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Evidence That Ouabain Binds to the Same Large Polypeptide Chain of Dimeric Na,K-ATPase That Is Phosphorylated from P_i†

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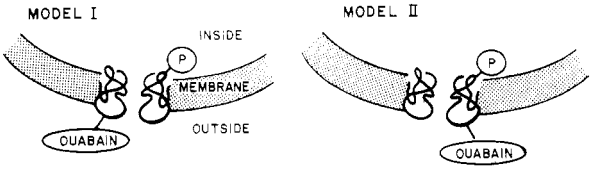
ABSTRACT: The functional unit of the Na,K-ATPase has previously been shown to include two large polypeptide chains (mol wt ≃ 95 000), only one of which can bind ouabain or be phosphorylated at one time. We have investigated the problem as to whether, when ouabain is bound and the enzyme is simultaneously phosphorylated from inorganic phosphate (P_i), both ligands are on the same large polypeptide chain or if they are on different 95K chains. We covalently labeled Na,K-ATPase purified from pig kidney outer medulla by using [^{3}H]-2-nitro-5-azidobenzoylouabain ([^{3}H]NAB-ouabain) and [^{32}P]phosphate, solubilized it with sodium dodecyl sulfate, and isolated the 95K polypeptide chains by using polyacrylamide gel electrophoresis. Large polypeptide chains labeled with [^{3}H]NAB-ouabain were separated from unlabeled chains by binding to a ouabain antibody and precipitation with immobilized protein A of *Staphylococcus aureus*. It was found that, for each chain thus separated, an equivalent amount of ^{32}P was precipitated. In controls, when two different samples were independently labeled with [^{3}H]NAB-ouabain or phosphorylated from $^{32}P_i$ and mixed before analysis, coprecipitation of ^{32}P with [^{3}H]NAB-ouabain-labeled chains was not observed. The results are thus in quantitative agreement with a model in which [^{3}H]NAB-ouabain binds at the same time to the same 95K polypeptide chain of a Na,K-ATPase functional unit that is phosphorylated from $^{32}P_i$.

The Na,K-ATPase[1] has been shown by electrophoresis on $NaDodSO_4$ polyacrylamide gels to consist of a large polypeptide of mol wt ~95 000, a glycoprotein of mol wt ~45 000, and a proteolipid component of mol wt ~12 000 (cf. Hokin et al., 1973; Lane et al., 1973; Forbush et al., 1978). The 95K polypeptide is phosphorylated from [^{32}P]ATP (Kyte, 1971; Uesugi et al., 1971) or from $^{32}P_i$ (Steckhoven et al., 1976) and forms part of the ouabain binding site (Ruoho & Kyte, 1974; Forbush et al., 1978); the functional roles of the glycoprotein and proteolipid are as yet unknown. The stoichiometry of ligand binding to purified Na,K-ATPase is such that for each pair of 95K polypeptide chains one ouabain molecule or one ATP molecule may be bound (Lane et al., 1973; Jorgensen, 1974b), or one ^{32}P may be incorporated (Hokin et al., 1973; Jorgensen, 1977). Thus the functional unit of the enzyme appears to be a dimer of 95K chains and to contain glycoprotein and proteolipid as well; cross-linking studies are also in agreement with a dimeric arrangement (Kyte, 1975; Giotta, 1976). Since under certain conditions both ouabain and ATP, or both ouabain and phosphate, may be bound to a functional unit of Na,K-ATPase at the same time, the question arises as to whether both ligands are on the same 95K polypeptide chain (model II) or whether they are on separate chains (model I). This problem is depicted in Chart I in which only the large polypeptides are shown traversing the membrane.

Chart I



The binding of [^{3}H]ouabain to Na,K-ATPase occurs most readily in the presence of either Mg, Na, and ATP, or in the presence of Mg and P_i; phosphorylation of the enzyme appears to be involved in both routes (Matsui & Schwartz, 1968;

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[1] Abbreviations used: Na,K-ATPase, magnesium dependent, sodium plus potassium stimulated adenosinetriphosphatase (EC 3.6.1.3); 95K chains, the 95 000 dalton polypeptide chains of Na,K-ATPase; P_i, inorganic phosphate; $NaDodSO_4$, sodium dodecyl sulfate; ATP, adenosine triphosphate; NAB-ouabain, 2-nitro-5-azidobenzoylouabain; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetate; K_d, dissociation constant.

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Albers et al., 1968; Tobin et al., 1974; Steckhoven et al., 1976). Whereas the phosphorylated intermediate formed from ATP breaks down and cannot be re-formed when ouabain is bound (Sen et al., 1969), the phosphorylated intermediate from P_i is formed more readily in the presence of ouabain than in its absence (Post et al., 1975; Steckhoven et al., 1976). We have, therefore, chosen to investigate as a first case the problem presented in Chart I in which ouabain is bound in the presence of P_i.

The approach used in this investigation is one of simultaneous covalent labeling of the Na,K-ATPase functional unit with a photoaffinity analogue of ouabain, [^{3}H]NAB-ouabain (Forbush et al., 1978), and with $^{32}P_i$, solubilizing and isolating the 95K polypeptide chains, and separating the 95K chains that are labeled with [^{3}H]NAB-ouabain by means of an antibody to ouabain. The results will be seen to be in quantitative agreement with model II above, in which both ligands are on the same 95K polypeptide chain. A brief report of this work has been presented previously (Forbush & Hoffman, 1979).

Methods

General. Preparation and assay of Na,K-ATPase from pig kidney outer medulla, [^{3}H]NAB-ouabain binding and photolysis, and gel electrophoresis were performed as previously described (Forbush et al., 1978). Purified Na,K-ATPase (Jorgensen, 1974a) was used in all but two experiments; in these, NaI-treated microsomes (Lane et al., 1973) were used as noted below. In most of the experiments reported here, the concentration of [^{3}H]NAB-ouabain and of enzyme functional units (determined by [^{3}H]NAB-ouabain binding) was 1–10 μM in the binding and photolysis medium, with a slight excess (~10%) of enzyme binding sites compared with [^{3}H]-NAB-ouabain in order to keep the free concentration of [^{3}H]NAB-ouabain low and thus minimize nonspecific binding.

$^{32}P_i$ (New England Nuclear) was freed of contaminating polyphosphates by adsorption to DEAE-cellulose, followed by elution with 0.6 M triethylammonium bicarbonate which was then removed by evaporation. Na,K-ATPase which was inhibited with ouabain or [^{3}H]NAB-ouabain (see below) was phosphorylated by addition of $^{32}P_i$ to a mixture which then contained ~1 mg/mL Na,K-ATPase, 5 mM Mg, 120 mM NaCl, 2 mM EDTA, ~0.1 mM PO_4 (~0.5 Ci/mmol), 30 mM Tris, pH 7.25 (at 37 °C). After 1–5 min at 22 °C (and in some cases photolysis), the protein was denatured by addition of 0.25 volume of 1 M sodium phosphate, pH 2.4. Phosphorylated intermediate was determined by filtration (0.08 μm Nucleopore filters or Gelman GN-6) after dilution of small samples (e.g., 5 μL) into 1 mL of 5% trichloroacetic acid. For purification of phosphorylated chains, the remainder was solubilized by the addition of 0.2 volume of 15% $NaDodSO_4$, 10% sucrose and incubation at 37 °C for 5 min prior to electrophoresis. Gel electrophoresis was also useful as an alternative to filtration for determination of phosphorylated intermediate when a very low background was needed; in this case, thin gels (1 mm) were rapidly stained and destained prior to slicing and counting. Unlike the phosphorylated intermediate formed from [γ-^{32}P]ATP (Hokin et al., 1973; B. Forbush III and J. F. Hoffman, unpublished), the intermediate formed from $^{32}P_i$ is not stable to $NaDodSO_4$ solubilization unless it is first denatured with acid (unpublished results).

[^{3}H]NAB-ouabain II was prepared as described previously (Forbush et al., 1978) except that tritium was incorporated from [^{3}H]$NaBH_4$ at the stage of reduction of the ethylenediamine–ouabain adduct. A significant isotope effect was noted on ion-exchange chromatography (see Klein, 1966, for review of such effects), with [^{3}H]ethylenediamine–ouabain I and II eluting on the leading edge of the nontritiated compounds. Thus [^{3}H]NAB-ouabain II was prepared with a higher specific activity (1.56 Ci/mmol) than that of the overall specific activity of reduced material (0.063 Ci/mmol). The purity of the [^{3}H]NAB-ouabain II was 89% by thin-layer chromatography, and it was 96% cardiac steroid as determined by the method of Hansen & Skou (1973). "[^{3}H]NAB-ouabain" is used to refer to this preparation of [^{3}H]NAB-ouabain II elsewhere in this paper.

"Chains" Experiments. The principal experiments of this investigation ("chains" experiments) involved simultaneous phosphorylation of the NaK-ATPase from $^{32}P_i$ and covalent labeling with [^{3}H]NAB-ouabain followed by solubilization with $NaDodSO_4$, isolation of the phosphorylated labeled 95K polypeptide chains on a polyacrylamide gel, and separation of [^{3}H]NAB-ouabain-labeled chains by means of an antibody to ouabain. Labeling and phosphorylation were done at the same time in four experiments (type A); in another six experiments (type B), labeling with [^{3}H]NAB-ouabain was performed first, and an attempt was made to remove noncovalently bound label by dilution and centrifugation; phosphorylation was then performed under conditions which favored phosphorylation only of labeled functional units. Individual experiments of the two types are distinguished below by the prefix A or B (e.g., B0815).

In type A experiments, one sample (Ia) of Na,K-ATPase was simultaneously phosphorylated from $^{32}P_i$ and photolabeled with [^{3}H]NAB-ouabain, and a second sample (Ib + II) was prepared by mixing, after acid denaturation, two samples that were either photolabeled with [^{3}H]NAB-ouabain (Ib) or phosphorylated from $^{32}P_i$ (II). The protocol is as follows: Two samples of Na,K-ATPase (final 1.5 mg/mL) were incubated in 5 mM Mg, 1 mM EDTA, 40 mM imidazole (pH 7.5) at 37 °C for 15 min. Sample I contained 1.7 μM [^{3}H]NAB-ouabain and 75 μM P_i in 375 μL, whereas sample II contained 100 μM ouabain and 30 μM $^{32}P_i$. To half of sample I (Ia) were added NaCl (final 50 mM) and $^{32}P_i$ (to ~0.5 Ci/mmol) and, after 2 min (20 °C), the sample was photolyzed for 1 min, immediately denatured by acid (pH 2.4 phosphate, 0.2 M final), and further processed as described under Isolation of Labeled 95K Polypeptide Chains. The rest of sample I (Ib) was photolyzed, denatured with acid, and added to sample II which had also been denatured; the combined sample (Ib + II) was then processed in parallel with sample Ia. At each step of the above sequence, 5-μL samples (in duplicate) were diluted into 1 mL of either 10 mM Tris (pH 7.5) or 5% trichloroacetic acid and filtered to determine the amount of [^{3}H]NAB-ouabain binding or covalent [^{3}H]NAB-ouabain labeling and phosphorylation.

The protocol for three type B experiments (B0815, B0824, B1114) is given here in detail; the important differences in three other experiments are then presented. Na,K-ATPase (NaI microsomes, 0.96 mg/mL in experiments B0815, B0824; purified Na,K-ATPase, 0.5 mg/mL in B1114) was incubated with [^{3}H]NAB-ouabain (0.51 μM) in 120 mM NaCl, 3 mM NaATP, 3 mM $MgCl_2$, 0.5% BSA, pH 7.5 for 15 min at 37 °C, and photolyzed for 1 min. It was then diluted into 35 mL of 60 mM imidazole, 1 mM EDTA, pH 7.5, and incubated at 37 °C for 70 min to allow noncovalently bound [^{3}H]-NAB-ouabain to dissociate (Forbush et al., 1978); the enzyme was centrifuged for 1 h at 45000*g*, and the pellet was resuspended in 0.5 mL of 10 mM Tris, pH 7.5 (20 °C), and stored overnight at 4 °C. A 75-μL sample was phosphorylated with $^{32}P_i$ (~0.5 Ci/mmol, 82 μM) in the presence of 5 mM

Mg, 75 mM Na (see Results), and a control sample (see Results) was phosphorylated from [γ-^{32}P]ATP (~0.1 Ci/mmol, 100 mM) in the presence of 75 mM Na, 5 mM Mg, 1.3 mM P_i. The two samples were denatured with acid (pH 2.4, phosphate, 0.2 M final) and processed in parallel as described below. In two experiments (B0615, B0620), binding of [^{3}H]NAB-ouabain to purified Na,K-ATPase was done in MgP_i binding medium and the noncovalently bound label was reduced by dilution into, and incubation for 60 min in, 120 mM Na, 3 mM ATP, 30 mM Tris prior to centrifugation and resuspension. In these experiments, the control sample was prepared by mixing a fraction of the [^{3}H]NAB-ouabain labeled pellet (after acid denaturation) with a fresh sample of Na,-K-ATPase that had been separately phosphorylated from $^{32}P_i$ and denatured with acid (as in type A experiments). In one experiment (B0808), all steps subsequent to binding of [^{3}H]NAB-ouabain (in NaMgATP medium) were carried out after the enzyme had been filtered onto the surface of a Nucleopore filter; solutions were changed by drawing them through the filter or removing them from the top with suction. The control consisted of two samples on separate filters labeled with [^{3}H]NAB-ouabain or phosphorylated with $^{32}P_i$ and combined after acid denaturation and $NaDodSO_4$ solubilization.

The fraction of bound [^{3}H]NAB-ouabain that was covalently incorporated into the 95K chain was determined as the ratio of the amount of label migrating with the 95K polypeptide on polyacrylamide gels (either the 2-cm pH 2.4 gel used in 95K chain purification described below, or a 10-cm pH 7.4 gel) to the total amount of bound [^{3}H]NAB-ouabain. In the two experiments (B0615, B0628) gel tracks were not digested, and the labeling efficiency of the large chain was estimated as 54% of the label that was stable to trichloroacetic acid in filtration assays of binding, the other 46% being labeling of the proteolipid; 54% was the mean ratio in the other type B experiments (range ~42–65%).

Isolation of Labeled 95K Polypeptide Chains. [^{3}H]-NAB-ouabain-labeled and ^{32}P-phosphorylated Na,K-ATPase, solubilized in $NaDodSO_4$, was applied to the top of a 9% acrylamide (0.045% bisacrylamide)–17% acrylamide (0.15% bisacrylamide) gradient slab gel (3-cm running length; well 0.3 cm thick × 5 cm width) and electrophoresed for 1 h at 14 °C, pH 2.4, sodium phosphate buffer system (Fairbanks et al., 1971). A 2-mm central width of the gel was sliced and digested in H_2O_2 and counted for ^{3}H and ^{32}P to confirm the labeling profile, an example of which is shown in Figure 1A. Note that the proteolipid (marked c, see Forbush et al., 1978) is not resolved from the photolyzed NAB-ouabain (marked d) on the short gel. The remainder of the gel track (~48 mm width) was immediately sliced and Cherenkov radiation from ^{32}P was counted, with the slices in the bottom of empty vials. The slice(s) containing the ^{32}P peak corresponding to the labeled 95K chains (marked a in Figure 1A) was homogenized with 3 mL of 10 mM sodium phosphate (pH 2.4) in a Teflon pestle homogenizer, 0.2 mL of 20% $NaDodSO_4$ was added, and the suspension was incubated for 10 min at 37 °C to extract the solubilized large polypeptide chains. The gel particles were removed by centrifugation at 10000g (5 min) and the supernatant containing solubilized 95K chains was removed and concentrated by ultrafiltration to 0.5–1 mL, washed once with 4–6 mL of 10 mM sodium phosphate (pH 2.4), and reconcentrated to about 2 mL by ultrafiltration (Amicon Model 12, PM 10 or PM 20 filters, 20–40 psi, total 0.3–1 h, 22 °C). This solution contained ^{3}H and ^{32}P label on only the 95K polypeptide chains as shown by reelectrophoresis:

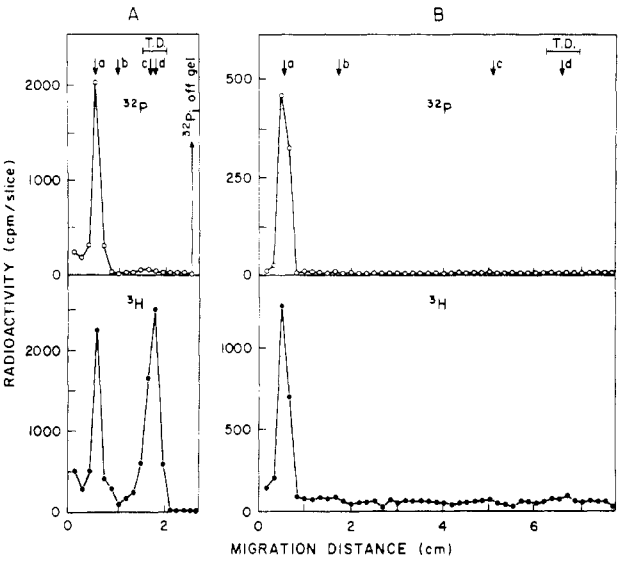

FIGURE 1: Gel electrophoretic profile of ^{32}P and [^{3}H]NAB-ouabain labeled Na,K-ATPase. Samples were from experiment B1114, prepared as described in text. Marks a–d and T.D. denote the running positions of the 95K polypeptide, the glycoprotein, the proteolipid, photolyzed NAB-ouabain, and the tracking dye, respectively. (A) Separation of labeled 95K chains on short pH 2.4 gel. The arrow at the end of the ^{32}P profile notes the fact that $^{32}P_i$ ran ahead of the tracking dye, off of the gel. (B) Reelectrophoresis (on pH 7.8 gel) of 95K chains isolated from peak a on the short gel (panel A). Hydrolysis of about 40% of the phosphorylated intermediate between the time of running gel A and gel B is reflected in the decrease in $^{32}P/^3H$ ratio in peak a.

as seen in Figure 1B, only a single peak of ^{3}H and of ^{32}P was detected corresponding to a single protein peak (not shown). The final solution contained 0.1–0.4% $NaDodSO_4$, measured by the method of Hayashi (1975). The hydrolysis of ^{32}P-labeled phosphorylated intermediate during the electrophoresis, extraction, and concentration procedures was estimated to be 10–30%.

Antibody Technique. Ouabain antibody in rabbit serum was the generous gift of Dr. Thomas W. Smith (Smith, 1972). The antibody was stored frozen as serum or diluted 100-fold in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.02% sodium azide, pH 7.4; the concentration of ouabain binding sites calculated for undiluted serum was found to be 4.6×10^{-5} M, and the association constant was about 4×10^9 M^{-1} (data not shown) in agreement with values for the fresh serum (Smith, 1972; Smith & Skubitz, 1975). The association constant for [^{3}H]NAB-ouabain was about twice that for [^{3}H]ouabain, as determined by competition with nonradioactive ouabain. *Staphylococcus aureus* protein A was purchased in the form of lyophilized heat-treated bacteria (New England Enzyme Center, IgGSORB) and was stored and washed as described by Kessler (1975). Conditions for optimum precipitation of [^{3}H]NAB-ouabain labeled chains by the antibody are discussed below; the protocol used in most of the experiments is as follows: To a 0.05–0.1-mL sample containing solubilized, labeled 95K chains (and optionally 10^{-4} M cold ouabain) was added 0.95 mL of a suspension containing 0.5% *S. aureus* protein A, 0.16% BSA, and a 2000-fold dilution of ouabain antiserum in 110 mM NaCl, 3 mM EDTA, 30 mM Tris, 30 mM Hepes, pH 7.1. After incubation at room temperature for 20 min, the *S. aureus* protein A–antibody–NAB–ouabain–95K chain complex was pelleted by centrifugation for 2 min (Model 3200 microfuge, Eppendorf), and 0.8 mL of the supernatant was decanted and transferred to scintillation vials for counting of ^{3}H and ^{32}P. The pellet was washed twice (with vortexing to diperse it) in the original medium containing in

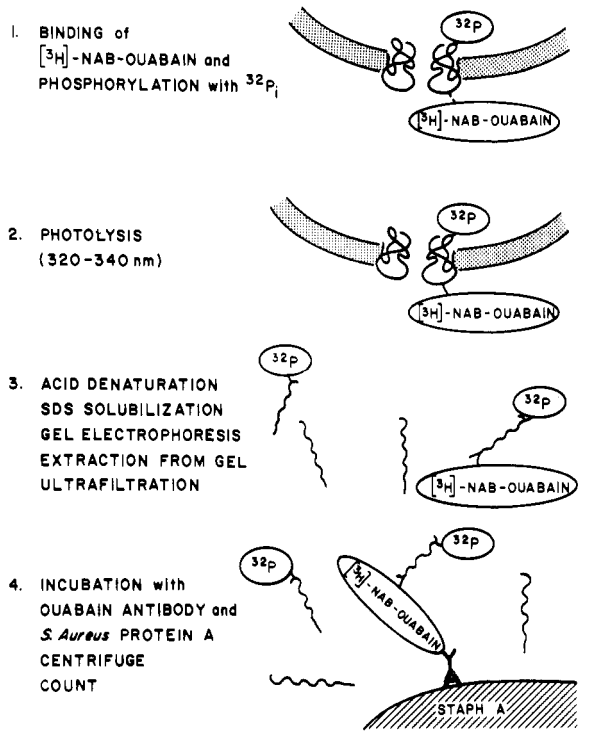

FIGURE 2: Schematic representation of "chains" experiment for model II of Chart I. Procedures are described in detail in text. This diagram represents the protocol of type A experiments. In type B experiments, the incorporation of $^{32}P_i$ took place after photolabeling with [^{3}H]-NAB-ouabain (panel 2).

addition 0.5% Triton X-100. ^{3}H and ^{32}P on the precipitated 95K chains were counted in a scintillation counter after addition of 1 mL of 30% H_2O_2 to the pellet to break up the complex (2% $NaDodSO_4$ was also effective) and addition of 10 mL of scintillant (ACS, New England Nuclear).

Results

The major finding of this investigation is that ouabain binds to the same 95K polypeptide chain of Na,K-ATPase that is phosphorylated from P_i. This is the result of ten experiments the data from which will be presented in Tables II and III below. In each of these "chains" experiments, the Na,K-ATPase was covalently labeled with [^{3}H]NAB-ouabain and phosphorylated from $^{32}P_i$, the enzyme was denatured in acid and solubilized in $NaDodSO_4$, 95K polypeptide chains were isolated by polyacrylamide gel electrophoresis, and [^{3}H]-NAB-ouabain-labeled chains were separated by means of a ouabain antibody. This procedure is outlined schematically in Figure 2 in which model II of Chart I was selected for illustration.

Phosphorylation by P_i and Binding of [^{3}H]NAB-ouabain. [^{3}H]NAB-ouabain was found to support phosphorylation from $^{32}P_i$ in the same way as ouabain, as shown in Table I (lines 2 and 4). The K_d of the inhibited Na,K-ATPase for $^{32}P_i$ was about 10 μM, in rough agreement with Stekhoven et al. (1976, 5.6 μM); the presence of 120 mM NaCl or 30 mM KCl in the phosphorylation medium had no significant effect on the level of phosphorylation or on the K_d for P_i when Na,K-ATPase had been preinhibited with ouabain (data not shown). On the other hand, phosphorylation of the uninhibited enzyme by $^{32}P_i$ requires much higher concentrations of P_i (Stekhoven et al., 1976; Post et al., 1975; B. Forbush III and J. F. Hoffman, unpublished observation) and is prevented by Na (Post et al., 1975). Therefore in this study functional units of Na,K-ATPase that were inhibited by [^{3}H]NAB-ouabain could be selectively phosphorylated by using a low concentration of $^{32}P_i$ (50–100 μM), and by including 120 mM NaCl in the phosphorylating medium in most experiments. In three experiments (A0622, B0615, B0620), Na was omitted from the phosphorylation medium, without significantly affecting the results.

Table I: Effect of NAB-Ouabain and Photolysis on Phosphorylation[a]

	phosphorylation (% of control)
no additions	0.4 ± 0.4
5 mM ouabain (control)	100
5 mM ouabain + light	112 ± 9
5 mM NAB-ouabain	108 ± 17
5 mM NAB-ouabain + light	103 ± 19

[a] Mean result (± SD) from three experiments. Na,K-ATPase (0.9–1.7 mg/mL, in 10 μL) was incubated with no additions, ouabain, or [^{3}H]NAB-ouabain for 15 min at 37 °C in the presence of 5 mM Mg, 120 μM P_i and cooled to room temperature. Phosphorylation was initiated by the addition of $^{32}P_i$ (final 80 μM) and NaCl (final 110 mM) and after 2 min 10 mL of 5% trichloroacetic acid was added and ^{32}P intermediate was determined by Millipore filtration. The results are expressed as % of the + ouabain samples, after subtraction of background incorporation in a sample containing 1 mM P_i (17–25% of control).

Table II: Stoichiometry of Ligand Binding[a]

(1)	(2)	(3)	(4)	(5)	(6)
	ligand binding (nmol/mg)				
	[^{3}H]NAB-ouabain		^{32}P		covalent labeling, fraction of [^{3}H]NAB-ouabain
expt	prebound	at phos	at phos	^{32}P/^{3}H	
A0622		1.08	1.03‡	0.95	0.21
A0802		1.06	0.68	0.64	0.21
A0906		0.64	0.49	0.76	0.17
A1108		0.91	0.54	0.59	0.21
B0615	1.26	0.77	0.79‡	1.04	0.27*
B0620	1.02	0.60	0.52‡	0.87	0.31*
B0808	0.79	0.44	0.39	0.89	0.35
B0815ϕ	0.29	0.10	0.10	1.0	0.51
B0824ϕ	0.33	0.16	0.17	1.06	0.46
B1114	0.93	0.51	0.40	0.78	0.27

[a] "Chains" experiments as described in text. Column 1: first letter of experiment number designates type A or type B experiment. Columns 2 and 3: [^{3}H]NAB-ouabain binding by Nucleopore filtration at the time of phosphorylation (3) and at the time of ouabain prebinding in type B experiments (2). Column 4: phosphorylated intermediate determined by trichloroacetic acid precipitation and Nucleopore filtration. Phosphorylation took place in the presence of Na except in experiments denoted with a ‡. Column 5: column 4/column 3. Column 6: ^{3}H label appearing at the position of the 95K chain on polyacrylamide gels as a fraction of total ligand binding at phosphorylation (column 3) except where covalent labeling is estimated as 0.54 times the amount of bound [^{3}H]NAB-ouabain stable to trichloroacetic acid (denoted by *). In experiments marked with an ϕ, NaI microsomes were used instead of purified enzyme.

The light used for photolysis of NAB-ouabain had little effect on phosphorylation as seen in Table I, line 3. Furthermore, when NAB-ouabain that was on the Na,K-ATPase inhibitory site was photolyzed, and 30–40% of the inhibitor became covalently bound, the amount of phosphorylation was unaffected (Table I, line 5). Thus, simultaneous phosphorylation and covalent [^{3}H]NAB-ouabain labeling of the same Na,K-ATPase functional unit were feasible.

The stoichiometry of ligand binding in the "chains" experiments is given in columns 2–5 of Table II. In order to eliminate nonspecific photolabeling, the total amount of [^{3}H]NAB-ouabain added was slightly less than the number of binding sites (see Methods); therefore, the amount of

Table III: Precipitation of Labeled 95 K Chains[a]

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	fraction of label precipitated by antibody							
	no additions		+ ouabain					
expt	[^{3}H]NAB-ouabain	^{32}P	[^{3}H]NAB-ouabain	^{32}P	fraction "precipitable" ^{32}P	covalent labeling, fraction of bound [^{3}H]NAB-ouabain	^{32}P/[^{3}H]NAB-ouabain "chains" samples	^{32}P/[^{3}H]NAB-ouabain control samples
A0622	0.85	0.15	0.01	0.003	0.18	0.21	0.86	0.004
A0802	0.77	0.15	0.012	0.005	0.19	0.21	0.90	0.001
A0906	0.82	0.12	0.016	0.004	0.15	0.17	0.88	
A1108	0.82	0.16	0.02	0.006	0.19	0.21	0.91	0.007
B0615	0.70	0.19	0.003	0.002	0.29	0.29	1.0	0.005
B0620	0.45	0.12	0.005	0.002	0.27	0.33	0.82	0.013
B0808	0.84	0.32	0.03	0.005	0.38	0.35	1.08	0.004
B0815	0.73	0.39	0.006	0.003	0.54	0.51	1.06	0.005
B0824	0.78	0.35	0.007	0.014	0.48	0.46	0.98	0.011
B1114	0.82	0.25	0.008	0.003	0.31	0.27	0.87	
							0.94 ± 0.09	0.006 ± 0.004

[a] Experiments described in text. Column 1: Experiments are those presented in Table II. Columns 2–5: fraction of ^{3}H and ^{32}P precipitated by antibody-protein A system. In columns 4 and 5, 10^{-4} M ouabain was included in the antibody incubation. Column 6: col 3/col 2. Column 7: From Table II, col 6. Column 8: col 6/col 7. Column 9: Data from mixed chain control samples, described in text. The result given here was calculated in the same way as the result in col 8, except that nonspecific ^{32}P precipitation (similar to col 5) was subtracted from specific ^{32}P precipitation (similar to col 3).

[^{3}H]NAB-ouabain bound, and thus of ^{32}P incorporated, was only 70–90% of the maximum levels attainable. In type B experiments, some noncovalently bound [^{3}H]NAB-ouabain was removed by dilution, incubation, and centrifugation prior to the phosphorylation step, and this is reflected in the difference between columns 2 and 3. Also note that the ratio of ^{32}P incorporated to [^{3}H]NAB-ouabain bound (column 5) was close to 1 in most experiments, confirming the previous reports of an equal number of sites for phosphate and ouabain (Hokin et al., 1973; Lane et al., 1973; Jorgensen, 1977). The lower level of phosphorylation in some experiments (particularly A0802, A1108) may reflect aging of the enzyme preparation; inactivation of the Na,K-ATPase results in more rapid loss of phosphorylation than ouabain binding (Erdman & Schoner, 1973; Jorgensen, 1977).

Photolabeling with [^{3}H]NAB-ouabain. When [^{3}H]-NAB-ouabain molecules bound to the Na,K-ATPase are photolyzed, the fraction that covalently label the 95K chains of the enzyme is about 15–20% (Table II, column 6, type A experiments), the remainder being comprised of 15–20% that covalently label the proteolipid and 60–70% that fail to react covalently with the Na,K-ATPase (Forbush et al., 1978). The fraction of the bound [^{3}H]NAB-ouabain that covalently labels the 95K chain is important because it represents the fraction of inhibited functional units that are sampled by antibody in the last step of the "chains experiments". As depicted qualitatively in Figure 2 (panels 3 and 4), since the functional units that were inhibited with [^{3}H]NAB-ouabain were also phosphorylated from $^{32}P_i$, an equal fraction (15–20%) of the phosphorylated chains will be separated by the antibody if model II (Chart I) is correct. Type B experiments were designed to increase this fraction by decreasing the amount of noncovalently bound [^{3}H]NAB-ouabain prior to phosphorylation: after photolabeling the Na,K-ATPase with [^{3}H]NAB-ouabain the enzyme was diluted and incubated, then centrifuged, resuspended, and phosphorylated. The resulting decrease in bound [^{3}H]NAB-ouabain was pointed out above (Table II, column 3); the effect on the ratio of covalent labeling of the 95K chain to total bound [^{3}H]NAB-ouabain is seen in the higher values for type B experiments in column 6. The dissociation step was particularly effective in experiments B0815 and B0824, where covalent label on the 95K chain is seen to represent ~50% of [^{3}H]NAB-ouabain remaining bound, most of the rest being attached to the proteolipid (data not shown).

Performance of Ouabain Antibody–Protein A System. The ouabain antibody used in this study was raised against ouabain that was conjugated to BSA through oxidation of the rhamnose of the ouabain molecule (Smith, 1972). Since the covalent linkage between [^{3}H]NAB-ouabain and Na,K-ATPase is also through a modification of the rhamnose, the antibody was expected to recognize [^{3}H]NAB-ouabain-labeled Na,K-ATPase after unfolding of the ouabain binding site by $NaDodSO_4$ solubilization. As shown in Table III, column 2, it was found that about 75–85% of the purified [^{3}H]NAB-ouabain-labeled 95K chains were precipitated by the antibody system after a 20-min incubation at 22 °C, pH 7.1. The effects of pH, time, and temperature on the precipitation method were investigated, since decreasing any of these three variables would lead to decreased hydrolysis of phosphorylated intermediate. It was found that 80% precipitation could be achieved at pH 5.8 (cf. Figure 3); at pH 5.0 only ~40% was precipitated (not shown). Little difference was noted between precipitation at 10 and 25 °C. Up to 0.2% $NaDodSO_4$ could be included in the antibody incubation medium without affecting efficiency as long as 0.3% BSA was also included; presumably BSA protects the antibody by binding $NaDodSO_4$ and keeping the free concentration low.

Nonspecific precipitation of labeled large chains in the presence of excess ouabain was very low, about 1% after two washes as seen in column 4 of Table III. In control experiments excess rabbit IgG was also found to prevent precipitation of labeled large chains by competition with ouabain antibody for protein A binding sites, shown in Table IV. It should be pointed out that utilization of protein A of *S. aureus* is markedly superior to double-antibody methods (cf. Skelley et al., 1973); in preliminary experiments by using double-antibody precipitation or a second antibody on an agarose support, we achieved only 50–60% precipitation after several hours of incubation, with nonspecific background precipitation of 5–15%.

^{32}P and [^{3}H]NAB-ouabain Are on the Same Chain. For the case of model I in Chart I, in which [^{32}P]- and [^{3}H]-NAB-ouabain are hypothesized to be on different large po-

Table IV: Antibody Precipitation of [^{3}H]NAB-Ouabain-Labeled Chains[a]

additions		
before incubation	after incubation	precipitation (% of total label)
		84.0 ± 1.0
no antibody		4.0 ± 0.3
ouabain		5.0 ± 0.9
IgG		6.9 ± 0.5
	ouabain	82.4 ± 1.4
	IgG	80.1 ± 0.2

[a] Mean result (± SD) from three experiments. Antibody–protein A precipitation as described under Methods, with the optional omission of antibody, addition of 10^{-5} M ouabain or 2 mg/mL rabbit IgG, before the incubation or immediately before the first centrifugation to pellet the *S. aureus*. Samples were from B0809 and B0815, stored at −20 °C for 3 months; the high background in these experiments (4–5%) may be related to storage, as it was not seen in the original "chains" experiments (Table III, col 3).

lypeptide chains in the functional unit, when the [^{3}H]-NAB-ouabain labeled chains are selected by antibody–protein A precipitation, no ^{32}P chains would be selected. For model II on the other hand, we expect that, with each [^{3}H]NAB-ouabain labeled chain precipitated, there would be an equal amount of ^{32}P. Since the fraction of chains with covalently bound [^{3}H]NAB-ouabain is only ~18% in the type A experiments and ~30–50% in type B experiments, a similar fraction of incorporated ^{32}P would be precipitated. Table III, column 3, shows that a significant percentage of ^{32}P was precipitated by the antibody–protein A, which is in support of model II of Scheme I. Column 5 shows that precipitation of ^{32}P is prevented by excess ouabain, so it is not simply due to nonspecific adsorption of ^{32}P-containing chains to the antibody–protein A complex.

If the fraction of [^{3}H]NAB-ouabain labeled chains precipitated is taken as the efficiency of the selection method (Table III, column 2, usually 70–85%), then the amount of ^{32}P precipitable at 100% efficiency can be extrapolated from the value in column 3 by dividing by the efficiency (column 2); the result is given in column 6. The extrapolation makes the assumption that the affinity of the antibody for a [^{3}H]-NAB-ouabain-labeled chain is not affected by whether or not the chain is phosphorylated. As an empirical test of the extrapolation, labeled, phosphorylated 95K polypeptide chains from experiment B1114 were incubated with antibody–protein A for different periods of time at pH 5.8 or 7.1 in the presence of different amounts of ouabain and the antibody–protein A complex was pelleted. The results in Figure 3 show that to a first approximation the ratio of ^{3}H to ^{32}P in the pellet is independent of the efficiency of precipitation.

A quantitative test of how well the data fit model II is obtained by comparing (a) the fraction of inhibited (and therefore phosphorylated) large chains which are covalently labeled with [^{3}H]NAB-ouabain and which are thus selected by the antibody–protein A system (Table III, column 7) and (b) the fraction of ^{32}P that is precipitable by antibody–protein A (Table III, column 6). The ratio of a to b is given in Table III, column 8, and is seen to be not different from 1.0 within experimental error. This represents conclusive evidence that [^{3}H]NAB-ouabain labels the same 95K chain that is phosphorylated from $^{32}P_i$.

Mixed Chain Controls. It is important to eliminate the possibility of aggregation of the large polypeptide chains after extraction from the polyacrylamide gel and partial removal of $NaDodSO_4$ by ultrafiltration; this would result in erroneous coprecipitation of ^{32}P and [^{3}H]NAB-ouabain by the antibody

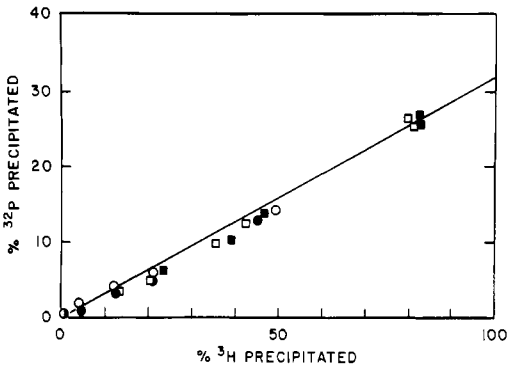

FIGURE 3: Precipitation of ^{32}P with [^{3}H]NAB-ouabain-labeled 95K chains. Aliquots of experimental sample B1114 and 10^{-4}, 10^{-6}, 3.8×10^{-7}, 2.3×10^{-7}, or 1.4×10^{-7} M ouabain were incubated for 20 min at room temperature with the antibody–protein A system as described under Methods (○, ●). Other aliquots were incubated with antibody without ouabain for 1, 2, 4, 6, and 20 min (□, ■). The amount of ^{3}H and ^{32}P in the pellet after centrifugation and two washes is plotted. (Solid symbols) Incubation at pH 7.1; (open symbols) incubation at pH 5.8. The straight line indicates the extrapolation for Table III, column 6, made through the points at 85% on the abscissa.

system. To test this, two kinds of mixed chain control samples were prepared and treated in parallel with the "chains" samples. In six experiments, separate aliquots of Na,K-ATPase were either phosphorylated from $^{32}P_i$ in the presence of cold ouabain *or* labeled with [^{3}H]NAB-ouabain in the presence of cold P_i; both aliquots were denatured with acid, and then mixed and solubilized with $NaDodSO_4$. In two other experiments (B0815, B0824), Na,K-ATPase that was labeled with [^{3}H]NAB-ouabain was phosphorylated from [γ-^{32}P]ATP in the presence of Na and Mg. Since ouabain prevents phosphorylation of Na,K-ATPase under these conditions (Post et al., 1975), only functional units from which [^{3}H]NAB-ouabain had dissociated could incorporate ^{32}P; therefore ^{32}P and [^{3}H]NAB-ouabain were on 95K chains of different functional units. As seen in column 9 of Table III, when 95K polypeptide chains of either type of sample described above were isolated and incubated with antibody, less than 1% of the ^{32}P was precipitated, although 30–85% of the [^{3}H]-NAB-ouabain labeled chains were bound to the antibody (not shown). Thus the antibody–protein A system is very effective at separating [^{3}H]NAB-ouabain labeled chains from unlabeled chains.

Stability of Phosphorylated 95K Chains. During the course of these experiments, the ^{32}P that was precipitable by antibody–protein A was examined to see if it had the expected sensitivity to high pH, temperature, and hydroxylamine that characterizes the acyl phosphate intermediate of the Na,K-ATPase (Hokin et al., 1965). Samples for "chains" experiments that had been stored at −20 °C for 24–48 h were thawed and incubated for various times and under various conditions, prior to dilution and assay with antibody–protein A. The half-time of loss of precipitable ^{32}P is given in Table V. As expected, the ^{32}P incorporated into 95K polypeptide chains is relatively unstable at pH >8.5, or at T = 37 °C, or in the presence of hydroxylamine. The rate of hydrolysis is almost constant between pH 5.0 and 7.5 (not shown), in agreement with the pH stability profile of $NaDodSO_4$ solubilized phosphorylated intermediate reported by Alexander & Rodnight (1974) and Steckhoven et al. (1976).

Discussion

The results in this paper support the conclusion that, when the Na,K-ATPase is simultaneously inhibited with ouabain

Table V: Stability of ^{32}P on 95K Chains

	half-time of hydrolysis (min)[a]		
	0–10 °C	20–22 °C	37 °C
pH 3.0–3.5	>600[b]	300–600[b]	40–50
pH 7.5–7.7	300–600[b]	60–180	30–40
pH 8.5–8.6[c]			14–18
+0.2 M hydroxylamine (pH 7.5)			2

[a] Aliquots (25–100 μL) of samples from "chains" experiments were adjusted to the desired pH with Tris, Hepes, or phosphate buffers (~20 mM final), incubated at the desired temperature for 0–4 h, readjusted to pH 7.1 by the addition of 0.9 mL of 110 mM NaCl, 3 mM EDTA, 30 mM Tris, 30 mM Hepes, and either immediately or after 1–4 h at −20 °C were incubated with antibody–protein A as described under Methods. The half-time for loss of precipitable ^{32}P is given; results from a number of experiments showed considerable variation and the range is given. [b] Values extrapolated from 5–20% hydrolysis at 1–4 h. [c] At pH 8.5, the zero-time samples, in which the pH was immediately readjusted, showed 30–60% hydrolysis regardless of temperature.

and phosphorylated from P_i, both ligands are on the same large polypeptide chain of the functional unit. The quantitative finding is that for each large polypeptide chain labeled with [^{3}H]NAB-ouabain and selected by the antibody system, a [^{32}P]phosphate is also selected. Use of the ouabain antibody for separation of labeled large chains has proved to be very effective, largely due to the *S. aureus* protein A technique.

The cardiac glycoside binding site of the Na,K-ATPase apparently consists of a primary region that recognizes the lactone ring and steroid portion of the inhibitors, and a secondary region that binds the sugar group(s) (cf. Yoda, 1974). It is possible, although unlikely, that the primary and secondary regions of the binding site are on opposite 95K chains of the dimer, and that a bound inhibitor molecule spans the two chains. Because the NAB group in NAB-ouabain is approximately at the position of the terminal sugar in a cardiac triglycoside, it is expected to photolabel the secondary region of the binding site (Forbush et al., 1978). Thus we conclude that the *secondary* region of the ouabain binding site is on the same 95K polypeptide chain as the ^{32}P from $^{32}P_i$. Within experimental error, our results do not support the hypothesis that the NAB group in the NAB-ouabain is free to label either of the two 95K chains, as then the ratio of precipitable ^{32}P to [^{3}H]NAB-ouabain covalent labeling (Table III, column 8) would be less than 1.0.

In terms of the present result, only a particular one of the two large chains may have binding sites for both P_i and ouabain; alternatively, and we feel more likely, during turnover of the enzyme the two chains may alternate in having both sites available first on one chain and then on the other. Thus while this investigation has answered the question posed in Chart I, at least for one case, it does not address the question as to whether the two 95 000 mol wt chains in a functional unit are functionally and structurally identical. Repke et al. (1974) and Stein et al. (1973) have discussed the implications of a Na,K-ATPase that is dimeric with regards to the large subunits in terms of a flip-flop mechanism, whereas Jorgensen (1977) has proposed that the two large subunits may be slightly different, one binding ATP and the other being phosphorylated.

The present investigation is a first step toward understanding the reaction mechanism and interactions of the 95K polypeptide chains of the Na,K-ATPase, as regards ligand binding sites. We are now pursuing the same question in the case of [^{3}H]NAB-ouabain binding and phosphorylation from [γ-^{32}P]ATP; as noted in the introduction, this is technically more difficult than ouabain binding and phosphorylation from $^{32}P_i$. Because the intermediates from $^{32}P_i$ and [γ-^{32}P]ATP appear to involve very different conformational states of the enzyme, it is possible that the alternative answer (Chart I, model I) will be obtained when this experiment is performed. The relationship of the ouabain binding site and the ATP binding site will also be examined, by using [^{14}C]-8-azido-ATP (Haley & Hoffman, 1974), as well as the sites of action of covalent group-specific modifying reagents.

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Manganese Electron Paramagnetic Resonance Studies of Sheep Kidney $(Na^+ + K^+)$-ATPase. Interactions of Substrates and Activators at a Single Mn^{2+} Binding Site†

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ABSTRACT: The interactions of Mn^{2+}, inorganic phosphate, and nucleotide substrate and substrate analogues with highly purified membrane-bound sodium plus potassium ion transport adenosine triphosphatase from sheep kidney medulla have been examined by using electron paramagnetic resonance techniques. EPR studies of both native and partially delipidated ATPase preparations indicate that the enzyme binds Mn^{2+} at one tight site with a K_D of 0.21×10^{-6} M. A second class of 24 ± 3 weaker binding sites for Mn^{2+} is also observed in the native enzyme, but these are removed upon removal of 50% of the essential phospholipids in the enzyme preparation, confirming that these are lipid binding sites for Mn^{2+} as had been previously suggested [Grisham, C., & Mildvan, A. (1974) *J. Biol. Chem. 249*, 3187]. The X-band EPR spectrum of the binary Mn^{2+}–ATPase complex exhibits a powder or quasi-solid state line shape consisting of a broad transition with partial resolution of the ^{55}Mn nuclear hyperfine structure, as well as a broad component to the low-field side of the main pattern. The spectrum of the delipidated enzyme is significantly broadened compared with the native enzyme, with a loss in resolution of the hyperfine lines. Low concentrations of phosphate produce a shift toward the center of the spectrum for the broad low-field component, with additional smaller effects on the hyperfine structure of the spectrum. ATP, ADP, AMP–PNP, and high concentrations of inorganic phosphate all broaden the hyperfine lines of the Mn^{2+} spectrum, consistent with a change in coordination geometry of the bound Mn^{2+}, a change in accessibility of the Mn^{2+} site to solvent, or both. These spectra strongly suggest that the true substrate for the enzyme is ATP and not Mn^{2+}–ATP. In contrast to the above effects, AMP causes a substantial narrowing of the Mn^{2+} spectrum, including the narrowing and eventual disappearance of the broad, low-field signal. These changes are consistent with a greatly reduced axial distortion of the Mn^{2+} ion geometry in the ternary ATPase–Mn^{2+}–AMP complex. Addition of phosphate to this complex provides a very slight broadening, while the further addition of Na^+ ion restores the broad, asymmetric spectrum observed in the binary ATPase–Mn^{2+} complex. By contrast, the addition of AMP to the delipidated enzyme–Mn^{2+} complex produces much different spectra, indicating unusual Mn^{2+} distortions and suggesting that lipid removal greatly alters the ability of the enzyme to interact with nucleotides. These results suggest that, while the divalent metal and substrate sites are preserved in the delipidated enzyme, their conformations are substantially altered.

Characterization of the structure and function of $(Na^+ + K^+)$-ATPase,[1] a plasma membrane bound enzyme responsible for Na^+ and K^+ transport in mammals, will depend on the development of specific spectroscopic probes. In this regard, use can be made of the monovalent and divalent cation requirements of the system as well as the substrate and inhibitor binding sites. Thus our previous studies have partially characterized a Mn^{2+} binding site on the enzyme which is responsible for the divalent cation activation of the enzyme and the transport system (Grisham & Mildvan, 1974, 1975; Grisham et al., 1974). Nuclear magnetic resonance studies which use the $^{205}Tl^+$, ^{31}P, and $^7Li^+$ nuclei have also shown that this Mn^{2+} site is very near (a) a Na^+ site which is probably involved in enzyme activation and ion transport (Grisham et al., 1974), (b) a K^+ site which has not previously been detected in kinetic studies (Grisham, 1978; Grisham & Hutton, 1978), and (c) a noncovalently binding phosphate site (Grisham & Mildvan, 1975) which had not been detected previously on this enzyme. Since the report of our NMR data on this phosphate site, kinetic (Froelich et al., 1976) and calorimetric (Kuriki et al., 1976) data have also been reported, which implicate a noncovalent enzyme–phosphate intermediate in the mechanism of the $(Na^+ + K^+)$-ATPase.

Electron paramagnetic resonance (EPR) spectra of the bound Mn^{2+} ion can provide additional insight into molecular motion and structure at the active site of enzyme–Mn^{2+} complexes. Spectra for macromolecular complexes of Mn^{2+} in solution typically resemble those for the ion in the solid state because of the slow rotational motion of the complex (Reed & Ray, 1971; Reed & Cohn, 1973). Magnetic anisotropies

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[1] Abbreviations used: $(Na^+ + K^+)$-ATPase, sodium and potassium ion transport adenosinetriphosphatase; Tes, *N*-tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid; TMA, tetramethylammonium; EPR, electron paramagnetic resonance; PRR, proton relaxation rate; P_i, inorganic phosphate.

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