still another explanation for difficulty in defining the nature of hepatic β-subunits is that they might not be produced by rat hepatocytes. This possibility warrants consideration for several reasons. First, the functional role of the β-subunit in the renal α–β complex has not been delineated, although renal β-subunits copurify with α-subunits (Kyte, 1981; Jörgensen, 1982) and form crystalline arrays with α-subunits in membrane fragments (Zampighi et al., 1984). Second, studies with anti-β-subunit monoclonal antibodies suggest that certain types of cultured cells, such as embryonic chicken fibroblasts (Fambrough & Bayne, 1983) and dog MDCK epithelia (Smith et al., 1985), lack the antigens to these but not to anti-α-subunit antibodies. Third, a putative β-subunit cDNA probe has been constructed which hybridizes to β-like mRNA sequences on Northern blots of RNA extracted from rat kidney or rat brain, but not from rat liver (R. Levenson, personal communication).[5]

ACKNOWLEDGMENTS

We thank J. Kyte and Y. Israel for helpful comments and S. Dutky for word processing. Insulin and glucagon were generously provided by W. Bromer (Eli Lilly Research Laboratory, Indianapolis, IN).

Registry No. ATPase, 9000-83-3; Na, 7440-23-5.

REFERENCES

Averdunk, R., & Lauf, P. K. (1975) *Exp. Cell Res. 93*, 331–342.
Braughler, J. M., & Corder, C. N. (1977) *Biochim. Biophys. Acta 481*, 313–327.
Calton, G. J. (1984) *Methods Enzymol. 104*, 381–387.
Craig, W. S. (1982) *Biochemistry 21*, 2667–2674.
Elder, J. H., Jensen, F. C., Bryant, M. L., & Lerner, R. A. (1977) *Nature (London) 267*, 23–28.
Fambrough, D. M., & Bayne, E. K. (1983) *J. Biol. Chem. 258*, 3926–3935.
Farley, R. A., Ochoa, G. T., & Kudrow, A. (1986) *Am. J. Physiol.* (in press).
Fehlmann, M., & Freychet, P. (1981) *J. Biol. Chem. 236*, 7449–7453.
Goding, J. W. (1980) *J. Immunol. Methods 39*, 285–308.
Goldin, S. (1977) *J. Biol. Chem. 252*, 5630–5642.
Hood, L., Loh, E., Hubert, J., Barstad, B., Eaton, B., Early, P., Fuhrman, T., Johnson, N., Kronenberg, M., & Schilling, J. (1977) *Cold Spring Harbor Symp. Quant. Biol. 41*, 817–836.
Hubert, J. J., & O'Brien, J. S. (1983) *Biochem. J. 213*, 473–478.
Jörgensen, P. L. (1974) *Biochim. Biophys. Acta 356*, 36–52.
Jörgensen, P. L. (1982) *Biochim. Biophys. Acta 649*, 27–68.
Kaplan, J. G. (1978) *Annu. Rev. Physiol. 40*, 19–41.
Kennel, S. J. (1976) *J. Biol. Chem. 251*, 6197–6204.
Koch, K. S., & Leffert, H. L. (1979a) *Cell (Cambridge, Mass.) 18*, 153–163.
Koch, K. S., & Leffert, H. L. (1979b) *Nature (London) 279*, 104–105.
Kyte, J. (1971) *J. Biol. Chem. 246*, 4157–4165.

[4] Using enzyme-linked immunoadsorbant assays (Schenk & Leffert, 1983), K. Sweadner and co-workers found recently that monoclonal antibody 9-A5 binds preferentially to purified rat renal (Na^+,K^+)-ATPase α-subunits, in contrast to purified rat brain axolemma (Na^+,K^+)-ATPase α^+-subunits (K. Sweadner, personal communication). This observation raised the possibility that the 9-A5–Sepharose matrix selectively bound an α-subunit (and excluded an α^+-subunit) subpopulation from hepatic membranes. This was unlikely, however, since under conditions in which the higher molecular weight α^+-subunit subpopulation would have been detected, an α^+-specific polyclonal antiserum as well as silver nitrate gel staining failed to reveal α^+-subunits on immunoblots or on 5% polyacrylamide gels of crude hepatic and purified renal membrane proteins, respectively, following SDS–PAGE (K. Sweadner, personal communication).

[5] This finding has been confirmed recently by A. McDonough and co-workers (personal communication).

Kyte, J. (1981) *Nature (London) 292*, 201–204.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Leffert, H. L., & Koch, K. S. (1979) *Prog. Liver Dis. 6*, 123–134.
Leffert, H. L., & Koch, K. S. (1982) in *Ions, Cell Proliferation and Cancer* (Boynton, A. L., McKeehan, W. L., & Whitfield, J. F., Eds.) pp 103–130, Academic Press, New York.
Leffert, H. L., & Koch, K. S. (1985) in *Control of Animal Cell Proliferation* (Boynton, A. L., & Leffert, H. L., Eds.) Vol. 1, pp 367–413, Academic Press, Orlando, FL.
Leffert, H. L., Schenk, D. B., Hubert, J. J., Skelly, H., Schumacher, M., Ariyasu, R., Ellisman, M., Koch, K. S., & Keller, G. (1985) *Hepatology (Baltimore) 5*, 501–507.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lytton, J., Lin, J. C., & Guidotti, G. (1985) *J. Biol. Chem. 260*, 1177–1184.
March, S. C., Parikh, I., & Cuatrecasas, P. (1974) *Anal. Biochem. 60*, 149–152.
Martin, J. B., & Doty, D. M. (1949) *Anal. Chem. 21*, 965–967.
Morrisey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Neville, D. M. (1968) *Biochim. Biophys. Acta 154*, 540–552.
Schenk, D. B. (1984) Ph.D. Thesis, University of California, San Diego, La Jolla, CA.
Schenk, D. B., & Leffert, H. L. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 5281–5285.
Schenk, D. B., Hubert, J. J., & Leffert, H. L. (1984) *J. Biol. Chem. 259*, 14941–14951.
Schwartz, A., Allen, J. C., & Harigaya, S. (1969) *J. Pharmacol. Exp. Ther. 168*, 3–41.
Shull, G. E., Schwartz, A., & Lingrel, J. B. (1985) *Nature (London) 316*, 691–733.
Smith, Z. D. J., Caplan, M. J., Forbush, B., & Jamieson, J. D. (1985) *J. Cell Biol. 101*, 434a.
Sweadner, K. J. (1979) *J. Biol. Chem. 254*, 6060–6067.
Sweadner, K. J., & Gilkeson, R. C. (1985) *J. Biol. Chem. 260*, 9016–9022.
White, A. A., Northup, S. J., & Zenser, T. V. (1972) *Methods Mol. Biol. 3*, 125–167.
Zampighi, G., Kyte, J., & Freytag, W. (1984) *J. Cell Biol. 98*, 1851–1864.

Electrostatic Interaction between Anions Bound to Site I and the Retinal Schiff Base of Halorhodopsin†

Brigitte Schobert and Janos K. Lanyi*
Department of Physiology and Biophysics, University of California, Irvine, California 92717
Received November 13, 1985; Revised Manuscript Received January 21, 1986

ABSTRACT: The influence of different anions on the deprotonation of the retinal Schiff base of halorhodopsin in the dark was investigated. We find that a large number of anions cause a significant increase of the pK_a of the Schiff base, an effect attributed to binding to "site I" on the protein. The concentration dependencies of the spectroscopic shifts associated with the changes of the pK_a yielded dissociation constants (and thus binding energies) for the anions, which were related to the Stokes radii. The data fit the predictions of electrostatic interaction between the anions and the positive charge associated with site I, if the latter is located within a few angstroms from the surface of the protein. The specificity of site I toward various anions is quantitatively explained by the differences in the change of Born energy upon transfer of the anions from water to the binding site. The changes in the deprotonation energy of the Schiff base upon the binding of anions, $\Delta\Delta G_{deprot}$, could be calculated from the ΔpK_a at infinite anion concentration. Unexpectedly, the $\Delta\Delta G_{deprot}$ values were remarkably close to the energies of binding to site I. Thus, site I and the Schiff base are strongly electrostatically coupled, either because of close proximity or because of the possibility of allosteric energy transfer between them.

The existence of three different anion binding sites in halorhodopsin, the light-driven chloride ion pump of *Halobacterium halobium*, has been demonstrated previously (Lanyi & Schobert, 1983; Schobert et al., 1983, 1986; Steiner et al., 1984; Falke et al., 1984). One of these sites, which is specific for chloride, bromide, and iodide, and whose occupancy appears to be required for transport, was termed site II. Another site, which is less specific, binds a variety of monovalent anions and was termed site I. Binding of the respective anions to these sites can be detected by small but characteristic shifts in the absorption band of halorhodopsin, a red shift in the case of anions which bind to site II (Ogurusu et al., 1982; Steiner et al., 1984; Schobert et al., 1986), and a blue shift in the case of anions which bind to site I (Schobert et al., 1986).

The chromophore of halorhodopsin (absorption maximum at 565–578 nm) is in a pH-dependent equilibrium with a species containing a deprotonated Schiff base, whose absorption maximum is near 410 nm (Lanyi & Schobert, 1983; Steiner et al., 1984; Hegemann et al., 1985; Schobert et al., 1986). Occupancy of site I by various anions raises the pK_a of the Schiff base by up to 2 pH units, as detected either by a shift of the equilibrium in favor of the protonated species at a pH near the pK_a (Lanyi & Schobert, 1983; Steiner et al., 1984) or by a shift of the pH titration curve of the absorption at 570 nm toward higher pH (Schobert et al., 1986). Quantitation of the dependency of the apparent pK_a on anion

† This work was supported by grants from the National Institutes of Health (NIH GM 29498) and the National Aeronautics and Space Administration (NAGW-212).

0006-2960/86/0425-4163$01.50/0