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Structure–Function Relationships in the Na,K-ATPase α Subunit: Site-Directed Mutagenesis of Glutamine-111 to Arginine and Asparagine-122 to Aspartic Acid Generates a Ouabain-Resistant Enzyme†

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ABSTRACT: Na,K-ATPases from various species differ greatly in their sensitivity to cardiac glycosides such as ouabain. The sheep and human enzymes are a thousand times more sensitive than the corresponding ones from rat and mouse. To define the region of the α1 subunit responsible for this differential sensitivity, chimeric cDNAs of sheep and rat were constructed and expressed in ouabain-sensitive HeLa cells. The construct containing the amino-terminal half of the rat α1 subunit coding region and carboxyl-terminal half of the sheep conferred the ouabain-resistant phenotype to HeLa cells while the reverse construct did not. This indicates that the determinants involved in ouabain sensitivity are located in the amino-terminal half of the Na,K-ATPase α subunit. By use of site-directed mutagenesis, the amino acid sequence of the first extracellular domain (H1–H2) of the sheep α1 subunit, Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn, was changed to that of the rat, Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp. When expressed in HeLa cells, this mutated sheep α1 construct, like the rat/sheep chimera, was able to confer ouabain resistance to these cells. Furthermore, similar results were observed when HeLa cells were transfected with a sheep α1 cDNA containing only two amino acid substitutions. This double mutation was a Gln-111 → Arg and Asn-122 → Asp change at the amino terminus and carboxyl terminus, respectively, of the H1–H2 extracellular region. The resistant cells, whether transfected with the rat α1 cDNA, the rat/sheep chimera, or the mutant sheep α1 cDNAs, exhibited identical biochemical characteristics including ouabain-inhibitable cell growth, $^{86}Rb^+$ uptake, and Na,K-ATPase activity. These results demonstrate that the presence of arginine and aspartic acid on the amino end and carboxyl end, respectively, of the H1–H2 extracellular domain of the Na,K-ATPase α subunit together is responsible for the ouabain-resistant character of the rat enzyme and the corresponding residues in the sheep α1 subunit (glutamine and asparagine) are somehow involved in ouabain binding.

The plasma membrane derived Na,K-ATPase is an ubiquitous enzyme which establishes and maintains the Na^+ and K^+ electrochemical gradient across the plasma membrane of animal cells (Jorgensen, 1982). This gradient serves as the energy source for numerous cellular activities such as active transport of certain solutes (Ullrich, 1979), regulation of cell volume (Macknight & Leaf, 1977), and restoration of the membrane potential in electrically excitable membranes (Thomas, 1972). The enzyme exists as a heterodimer consisting of a large catalytic α subunit and a smaller glycoslated β subunit whose function has not yet been determined. In addition to its role as a ion transporter, Na,K-ATPase is the target enzyme for a pharmacologically important class of drugs known as cardiac glycosides, such as digitalis and ouabain (Schwartz et al., 1975; Hansen, 1984). It is known that ouabain binds avidly to and subsequently inhibits the enzyme from a variety of sources including sheep and human whereas the corresponding rodent enzyme is virtually resistant to the drug (Repke et al., 1965; Wallick et al., 1980; Gupta et al., 1986; Schonfeld et al., 1986), apparently due to an increase in the dissociation rate of the drug from the binding site on the enzyme (Tobin & Brody, 1972; Wallick et al., 1980). Immunological (Ball & Lane, 1986) and biochemical (Peri-

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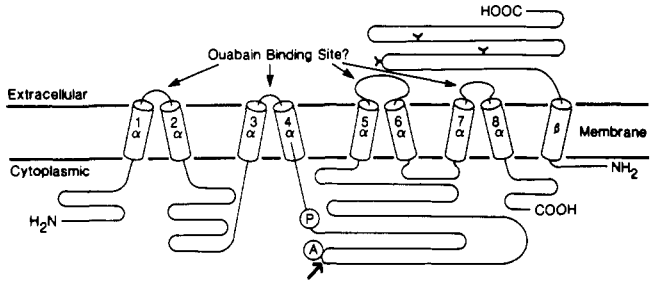

FIGURE 1: Hypothetical model of structural domains of the Na,K-ATPase (Shull & Lingrel, 1988). P and A represent the location of the phosphorylation and putative ATP binding region, respectively. The arrow indicates the location of the *Bam*HI site that is common to the cDNAs of both rat α1 and sheep α1.

yasamy et al., 1983) studies indicate that the underlying reason for the observed differences in ouabain sensitivities between rat and sensitive forms of the enzyme is a difference in the structure of the ouabain binding site as other characteristics of the two types of enzymes were found to be identical. Studies using photoaffinity and fluorescent derivatives of ouabain indicate that this binding site from sensitive enzymes is complex and may consist of several extracellular regions from both the carboxyl- and amino-terminal halves of the α subunit (Ruoho & Kyte, 1974; Fortes, 1977; Forbush et al., 1978; Goeldner et al., 1983; Jorgensen et al., 1982b) as well as regions from the β subunit (Hall & Ruoho, 1980). With chromosome-mediated gene transfer (Fallows et al., 1987) and cDNA expression (Kent et al., 1987), it was determined that the α1 subunit from insensitive enzymes (mouse or rat) can mediate ouabain resistance.

The complete amino acid sequence of the α subunit has been deduced from the cDNAs isolated from a number of species including sheep (1016 amino acids; Shull et al., 1985), human (1018 amino acids; Kawakami et al., 1986a), and rat (1018 amino acids; Shull et al., 1986a). The sequence of the β subunit from sheep (Shull et al., 1986b), human (Kawakami et al., 1986b), and rat (Young et al., 1987) has been obtained by similar techniques. Hydropathy analysis of the sequences has identified the stretches of amino acids that are of both sufficient length and hydrophobicity to traverse the plasma membrane. These data indicate that the α subunit possesses eight transmembrane domains (and therefore four extracellular regions) and the β subunit only one (Figure 1; Shull & Lingrel, 1988). Although the experimental verification of the membrane organization of the last four transmembrane domains (α5–8, Figure 1) has yet to be achieved, convincing evidence for the orientation of the first four transmembrane domains (α1–4) exists (Jorgensen et al., 1982).

In order to define the basis for the species differences in ouabain sensitivity, chimeric α1 subunit cDNAs were constructed by utilizing coding sequences from sensitive and resistant forms, and these were expressed in human tissue culture cells (HeLa). In addition, using site-directed mutagenesis of judiciously selected amino acids, we have identified residues responsible for the differential sensitivity.

Materials and Methods

Reagents. Dulbecco's modified Eagle's media and fetal bovine serum were purchased from Gibco Laboratories, Grand Island, NY. Ouabain was from Calbiochem, Behring Diagnostics, La Jolla, CA. Tissue culture supplies were purchased from Costar, Cambridge, MA. Restriction enzymes, T4 DNA polymerase and T4 DNA ligase, were from either New England Biolabs, Beverly, MA, or Boehringer Mannheim, Indianapolis, IN. $^{86}Rb^+$, as $RbCl_2$, was from New England Nuclear, Wilmington, DE. Dinonyl phthalate and silicone oil were from Fluka, Ronkonkoma, NY, and Aldrich Chemical Co., Milwaukee, WI, respectively. All other reagents were of the highest quality available.

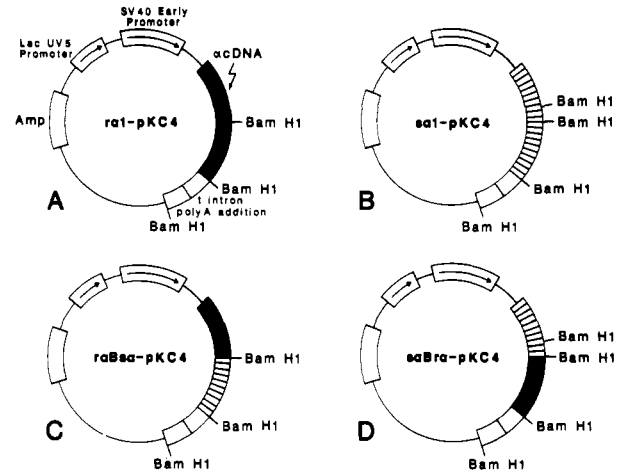

FIGURE 2: Expression vectors used in structure–function studies of the Na,K-ATPase α1 subunit. A description of these vectors and their construction appears in the text.

Expression Vector Constructions. The entire coding region of rat α1 or sheep α1, cloned into pBR322 (Shull et al., 1985, 1986a), was excised by double digestion with *Nhe*I and *Sac*I or *Eag*I and *Sac*II, respectively. The resulting fragments (3301 bp from rat α1 or 3170 bp from sheep α1) were gel purified, blunt-ended with T4 DNA polymerase, and ligated into the *Xba*I site of the polylinker region of the eukaryotic expression vector pKC4 [pKC4, a gift from Douglas Hanahan, Cold Spring Harbor, NY, is a derivative of pKO-neo (Van-Doren et al., 1984) containing the SV40 early promoter, small t intron, and early transcription terminator and polyadenylation sequences], which itself had been similarly blunt-ended. Following transformation of HB101 bacterial cells to ampicillin resistance, the constructs rα1-pKC4 and sα1-pKC4 (Figure 2, panels A and B) were identified via restriction endonuclease mapping. The chimeras rαBsα-pKC4 and sαBrα-pKC4 (Figure 2, panels C and D) were generated by digesting rα1-pKC4 and sα1-pKC4 to completion with *Bam*HI. The appropriate DNA fragments were isolated and ligated to the appropriate plasmid, which itself had been partially digested with *Bam*HI. The resulting construct rαBsα-pKC4 encodes a polypeptide whose amino-terminal half is derived from the rat α1 cDNA and carboxyl-terminal half is derived from the sheep α1 cDNA. The opposite chimera, sαBrα-pKC4, encodes a polypeptide whose amino-terminal half is derived from the sheep α1 cDNA and carboxyl-terminal half is derived from the rat α1 cDNA. All vectors used in this study were purified via cesium chloride gradient centrifugation.

Site-Directed Mutagenesis. A 430-bp fragment, which contains the coding region for residues 87–229 of the sheep α1 subunit, was excised from sα1-pKC4 by the concerted actions of *Sal*I and *Xba*I. Both fragments (the 430-bp fragment and the remainder of the vector) were gel purified, and the 430-bp cassette was subcloned into *Sal*I/*Xba*I-digested M13mp18 to yield M13mp18-430. The oligonucleotides used to generate the mutations of interest typically extended at least nine nucleotides in either direction from the location of the point mutation(s) and usually possessed a G or C base at both ends. The oligonucleotides were made on an Applied Biosystems Model 300A synthesizer. The mutagenesis reaction was performed by first kinasing 200 pmol of the appropriate oligonucleotide with 5 units of T4 polynucleotide kinase, and

20 pmol of this was annealed to 1 pmol of uracil-rich M13mp18-430 (Kunkel, 1985) at 95 °C. The solution was allowed to slowly cool to 22 °C, and deoxynucleotides were added to a final concentration of 0.4 mM. Klenow fragment and ligase (3 and 2 units, respectively) were added, and after 2 h at 37 °C an additional unit of ligase was added. After an hour at 22 °C the reaction mixture was used to transform JM103 cells. Numerous plaques from each reaction were picked and sequenced in order to identify the mutants of choice. Those cassettes found to possess the mutated bases were sequenced (Sanger et al., 1980) in their entirety. The double-stranded (rf) form of the mutant M13mp18-430 phage was cesium chloride gradient purified and doubly digested with *Sal*I and *Xba*I, and the mutant cassette was gel purified and ligated back into the parental, gel-purified *Sal*I/*Xba*I-digested sα1-pKC4. Following transformation of HB101 bacterial cells to ampicillin resistance, colonies were picked, and the plasmids were characterized by restriction endonuclease digestions and double-stranded sequence analysis to verify the presence of the appropriate mutation(s). The plasmids were cesium chloride gradient purified prior to their use in transfection experiments.

Transfection of HeLa Cells. Confluent dishes (100 mm) of HeLa cells were harvested via trypsinization, washed once with media, and resuspended in 272 mM sucrose, 7 mM sodium phosphate, pH 7.4, and 1 mM $MgCl_2$ (buffer A) at a cell density of 2.5×10^6 cells/mL. The appropriate vector (20 μg) was linearized with the restriction enzyme *Sca*I (40 units, 37 °C for 2 h), phenol/chloroform extracted, and ethanol precipitated. The DNA pellet was dissolved in 400 μL of buffer A and mixed with 400 μL of cells. Following a 2-min incubation a 22 °C the cells were electroporated with a 500-V pulse delivered at 25 μF (Bio-Rad gene pulser). The suspension was incubated 10 min more and then plated into duplicate 100-mm tissue culture dishes containing Dulbecco's modified Eagle's media, 10% fetal calf serum, and antibiotics. After 2 days, ouabain was added to 1 or 0.1 μM, and after 14 days, the number of resistant colonies was visually quantitated. Numerous colonies from each transfection were isolated with cloning cylinders and expanded into stable cell lines. These lines were maintained in duplicate dishes: one set was maintained on ouabain-free media and the other set was grown in 1 or 0.1 μM ouabain, depending on the transfectant. The media was changed every 2–3 days, and when the cells reached confluency (ca. 7–10 days), they were harvested via trypsinization, and about 10^5 cells were used to seed a fresh 100-mm tissue culture dish.

RNA Analysis. Total RNA was isolated from confluent 100-mm tissue culture dishes by a modification of a previously described procedure (Chomczynski & Sacchi, 1987). Briefly, the media was aspirated from the cells, and the monolayer was solubilized by the addition of 1.5 mL of 4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.2 M 2-mercaptoethanol. Following the addition of 0.2 mL of 2.0 M sodium acetate, pH 4.0, the solution was phenol (water saturated)/chloroform extracted. The aqueous phase was collected and the RNA precipitated with an equal volume of 2-propanol. The RNA pellet was dissolved in 0.5 mL of 10 mM HEPES, pH 7.5, 1 mM EDTA, and 0.1% SDS and phenol/chloroform extracted twice. The RNA was precipitated from the aqueous phase via 0.10 volume of 3.0 M sodium acetate, pH 5.2, and 2.5 volumes of ethanol. The pellet was washed once with 75% ethanol, dried, and dissolved in 50 μL of 0.5% SDS. Yields of 100–200 μg of RNA per dish were usually obtained. A total of 20 μg of RNA from each cell line was denatured in 1.5 M glyoxal, 65% DMSO, and 13 mM NaH_2PO_4, pH 7.0, with heating to 50 °C for 60 min. The RNA was fractionated by electrophoresing through a 1% agarose gel and transferred to a Nytran filter and probed according to the manufacturer's instructions (Schleicher & Schuell). After hybridization, the filter was washed successively in 2× SSC (22 °C), 1× SSC (37 °C), and 0.1× SSC (55 °C) (Maniatis et al., 1982), all containing 0.5% SDS. After being blotted dry, the signals were detected via autoradiography at −70 °C.

Ouabain-Inhibitable Cell Growth. Stably transfected or wild-type HeLa cells were harvested via trypsinization and resuspended at $(1.0–2.0) \times 10^5$ cells/mL. Cells (0.5 mL) were seeded into each well of a 24-well tissue culture plate which contained 0.5 mL of media plus twice the appropriate concentration of ouabain and incubated for 36–48 h at 37 °C in 5% CO_2. At this point, prior to the time at which the ouabain-free control cells attained confluency, the media was aspirated, and the cells were washed two times with phosphate-buffered saline and solubilized with 100 μL of 1.0 N NaOH. After the addition of 95 μL of 1.0 N HCl, 500 μL of Bio-Rad protein assay dye reagent was added, and the absorbance at 595 nm was recorded. The data are presented as percent of cell growth (as material absorbing at 595 nm) compared to growth in the absence of ouabain.

Ouabain-Inhibitable $^{86}Rb^+$ Influx. Influx studies were performed as described (Smith et al., 1982). Briefly, harvested cells were resuspended in media plus 5 mM HEPES, pH 7.4, at a density of 1×10^6 cells/mL. The 0.5-mL aliquots were incubated at 37 °C for 5 min in the presence of the appropriate concentration of ouabain. After the addition of 2–5 μCi of ^{86}Rb, duplicate 0.2-mL aliquots were removed after 2 min and centrifuged through ice-cold oil (300 μL; 1:1 dinonyl phthalate:silicone oil) layered with media (800 μL) in a microcentrifuge tube. The tubes were drained by inversion, and the pellet-containing tip of the tube was excised and mixed with 400 μL of 1% SDS. After several hours with occasional vortexing, 5 mL of Scintiverse BD (Fisher) was admixed with the sample, and the cell-associated radioactivity was quantitated via scintillation counting. The data were corrected for nonspecific absorption of $^{86}Rb^+$ by subtracting the radioactivity associated with the cells at 4 °C from that associated with the cells at the time of sampling and presented as percent of $^{86}Rb^+$ influx compared to influx in the absence of inhibitor.

Ouabain-Inhibitable Na,K-ATPase Activity. Na,K-ATPase activity in crude plasma membranes was assayed spectrophotometrically as described (Schwartz et al., 1969). The crude plasma membranes were isolated from the transfectants and wild-type HeLa cells as follows. Three to five confluent 100-mm tissue culture dishes or two to three confluent 175-cm^2 tissue culture flasks were washed two times with phosphate-buffered saline. The cells were harvested via scraping, incubated for 10 min at 4 °C in 1 mM $NaHCO_3$, 2 mM $CaCl_2$, and 5 mM $MgCl_2$ (approximately 10^8 cells/mL), and homogenized via 40 strokes in a glass (Dounce-type) homogenizer. The intact cells and nuclei were removed with a 3000*g* centrifugation (5 min), and the supernatant was treated with NaI as described (Lane et al., 1973). The solution was centrifuged at 48000*g* (45 min), and the pellet was washed two or three times with 10 mM Tris and 1 mM EDTA pH 7.4, and finally resuspended in this buffer at a protein concentration of about 1 mg/mL [determined by the method of Bradford (1976)]. The total Na,K-ATPase activity was determined by subtracting the ATPase activity obtained in the presence of 8×10^{-3} M ouabain from that observed in the absence of inhibitor.

Table I: Transfection of HeLa Cells with Rat α1 and Rat/Sheep Chimera

vector	no. of ouabain-resistant colonies[a] trial 1	trial 2
rα1-pKC4	230	170
rαBsα-pKC4	120	200
sαBrα-pKC4	0	0
sα1-pKC4	0	0
pKC4	0	0
none	0	0

[a] Resistant cells were selected in 1 μM ouabain 48 h after transfection.

The data are presented as percent of activity remaining at the indicated ouabain concentration compared to total Na,K-ATPase activity. Specific ouabain-inhibitable Na,K-ATPase activity in the transfectants usually comprised 50% of the total ATPase activity detected in the preparations, and the specific activity was 6–30 μmol of P_i hydrolyzed (mg of protein)$^{-1}$ h^{-1}.

Results

Comparison of the amino acid sequences of the Na,K-ATPase α1 subunit from ouabain-resistant and -sensitive species reveals high homology between the different enzymes. Pairwise comparison of the amino acid sequence of the rat α1 (Shull et al., 1986a) subunit with that of sheep (Shull et al., 1985) and human (Kawakami et al., 1986a) reveals only 35 and 32 amino acid differences, respectively, between the sensitive and resistant Na,K-ATPase α1 subunit. These appear throughout the length of the molecule, with a slight preponderance occurring in the amino half of the protein. In addition, four of these amino acid differences between rat and sheep or human are found in the first H1–H2 extracellular domain, while the sequences of the other extracellular domains are identical between rat and human or sheep except for one substitution found in the sheep H5–H6 domain (Shull et al., 1986a; Kawakami et al., 1986a). To locate the region of the α1 subunit responsible for the resistant phenotype, cDNAs for the sheep and rat α1 subunit were used to construct chimeric cDNA molecules for expression studies. One of the constructs, rαBsα-pKC4 (Figure 2C), consists of 1682 nucleotides of DNA derived from the 5′ end of the rat α1 cDNA and 1552 nucleotides from the 3′ end of the sheep α1 cDNA. The amino-terminal half of the resultant polypeptide encoded by this cDNA chimera contains 508 amino acids derived from the rat amino-terminal sequences, and the carboxyl-terminal half contains 510 amino acids from the sheep carboxyl-terminal sequences. The opposite chimera, sαBrα-pKC4 (Figure 2D), was similarly constructed; accordingly, this encodes an α subunit polypeptide whose amino-terminal half consists of 506 amino acids from the sheep amino half and carboxyl-terminal half is made up of 510 amino acids from the rat carboxyl-terminal half. These constructs, as well as the parental vectors rα1-pKC4 and sα1-pKC4 (Figure 2, panels A and B), were used to transfect HeLa cells, and the ability of a particular construct to confer ouabain resistance was determined and correlated with the expression of an insensitive Na,K-ATPase species in those resistant cells.

HeLa cells were transfected via electroporation, and the expression of a cardiac glycoside insensitive form of Na,K-ATPase was initially measured by selecting for cells that proliferate in 1 μM ouabain (Table I). Numerous colonies, which grow well in this concentration of inhibitor, were generated when either the entire rat α1 cDNA or the chimeric rat/sheep construct was used to transfect the otherwise sensitive HeLa cells. When the transfection experiments were repeated with either the sheep α1 cDNA or the opposite chimera (sheep/rat), resistant cells were never visualized. In order to demonstrate that the observed phenotype is in fact due to the expression of the rat-derived cDNA, Northern analysis of RNA isolated from the transfectants as well as from untransfected HeLa cells was performed (Figure 3). When the blots are probed with a 396-bp DNA fragment derived from the sheep α1 coding region, which exhibits 86% and 88% similarity with the corresponding sequence in rat α1 and human α1 (HeLa), respectively, a single species of RNA is detected in preparations from either the transfectants or the wild-type HeLa cells (Figure 3A). This indicates that both the rat α1 and the chimeric rat/sheep RNA comigrate with the endogenous human mRNA. Quantitation of the hybridization signals detected in Figure 3A, corrected for loading differences by standardization with a β actin probe, shows that either cDNA is expressed 2–4-fold higher than is the endogenous α gene (lanes 1–6 vs lane 7). The identity of the high molecular weight species in lane 5 (Figure 3A) is unknown but is probably the result of the recombination and integration of multiple copies of the vector in this particular transfectant. When the same blot is stripped and subsequently reprobed with a 130-bp DNA fragment corresponding to the rat α1 5′ untranslated sequence, it is found that only those RNA samples from the transfectants contain hybridizable mRNA (Figure 3B). No such RNA species is observed in the samples prepared from the wild-type HeLa cells (Figure 3B, lane 7).

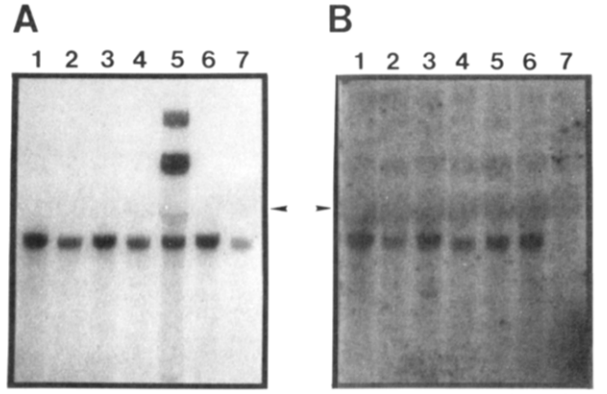

FIGURE 3: Detection of cDNA-encoded RNA in ouabain-resistant HeLa cells. Shown are the autoradiograms obtained, and the location of the 28S RNA is indicated (arrow). (A) Northern analysis of rα1-pKC4 transfectants (lanes 1–3), rαBsα transfectants (lanes 4–6), and wild-type HeLa cells (lane 7). The probe was a 396-bp *Xho*I/*Eco*RI fragment (50 ng) from the sheep α1 cDNA coding region that was gel purified and radiolabeled with the random priming method (Feinberg & Vogelstein, 1983). (B) Same as (A) except the probe was a 130-bp *Pst*I/*Apa*I fragment from the rat α1 cDNA 5′ untranslated region. A total of 20 μg of RNA was loaded onto each lane.

These data indicate that only when HeLa cells are transfected with a cDNA encoding an Na,K-ATPase α subunit whose amino-terminal half is the rat sequence can ouabain-resistant cells be generated. In addition, these data demonstrate that the resistance is not due to overexpression of the endogenous HeLa α RNA.

Additional experiments were performed to verify that the ability for the transfectants to survive high doses of ouabain is indeed a direct consequence of the expression of an insensitive form of Na,K-ATPase in these cells. This is necessary because recent reports indicate that some ouabain-resistant cells, either recipients of a ouabain-resistance "gene" (Levenson et al., 1984; Pressley & Edelman, 1986) or those selected in ouabain after chemical mutagenesis (Chopra & Gupta,

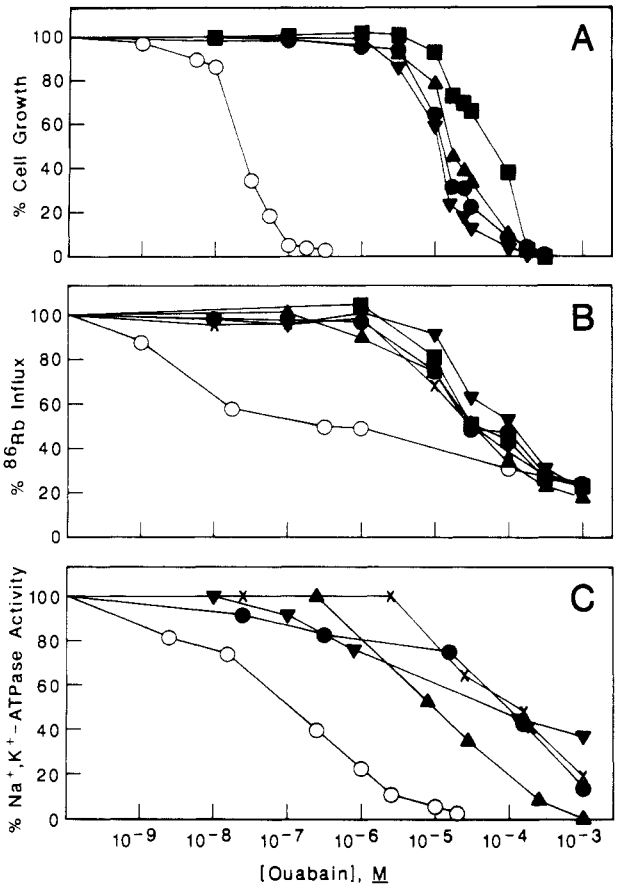

FIGURE 4: Biochemical properties of rα1-pKC4 and rαBsα-pKC4 transfectants. (A) Ouabain-inhibitable cell growth. Cells were grown in the presence of increasing concentrations of ouabain as described under Materials and Methods. Numerous cloned cell lines from each transfection were assayed in duplicate, and the average value is reported. (B) Ouabain-inhibitable $^{86}Rb^+$ influx. The ability for increasing amounts of ouabain to inhibit $^{86}Rb^+$ influx by various cloned cell lines was determined as described under Materials and Methods. Duplicate data points, corresponding to 0.2×10^6 cells, were taken at each ouabain concentration and averaged. (C) Ouabain-inhibitable Na,K-ATPase activity. Crude membranes were isolated from the cloned transfectants and assayed as described under Materials and Methods. About 30 μg of protein was used for each data point. (○) Wild-type HeLa cells; (■, ●, and ×) cell lines derived from rα1-pKC4 transfections; (▲ and ▼) cell lines derived from rαBsα-pKC4 transfections.

1986a), are not due to an alteration in Na,K-ATPase. The independently derived clonal lines from the transfected cells were biochemically characterized in terms of ouabain-inhibitable cell growth, $^{86}Rb^+$ influx, and Na,K-ATPase activity. While the wild-type HeLa cells are sensitive to relatively low doses of ouabain, an I_{50} of $(1 - 5) \times 10^{-8}$ M, the transfectants survived higher levels of the drug with an apparent I_{50} of about $(1 - 8) \times 10^{-5}$ M (Figure 4A). There were no differences observed in the extent of ouabain insensitivity regardless of whether the cells were transfected with either the rat α1 or the chimeric rat/sheep cDNA. Furthermore, the ouabain-resistant phenotype is stable, witnessed by the fact that transfectants maintained in ouabain-free media for more than 45 days still exhibit an I_{50} of $(1 - 8) \times 10^{-5}$ M ouabain (data not shown). The transfectants were also characterized with respect to $^{86}Rb^+$ influx. Reminiscent of the data described in Figure 4A, the sublines are all able to effectively concentrate $^{86}Rb^+$ in the presence of concentrations of ouabain that inhibit the uptake process in wild-type HeLa cells. As shown (Figure 4B), 50% inhibition of $^{86}Rb^+$ uptake into the transfectants occurs at an extracellular ouabain concentration of approximately 5×10^{-5} M, while similar inhibition of uptake into untransfected HeLa cells is achieved with 1×10^{-7} M ouabain. The presence of ouabain-insensitive $^{86}Rb^+$ uptake in the transfectants, relative to that observed in the untransfected HeLa cells, is indicative of the expression of a resistant form of Na,K-ATPase in those cells. This was experimentally verified by direct measurements of ouabain-inhibitable Na,K-ATPase activity in plasma membrane preparations from the transfectants (Figure 4C). Crude membranes from wild-type HeLa cells exhibit ouabain-inhibitable Na,K-ATPase activity with half-maximal inhibition occurring at approximately 1×10^{-7} M. Similar analysis of preparations from rat α1-transfected cells reveals the necessity for a 1000-fold higher concentration of ouabain to achieve half-maximal inhibition. Studies involving rαBsα-transfected cells also detect the presence of ouabain-resistant Na,K-ATPase activity, relative to that observed with wild-type HeLa cells (Figure 4C). In none of the assays (Figure 4B,C) were biphasic curves observed, as would be expected if both sensitive and insensitive forms of Na,K-ATPase were simultaneously present in the transfectants, regardless of whether the cells were maintained in ouabain or ouabain-free media. The reason for this is unclear, but perhaps the relative levels of the resistant enzyme are such that the activity contributed by the sensitive form remains undetectable with the methods employed here. In support of this is the finding that α1 subunit mRNA produced from the expression vector is two to four times higher than the endogenous α1 mRNA, as described earlier. Also, the ouabain-sensitive Na,K-ATPase activity and $^{86}Rb^+$ influx expressed by the wild-type HeLa cells are inhibited over a wide range of ouabain concentrations. Since these cells express a single class of Na,K-ATPase (Kawakami et al., 1986a), these data are probably due to the method of experimentation employed and not to heterogeneity of the enzyme in the HeLa cells. Similar inhibition profiles have also been reported elsewhere (Chopra & Gupta, 1986b).

As depicted in Figure 1 and as previously described (Shull et al., 1985, 1986a), the amino-terminal half of the Na,K-ATPase α subunit has four transmembrane domains and therefore two extracellular regions (H1–H2 and H3–H4). Since our studies using the rat/sheep chimera show that this half of the molecule possesses the determinants capable of mediating ouabain resistance, site-directed mutagenesis was applied to the question of precisely identifying those residues involved in this phenomenon. Because the ouabain binding site is extracellular in nature, it is reasonable to assume that those amino acids involved in binding, as well as those mediating ouabain resistance due to an absence of binding, are located in the H1–H2 and/or H3–H4 domains. The sequences of the H3–H4 domain of rat, sheep, and human α1 subunits are all identical (Glu-Tyr-Thr-Trp-Leu-Glu) and therefore, although possibly involved in ouabain binding, are not responsible for the species differences observed in ouabain sensitivity. However, comparison of the H1–H2 regions (Shull et al., 1986a) reveals four amino acid differences between the sequences of rat vs sheep or human (Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp vs Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn). The amino acids of this domain of the sheep α1 subunit (residues 111–122) were subsequently chosen for site-directed mutagenesis.

Five different sheep α1 mutants were generated, and by use of the same expression system as described for the rat α1 or rat/sheep chimera, the ability for the individual mutants to confer ouabain resistance to HeLa cells was evaluated (Table II). Expression in HeLa cells of either the sheep mutant

Table II: Transfection of HeLa Cells with Mutant Sheep α1 cDNAs

	Number of Ouabain-Resistant Colonies		
	1 μM ouabain		
construct[a]	trial 1	trial 2	0.1 μM ouabain
mutant A	240	140	
mutant B	170	150	
mutant C	0	0	70
mutant D	0	0	200
mutant E	0	0	0
sα1-pKC4	0	0	0

Sequences of the H1–H2 Extracellular Domain from Mutant and Wild-Type Sheep α1 cDNAs[b]

Mutant A: Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp
Mutant B: Arg-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asp
Mutant C: Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn
Mutant D: Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp
Mutant E: Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asn

Wild-type Sheep α: Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn
111 112 119 122

[a] sα1-pKC4 was mutagenized as described under Materials and Methods. A total of 20 μg of each construct was used to transfect HeLa cells. [b] Underlined residue(s) indicate(s) the location of mutation(s).

whose H1–H2 sequence is identical with the rat sequence (Gln-111 → Arg, Ala-112 → Ser, Gln-119 → Pro, Asn-122 → Asp) or the doubly mutated sheep α1 where the residues at either border of the H1–H2 extracellular region were both replaced by those found in the rat sequence (Gln-111 → Arg, Asn-122 → Asp) results in the generation of cells that are able to proliferate in 1 μM ouabain (Table II, mutants A and B). Transfection of HeLa cells with mutant sα1 cDNAs in which the residues at either border were independently mutated (Gln-111 → Arg, Ala-112 → Ser or Gln-119 → Pro, Asn-122 → Asp) did not give rise to cells that survived selection in 1 μM ouabain. Subsequently, when these transfectants were selected in 0.1 μM ouabain, several colonies were visualized (Table II, mutants C and D). No resistant cells were ever observed when the single mutant Gln-119 → Pro was transfected into the HeLa cells (Table II, mutant E). These data indicate that proliferation of the transfectants in 1.0 μM ouabain requires arginine and aspartic acid to both be present on the borders of the H1–H2 extracellular region of the expressed α subunit; the presence of only one or the other of these charged residues results in only partial resistance.

When the sensitivity of the transfectants toward ouabain was ascertained, it was found that those cells expressing either the mutants Sα1 (Arg-111, Ser-112, Pro-119, Asp-122, mutant A) or Sα1 (Arg-111, Asp-122, mutant B) were killed by the same levels of ouabain ($I_{50} = 1 \times 10^{-4}$ M) required to inhibit the growth of rα1- or rαBsα-transfected cells (Figure 5A). No difference in the I_{50} values was observed regardless of whether the cells were maintained in ouabain or ouabain-free media (data not shown). Similar experiments with cells transfected with the mutants Sα (Arg-111, Ser-112, mutant C) or Sα (Pro-119, Asp-122, mutant D) reveal a lower I_{50}, which shows that these cells are more sensitive to ouabain than any of the transfectants that were originally selected in 1 μM ouabain but are more resistant to the drug than are wild-type HeLa cells. These transfectants (mutants C and D) were not further characterized.

The cells transfected with the mutants Sα1 (Arg-111, Ser-112, Pro-119, Asp-122) or Sα1 (Arg-111, Asp-122) were further characterized in terms of RNA analysis and ouabain-inhibitable $^{86}Rb^+$ influx and Na^+,K^+-ATPase activity. Northern analysis of the RNA isolated from these mutants using the same 396-bp probe described in Figure 3A (derived from a portion of the sheep α1 coding region) reveals a hybridization pattern similar to that shown in Figure 3A: a single species of RNA comigrating with the endogenous mRNA is visualized (data not shown). Quantitation of the hybridization signals reveals that the mutant Sα1 cDNAs are expressed to levels comparable to those observed in the rat α1 or rat/sheep chimera transfectants (cf. Figure 3A). These cells also exhibit characteristics of a cardiac glycoside resistant Na,K-ATPase. Similar to the data shown for the rat α1 and rat/sheep chimera transfected cells (Figure 4B,C), these mutant Sα transfected cells exhibited ouabain-insensitive $^{86}Rb^+$ influx relative to that observed in untransfected HeLa cells with I_{50} values of approximately 5×10^{-5} M ouabain (Figure 5B). Analysis of crude plasma membranes isolated from these cells indicates that the Na,K-ATPase activity in those preparations is also relatively insensitive to ouabain, with I_{50} values again being about 1×10^{-5} M (Figure 5C).

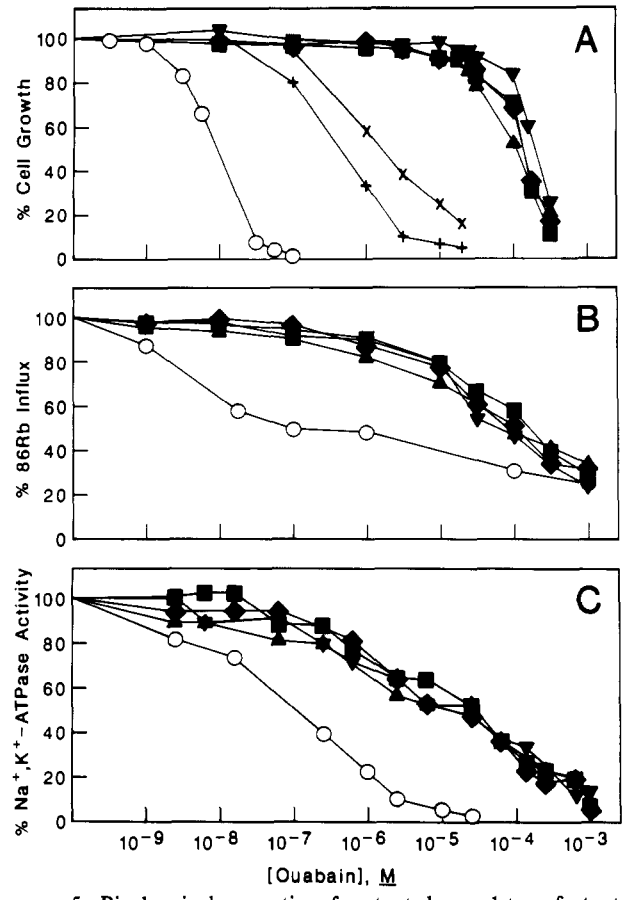

FIGURE 5: Biochemical properties of mutant sheep α1 transfectants. (A) Ouabain-inhibitable cell growth. (B) Ouabain-inhibitable $^{86}Rb^+$ influx. (C) Ouabain-inhibitable Na,K-ATPase activity. Various cloned cell lines from the transfections were assayed as described under Materials and Methods and in the legend to Figure 4. (▲ and ▼) Mutant A transfectants; (■ and ♦) mutant B transfectants; (×) mutant C transfectants; (+) mutant D transfectants; (○) wild-type HeLa cells.

It is important to note the similarities in the I_{50} values obtained in these biochemical studies between the rat α1, the rat/sheep chimera, and the mutant Sα- (Arg-111, Ser-112, Pro-119, Asp-122) or Sα1- (Arg-111, Asp-122) transfected cells. These I_{50} values correlate very closely with one another when the amount of ouabain required to inhibit either cell growth, $^{86}Rb^+$ uptake, or Na,K-ATPase activity is ascertained. When mutants containing arginine or aspartic acid separately, not simultaneously, at one or the other end of the H1–H2 extracellular regions (Arg-111, Ser-112 or Pro-119, Asp-122) are expressed in HeLa cells, one observes a degree of ouabain resistance that is intermediate to that measured in the other

transfectants (cf. Figure 5A).

Discussion

The determinants that mediate the species differences in Na,K-ATPase sensitivity toward cardiac glycosides have been identified. This study was facilitated by systematic cDNA expression studies involving the rat α1 subunit, a chimeric rat/sheep α1 subunit, and various mutant sheep α1 subunits. Cells transfected with the rat α1 cDNA or the rat/sheep chimera, which contains equal portions of the rat α1 amino half and the sheep α1 carboxyl half, proliferated in levels of ouabain that were 1000-fold higher than that which killed untransfected cells. In these cases, no differences are observed in the ouabain inhibition profiles of either the rat α1 or the chimeric rat/sheep transfectants. This indicates that the structural integrity of the chimeric α subunit has not been compromised. The enzyme present in rαBsα-pKC4 transfectants functions normally, as shown by $^{86}Rb^+$ uptake (Figure 4B) and ATP hydrolysis (Figure 4C), in a ouabain-inhibitable fashion, albeit at relatively high doses. The similarities observed in these I_{50} values for Na,K-ATPases containing either the rat α1 or the chimeric α subunit indicate that those amino acid residues present in the rat α1 subunit which are responsible for the ouabain insensitivity of the rodent enzyme are also present in the chimeric α subunit and are therefore contained in the amino-terminal half of the rat α1 subunit. It is assumed that the resistant enzyme present in the transfected cells is actually a hybrid molecule formed between the α subunit derived from the rat α1 or chimeric cDNA and the endogenous HeLa β subunit. Similar interspecies hybrids have been observed when murine cells were transfected with cDNA encoding an avian β subunit (Takeyasu et al., 1987). Furthermore, this study proves that rα1-pKC4 can be used in cotransfection experiments where ouabain resistance is used as a dominant selectable marker. When the cells were transfected with a mutant sheep α1 whose first extracellular domain (H1–H2) was specifically mutated to that sequence found in the rat, similarly resistant cells were generated. This shows that residues in the H1–H2 extracellular domain are involved in ouabain sensitivity, and when a sheep α1 was expressed in HeLa cells, which was mutated such that only the amino acids at the borders of the H1–H2 extracellular junction (Gln and Asn) were simultaneously changed to those found in the rat sequence (Arg and Asp), ouabain-resistant cells were again obtained. All of these cells exhibit ouabain-resistant $^{86}Rb^+$ uptake and Na,K-ATPase activity identical with that observed in rat α1 transfected cells, indicating that the presence of the charged amino acids arginine and aspartic acid on the amino-terminal and carboxyl-terminal borders of the H1–H2 extracellular region is responsible for the ouabain-resistant phenotype of the rat kidney Na,K-ATPase. This substantiates our recent hypothesis that these residues are indeed involved in resistance (Shull et al., 1986a).

Cardiac glycosides such as digitalis and ouabain possess three different structural regions, which together make up the intact molecule. These are an unsaturated lactone ring at position C17, a steroid body, and a terminal sugar residue(s), each of which has been postulated to bind to three unique sites on the Na,K-ATPase molecule of sensitive forms of the enzyme (Thomas et al., 1974; Akera et al., 1979). Since a number of studies indicate that the underlying reason explaining the species differences in cardiac glycoside sensitivity is that the actual binding site on the resistant enzyme is altered (Periyasamy et al., 1983; Ball & Lane, 1986; Schonfeld et al., 1986), it is reasonable to assume that the residues in the site that interacts with either the lactone, steroid, or sugar moiety of the glycoside are altered in this insensitive form. The work described here verifies that residues in the H1–H2 extracellular domain mediate ouabain sensitivity and therefore this region probably interacts specifically with one of the three structural features of the glycoside molecule. As Thomas et al. (1974) have postulated that the lactone ring may fit into an anionic site and because the H1–H2 domain contains three consecutive glutamate residues, perhaps this region of the α subunit interacts with the C17 side chain of cardiac glycosides. This hypothesis is corroborated by previous findings that the lactone ring or other substituents at the C17 position of the steroid mediate the potency of Na,K-ATPase inhibition (Repke & Portius, 1966; Ahmed et al., 1983).

Ouabain binding to sensitive forms of Na,K-ATPase is followed by a structural change that leads to a stable complex which is thought to be in the E_2P conformation (Jorgensen, 1983; Hansen, 1984). In resistant enzymes, this complex is unstable due to a rapid rate of dissociation of the inhibitor from the binding site (Tobin & Brody, 1972). The present findings show that substitution of the normally occurring residues that border the sheep α1 H1–H2 extracellular domain (glutamine and asparagine) with charged amino acids (arginine and aspartate) results in the generation of a ouabain-resistant Na,K-ATPase. We therefore postulate that the conformational change which effectively prevents ouabain dissociation from a sensitive enzyme is facilitated by the uncharged amino acids present at the borders of the H1–H2 extracellular domain of the α subunit. Such uncharged residues have been found at this location in ouabain-sensitive enzymes whose α subunit sequence is known (Shull et al., 1985; Kawakami et al., 1985, 1986a; Ovchinnikov et al., 1986; Takeyasu et al., 1988). Charged residues in this location in the corresponding resistant species (i.e., rat) could prevent this change in conformation, especially if this involved an interaction between the H1–H2 extracellular boundaries and the lipids of the plasma membrane. Such a hindrance, caused by a charged amino acid interacting with the surface of a membrane, is postulated as being involved in the rapid release of glycoside bound to insensitive forms of Na,K-ATPase. This is further corroborated by the observation that the α2 isoform of the rat enzyme, which has uncharged residues at the H1–H2 border (leucine and glutamine; Shull et al., 1986a), is sensitive to cardiac glycosides (Sweadner et al., 1979). Although yet to be demonstrated, one would assume that the α3 isoform is also sensitive to cardiac glycosides due to the presence of glutamine and asparagine at the H1–H2 borders (Shull et al., 1986a). Changing glutamine to arginine and asparagine to aspartic acid represents isosteric substitutions (Chothia, 1975; Knowles, 1987). The latter replacement (Asn → Asp) is especially isosteric as both residues have similar structures and hydrogen-bonding characteristics but are different by virtue of charge (Knowles, 1987). This indicates that the presence of a charged residue at the H1–H2 extracellular boundary is involved in resistance and not some other structural feature of arginine or aspartic acid. Replacement of these residues with other charged amino acids (glutamic acid or lysine) should also generate a ouabain-resistant enzyme, a possibility that is currently being explored.

Presumably the amino acids of the H1–H2 extracellular domain make up only a portion of the cardiac glycoside binding site, especially in light of studies with fluorescent and photoaffinity analogues of ouabain. Since compounds with a photoactivatable group on their terminal sugar moiety covalently modify the β subunit as well as the α subunit of the enzyme, the β subunit may be involved in the binding of this

portion of the molecule (Hall & Ruoho, 1980). The H3–H4 extracellular domain of the α subunit has also been implicated in ouabain binding (Shull et al., 1985) due to the presence of a tryptophan residue in this region (Fortes, 1977; Goeldner et al., 1983). A model describing the regions involved in ouabain binding to a sensitive Na,K-ATPase on the basis of these studies and the data presented here suggests that the initial binding event occurs between the residues of the H3–H4 domain and some structural feature of the cardiac glycoside, perhaps the steroid ring. This initial binding event should occur in both ouabain-sensitive (sheep or human) and -resistant (rat) forms of Na,K-ATPase, both of which have identical H3–H4 sequences, because the observed biochemical differences between such enzymes are mainly in dissociation and not association rates. That this specific interaction occurs is supported by preliminary evidence in which a photoaffinity analogue of ouabain covalently labeled the residues in the H3–H4 region (Ahmed et al., 1987). This binding interaction between the steroid body and amino acids in the H3–H4 region is postulated as being highly reversible and, in the case of sensitive enzymes, is stabilized by a conformational change involving the H1–H2 extracellular loop that effectively prevents the dissociation of the inhibitor from the binding site. That the steroid nucleus of the glycoside interacts with the residues in the H3–H4 domain (EYTWLE) is derived from the observation that there exists considerable homology between this sequence and a region postulated as being the steroid binding region of the human estrogen receptor (ECAWLE) and glucocorticoid receptor (QYSWMF) (Baker, 1986).

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UV-Induced Vanadate-Dependent Modification and Cleavage of Skeletal Myosin Subfragment 1 Heavy Chain. 1. Evidence for Active Site Modification†

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ABSTRACT: Ultraviolet irradiation above 300 nm of the stable MgADP–orthovanadate (V_i)–myosin subfragment 1 (S1) complex resulted in covalent modification of the S1 and in the rapid release of trapped MgADP and V_i. This photomodified S1 had Ca^{2+}ATPase activity 4–5-fold higher than that of the nonirradiated control S1, while the K^+EDTA-ATPase activity was below 10% of controls. There was a linear correlation between the activation of the Ca^{2+}ATPase and the release of both ADP and V_i with irradiation time. Analysis of the total number of thiols and the ability of photomodified S1 to retrap MgADP by cross-linking SH1 and SH2 with various bifunctional thiol reagents indicated that the photomodification did not involve these reactive thiols. Irradiation of the S1–MgADP–V_i complex caused a large increase in absorbance of the enzyme at 270 nm which was correlated with the release of V_i from the active site, suggesting an aromatic amino acid(s) was (were) involved. However, analysis by three different methods showed no loss of tryptophan. All the irradiation-dependent phenomena could be prevented by replacing Mg^{2+} with either Co^{2+}, Mn^{2+}, or Ni^{2+}. Unlike previous irradiation studies of V_i–dynein complexes [Lee-Eiford, A., Ow, R. A., & Gibbons, I. R. (1986) *J. Biol. Chem. 261*, 2337–2342], no peptide bonds were cleaved in photomodified S1. Photomodified S1 was able to retrap MgADP–V_i at levels similar to unmodified S1. Upon irradiation of the photomodified S1–MgADP–V_i complex, MgADP and V_i were again released from the active site, resulting in heavy chain cleavage to form NH_2-terminal 21-kDa and COOH-terminal 74-kDa peptides. All evidence indicates that this new photomodification and subsequent chain cleavage occur specifically at the active site.

We recently reported the photoaffinity labeling of gizzard myosin (Okamoto et al., 1986) by the photoreactive ADP analogue NANDP[1] (Nakamaye et al., 1985; Okamoto & Yount, 1985). In these experiments, NANDP was trapped at the active site as the stable noncovalent S1–MgNANDP–V_i complex which mimics the MgADP·P_i transition state for ATP hydrolysis (Goodno, 1982). In the early stages of these photoaffinity labeling experiments using NANDP, and in other experiments using 2- and 8-N_3ADP analogues, it was found that, in both skeletal and gizzard myosin, Mg–nucleotide diphosphate–V_i complexes were unstable to ultraviolet irradiation. In the dark, a typical half-life of a S1–Mg–nucleotide–V_i complex is about 2 days at 0 °C. This value decreases to minutes when the complex is irradiated with UV light above 300 nm. Fortunately, it was possible to prevent NANDP release in our earlier photolabeling studies of gizzard myosin by filtering out light below 400 nm (Okamoto et al., 1986). Although light above 400 nm will activate the azide of NANDP (Nakamaye et al., 1985), it does not cause V_i and nucleotide to be released from the active site. However, for other photoprobes which do not absorb radiation above 400 nm, the release of nucleotide from the active site caused by light >300 nm hindered specific photoaffinity labeling experiments.

It was of interest then to find conditions in which the binding of photoprobes could be stabilized and, at the same time, to investigate the properties of S1 to see if it was modified during the irradiation-dependent release of V_i and nucleotide. The latter possibility was suggested by the studies of Gibbons and co-workers (Lee-Eiford et al., 1986; Gibbons et al., 1987), who have found that, in attempts to use V_i to stabilize binding of 8-N_3ATP to dynein during photolabeling studies, both the α

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[1] Abbreviations: S1, myosin subfragment 1; PAR, 4-(2-pyridylazo)-resorcinol; MES, 2-(*N*-morpholino)ethanesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); pPDM, *N,N′-p*-phenylenedimaleimide; Co-Phen, $[Co^{III}phen_2CO_3]Cl$; V_i, orthovanadate; NANDP, diphosphate ester of (4-azido-2-nitroanilino)ethanol; NBS, *N*-bromosuccinimide; SDS, sodium dodecyl sulfate; A_1 and A_2, alkali light chains of skeletal myosin.

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