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Non-Hydrated State of the Acyl Phosphate Group in the Phosphorylated Intermediate of (Na^+,K^+)-ATPase

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The position in the acyl phosphate linkage of the phosphorylated intermediate of (Na^+, K^+)-ATPase that is cleaved by *N*-methylhydroxylamine was compared with that of the model compound acetylphosphate. The products of the cleavage of the phosphoenzyme by methylhydroxylamine were the active enzyme and a N-P compound, not the inhibited enzyme and inorganic phosphate. This means that the bond cleaved by methylhydroxylamine was the O-P bond, not the C-O bond. In contrast, methylhydroxylamine did not cleave the O-P bond of acetylphosphate in solution, at pH values from 0.3 to 7.0, whether or not the phosphoryl group formed a complex with magnesium. Acetylphosphate and hydroxylamine formed acetohydroxamic acid. Therefore, the state of the acyl phosphate bond in the native phosphoenzyme and in acetylphosphate in solution was different, and the difference was not due to different dissociation states of their phosphoryl groups or the binding of magnesium to the phosphoenzyme. Molecular orbital calculations for acetylphosphate revealed that the phosphorus atom charge is more positive than the carbon atom, irrespective of the dissociation state of the phosphoryl group. Similarly, the overlapping electron population of the O-P bond is always smaller than that of the C-O bond. Thus, the electronic structure of the acyl phosphate linkage of acetylphosphate under vacuum supports the results obtained with the native phosphoenzyme, rather than those obtained with acetylphosphate in solution. The linkage in the active site of the phosphorylated intermediate of (Na^+,K^+)-ATPase appeared to be equivalent to the non-hydrated state of the model compound acetylphosphate. The phosphoenzyme with bound ouabain, or without a tightly bound divalent cation was insensitive to methylhydroxylamine. The native phosphoenzyme of (Ca^{2+})-ATPase was not susceptible to methylhydroxylamine.

Key words: *ab initio* molecular orbital calculation, acyl phosphate bond, hydroxylamine, (Na^+,K^+)-ATPase, nucleophilic substitution.

The active transport of cations by (Na^+,K^+)-ATPase in cell membranes is thought to have a mechanical essence in the action of the phosphate group incorporated into the enzyme by ATP (*1, 2*). Passive movement of Na^+ or K^+ occurs independently of ATP, through the specific channel formed in the enzyme having two alternative conformations designated E_1 and E_2. The channel's conformations E_1 and E_2 favor Na^+ and K^+, respectively. Movement of Na^+ out of the cell against the electrochemical potential gradient requires phosphorylation of the Asp-369 residue of (Na^+, K^+)-ATPase by the transphosphorylation of the terminal phosphate group of ATP. This phosphoenzyme is designated E_1P, and its conversion to E_2P corresponds to the physiological translocation of Na^+ (*3-6*). Translocation of K^+ into the interior of the cell against the electrochemical potential gradient proceeds by the saturation of the nonhydrolyzing ATP binding site of (Na^+,K^+)-ATPase with ATP (*3-6*).

Thus it is the incorporation of a phosphate group, either covalently or noncovalently, into the enzyme molecule that is a common factor in these two elementary processes of active cation transport. The question of how the phosphate group works within the enzyme molecule must be answered in order to understand this essential mechanism of active transport *via* (Na^+,K^+)-ATPase or, more generally, of P-type ATPases.

The present experiments deal with the rather old question about the chemical nature of the covalently bound phosphate group. Until the early 1970s, it was controversial whether or not hydroxylamine inhibited (Na^+,K^+)-ATPase by attacking the carbonyl carbon atom of the phosphoryl aspartate residue to give its hydroxamic acid derivative (*7-9*). It was shown consistently that the carbon atom was the nucleophilic center by organic chemistry experiments (*10, 11*) using acid-denatured phosphoenzyme (*12*).

We compared the reaction modes of hydroxylamine acting on the active state phosphoenzyme of (Na^+,K^+)-ATPase and acetylphosphate (AcP), which is the classic model compound for the acyl phosphate linkage, C(=O)-O-

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Abbreviations: CDTA, 1,2-cyclohexylenedinitrilotetraacetic acid; AcP, acetylphosphate.

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P, of P-type ATPases (*7, 12-14*). We found that the nucleophilic center appeared to be the phosphorus atom in the active phosphoenzymes, whereas it appeared to be the carbon atom in AcP in solution. *Ab initio* molecular orbital calculations of AcP under vacuum without interactions with other molecules such as water, suggested that O-P bond cleavage is more likely than C-O bond cleavage.

These results led us to conclude that the state of the acyl phosphate group of the phosphorylated Asp-369 of (Na^+, K^+)-ATPase is analogous to the non-hydrated acyl phosphate linkage of AcP.

EXPERIMENTAL PROCEDURES

Preparation of Membrane-Bound ATPases—The membrane-bound (Na^+,K^+)-ATPase, supplied by Dr. Yutaro Hayashi, was prepared from dog kidney outer medulla microsomes by SDS treatment and sucrose density gradient centrifugation (*15, 16*). The preparation was stored at the temperature of an ice bath in the presence of 1 mM EDTA adjusted to pH 7.2 with imidazole. The specific activity of the ouabain-sensitive ATP hydrolysis was 5.9 to 30.7 μmol P_i/min/mg of protein, in the presence of 2 mM ATP·Na_2, 2 mM $MgCl_2$, 100 mM NaCl, 15 mM KCl, 0.5 mM EGTA, and 20 mM histidine (free form) at pH 7.3 and 37°C (*17*).

The membrane-bound (Ca^{2+})-ATPase was purified by sodium deoxycholate treatment (*18*) from rabbit skeletal muscle microsomes. The microsomes were a gift from Dr. Taibo Yamamoto. The purified enzyme was stored at −85°C, in the presence of 0.3 M sucrose, 50 mM KCl, and 10 mM histidine (free form, pH 7.2). The specific activity of the Ca^{2+}-dependent ATPase was 4.3 to 7.0 μmol P_i/min/mg of protein, in the presence of 4 mM ATP·Na_2, 4 mM $MgCl_2$, 0.6 mM $CaCl_2$, 0.1 M KCl, 0.5 mM EGTA, and 20 mM histidine (free form) at pH 7.2, and 37°C (*17*).

Determination of ATPase Activity—The ATPase reaction was performed in 1 ml of reaction mixture under optimum conditions as described above in "*Preparation of Membrane-Bound ATPases.*" The reaction was stopped by the addition of 0.1 ml of 20% SDS. Molybdate reagent (2.5 ml) was added, followed by the addition of 0.1 ml of the reducing reagent at 30°C, according to the method of Fiske and SubbaRow (*19, 20*). The total amount of P_i in the test tube was thus determined directly.

Phosphorylation and Dephosphorylation—The phosphorylation of (Na^+,K^+)-ATPase (0.01 mg) was carried out at approximately 0°C, in 1 ml of reaction mixture containing 20 μM [^{32}P]ATP·Na_2, 0.2 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM histidine (pH 7.3) (*17*). In order for the formation of sodium-occluding E_1P to predominate, 2 M NaCl was added to the reaction. When 1 mM nonradioactive ATP containing 1 mM ADP was added, 80% of the phosphoenzyme disappeared within 2 s. For the predominant formation of sodium-deoccluding E_2P, 0.1 M NaCl was added. Under these conditions more than 95% of the phosphoenzyme disappeared within 2 s by adding 1 mM nonradioactive ATP containing 15 mM KCl. The amount of the phosphoenzyme at steady state was 1.1 to 1.4 nmol/mg of protein. These levels were taken as the 100% value in each experiment, and the relative amounts of the phosphoenzyme are indicated in the figures.

The phosphorylation of (Ca^{2+})-ATPase (0.02 mg) was similar to that of (Na^+,K^+)-ATPase but carried out in the absence of NaCl and by replacing the sodium salt of ATP with the Tris salt. For the predominant formation of calcium-occluding E_1P, 1.5 mM $CaCl_2$, and 0.1 M KCl were present. Calcium-deoccluding E_2P was formed by the inclusion of 7 μM free Ca^{2+} using a $CaCl_2$/EGTA buffer system, and in the absence of KCl. The amount of the steady state phosphoenzyme was 1.7 to 4.3 nmol/mg of protein.

To follow the phosphoenzyme degradation, the radiolabeling was chased at 60 s in the case of E_1P and 40 s in the case of E_2P, by adding 0.05 ml of unlabeled ATP (21 mM) giving a 50-fold dilution of the radioactivity, or by adding 1 ml of the above-mentioned phosphorylation solution but replacing $MgCl_2$ with 10 mM CDTA to remove the free Mg^{2+} which is an absolute requirement of transphosphorylation. The reaction was stopped by adding 10 ml of ice cold 10% perchloric acid containing 0.1 M P_i and 10 mM unlabeled ATP. The latter was added to the perchloric acid solution just before use.

The radiolabeled phosphoenzyme was collected on a Millipore filter (0.45 μm pore size), and washed four times with 15 ml of 5% perchloric acid solution containing 0.1 M P_i. The Cherenkov radiation of the filter was counted to estimate the amount of phosphoenzyme.

Determination of the Amount of Acetohydroxamic Acid—According to the method of Lipmann and Tuttle (*21*), 3 ml of 10% $FeCl_3$ dissolved in 1 M HCl was added to 2 ml of the sample solution at room temperature, and the absorbance of Fe·acetohydroxamate complex at 540 nm was measured immediately. When 2 mM AcP and 1 M NH_2OH were incubated for 15 min at 37°C, pH 7.0, about 91% of the AcP was detected as acetohydroxamic acid by this method, confirming that the nucleophilic center of AcP in this reaction was the carbon atom at neutral pH (*10-12*).

Detection of N-P Compound by ^{31}P-NMR—(Na^+,K^+)-ATPase (1 mg/ml) was allowed to turnover *via* the intermediary phosphorylation and dephosphorylation by 1 mM ATP·Na_2 in the presence of 2 M or 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, and 20 mM histidine, at pH 7.3 and 37°C. After 1 min, 0.1 M CH_3NHOH was added, and the incubation was continued for several minutes during which all the ATP was hydrolyzed. To avoid the possible breakdown of unstable chemical products, the sample (4 ml) was quickly poured into a NMR sample tube (9 mm diameter) precooled to 4°C. The ^{31}P-NMR spectra were recorded at 161.7 MHz in a JEOL GSX-400 NMR spectrophotometer at 4°C. The chemical shift of P_i (85%) was determined in a separate measurement and was set to 0 ppm in all spectral data.

Ab Initio Calculations—The molecular orbitals of AcP were calculated at the STO-3G level using the IMSPACK program (IMS version of Gaussian 70) at the Computer Center of the Institute for Molecular Sciences, Okazaki. The initial geometry of the phosphoryl group was cited from the structural data of crystalline ATP (*22*), and the constructed geometry of AcP was optimized by energy minimization.

Hydroxylamine and Methylhydroxylamine—HCl salt of NH_2OH was purchased from Wako Pure Chemical Industries (Osaka). HCl salt of CH_3NHOH was purchased from Nacalai Tesque (Kyoto). They were used without further purification, since NH_2OH recrystalized from methanol did

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not change the action of phosphoenzymes in preliminary experiments. In one analysis of 2 M NH_2OH solution by ion chromatography, the contaminant NH_4^+, which can stimulate E_2P degradation by substituting for K^+, was present at 68.2 μM. According to the experiments of Fedosova *et al.* (*23*) when the enzyme is reconstituted into liposomes, CH_3NHOH does not act on the phosphoenzyme at the surface where K^+ acts, but is only effective on the opposite side, that is the same side the phosphoryl group is incorporated into the enzyme.

These solutions were adjusted to pH 7.0 with NaOH. NaCl was always added in control runs to give the same final Na^+ concentration. In experiments with (Ca^{2+})-ATPase, NaOH, and NaCl were replaced with Tris and Tris·HCl, respectively.

RESULTS

Cleavage of the Phosphoenzyme by Hydroxylamine—To confirm and also to compare the reactivity of NH_2OH with the two phosphoenzymes, E_1P and E_2P of (Na^+,K^+)-ATPase, the enzyme was labeled with 20 μM [^{32}P]ATP, in the presence of 2 M NaCl or in the presence of 0.1 M NaCl.

After the labeling of the enzyme was stopped by the addition of a 50-fold excess of nonradioactive ATP, 0.1 M NH_2OH was added to each of the phosphoenzymes. This accelerated the dephosphorylation of E_1P (Fig. 1A) and more effectively accelerated the dephosphorylation of E_2P (Fig. 1B). These results did not change in the presence of 5 mM CDTA added to chelate free Mg^{2+} (not shown), indicating that the reaction of the phosphoenzyme with NH_2OH did not require any additional divalent cations. Since the phosphorylation reaction mixture always contained 0.5 mM EGTA, the argument that metal ions such as Cu^{2+} or Ca^{2+} participate the action of NH_2OH on the enzyme is not relevant (*9*, see also Refs. *8* and *24*).

The *N*-methyl derivative of NH_2OH was more reactive than NH_2OH with both phosphoenzymes (Fig. 1). Since this was also the case for the acid-denatured phosphoenzyme (not shown), the increased reactivity of CH_3NHOH did not reflect greater accessibility to the enzyme's active site due to increased hydrophobicity of CH_3NHOH. This was probably a reflection of the stronger nucleophilicity of CH_3NHOH compared to the NH_2OH. The stronger nucleophile, CH_3NHOH, was used throughout this work.

No Irreversible Inhibition by Hydroxylamine—If cleavage of the phosphoenzyme (Fig. 1) proceeds by a substitution reaction of the nucleophilic attack by NH_2OH at the carbon atom in the acyl phosphate linkage at Asp-369 to release P_i, the residue should be derivatized as the hydroxamic acid, and the resulting enzyme should be irreversibly inhibited, since the derivative is stable (*10*) and the residue is essential for the ATPase activity (*1-7, 13, 14, 24*). Tacitly based on this mechanism, NH_2OH sensitivity has been repeatedly used to identify the phosphorylated intermediate of P-type ATPase after acid denaturation (*12-14*).

To test this substitution mechanism in the native form of the phosphoenzyme, the enzyme was cycled through phosphorylation and dephosphorylation in the presence of 0.1 M CH_3NHOH at 37°C and neutral pH, for periods of 1-60 min to allow all the enzyme molecules to interact with CH_3NHOH. The steady state form of the dominant phosphoenzyme during this incubation period was controlled by the concentrations of monovalent cations. In the presence of 2 M NaCl with 10 mM KCl, E_1P was the predominant phosphoenzyme, and in the presence of 0.1 M NaCl without KCl, E_2P was the predominant phosphoenzyme (*3-6*). After this cycling, the enzyme was washed to remove CH_3NHOH. Then the ATPase activity of the recovered enzyme was measured under optimum conditions. No inhibition of ATPase activity was found regardless of the main conformation of the phosphoenzyme during the CH_3NHOH treatment at neutral pH (Fig. 2, A and B). Preincubation with CH_3NHOH at pH 4.4 or 10 decreased the activity to about 40 or 60% respectively of that in the absence of CH_3NHOH (not shown), probably due to breaks in the peptide bonds (*25*).

Together with the results shown in Fig. 1, these results indicate that the peptide bonds of the enzyme are stable in the presence of 0.1 M CH_3NHOH at neutral pH, but that the acyl phosphate bond is unstable. Namely, CH_3NHOH cleaves the phosphoenzyme without the formation of an

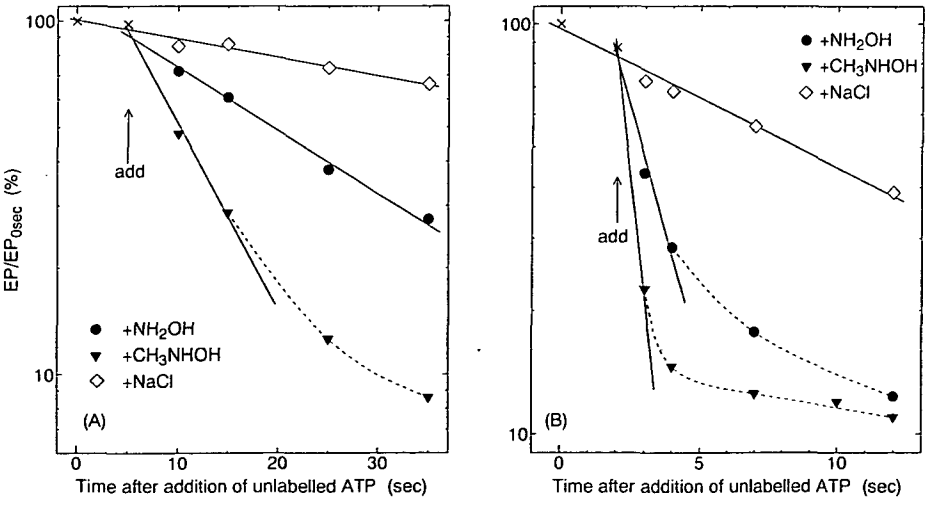

Fig. 1. **Acceleration of phosphoenzyme cleavage by hydroxylamines.** (Na^+,K^+)-ATPase (10 μg) was phosphorylated at 0°C and pH 7.3 in 0.95 ml of solution containing 20 μmol histidine (free form), 0.5 μmol EGTA, 0.2 μmol $MgCl_2$, and 2 mmol (A) or 100 μmol (B) NaCl. The phosphorylation was started by the addition of 0.05 ml containing 20 nmol [γ-^{32}P]ATP at 60 s (A) or 40 s (B) before zero time. At zero time, further formation of [^{32}P]phosphoenzyme was interrupted by the addition of 1.05 μmol unlabeled ATP (×) in 0.05 ml volume. At 5 s (A) or 2 s (B) as indicated by the arrows, 0.05 ml containing 100 μmol NH_2OH (●), CH_3NHOH (▼), or NaCl (◇) was added. The reaction was terminated by the addition of 10 ml of 10% $HClO_4$ containing 0.1 M P_i and 10 mM unlabeled ATP at the times indicated. Data points are the averages of the duplicate determinations, and are given as values normalized against 100% phosphoenzyme at zero time.

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inhibited form, such as a hydroxamic acid derivative of the essential aspartate residue, regardless of the conformation of the phosphoenzyme. Possibly, CH_3NHOH does not attack the carbon atom, but attacks the phosphorus atom in the acyl phosphate linkage (*24*). If this is so, a N-P compound should be released to reproduce the active enzyme.

When the ATPase reaction was performed with 0.1 M CH_3NHOH or NH_2OH in the presence of 2 M NaCl and no KCl, the production of P_i increased by about 1.3- to 1.8-fold (not shown). Most likely, nonenzymatic release of P_i from the phosphoenzyme (Fig. 1), upon its return to the initial free state (Fig. 2), coincided the enzymatic release of P_i (see also below Fig. 3).

Trial for Detection of the N-P Product—During the course of ATP hydrolysis experiments using the Fiske-SubbaRow method (*19, 20*), it was noticed that the color development of the P_i-molybdate complex gradually increased for a few hours or so after the reducing reagent was added, but only in the test tubes containing CH_3NHOH. As CH_3NHOH did not directly affect the reducing reaction, it was hypothesized that additional labile N-P compound (*19*) formed from the phosphoenzyme and CH_3NHOH had gradually dissociated to produce P_i during the reductive reaction under acidic pH.

To test this assumption, the enzyme was cycled through phosphorylation and dephosphorylation for 21 h at 2°C, to accumulate a large amount of the putative N-P compound, in the presence of 0.1 M CH_3NHOH and 2 M NaCl so that main form of the steady state phosphoenzyme was E_1P (*cf.* Fig. 1A). After stopping the ATPase reaction, the Fiske-SubbaRow reaction was initiated at room temperature. The color development continued for 2 h (Fig. 3). Thus we interpreted this phenomenon as indirect evidence for the formation of the N-P compound from E_1P and CH_3NHOH. This phenomenon was not seen in the presence of 0.1 M NaCl, where E_2P is the main phosphoenzyme, despite the expectation that a larger amount of N-P compound would accumulate (not shown, *cf.* Fig. 1B). Stability of the N-P bond depends on ionic strength (*26*).

To detect the N-P compound directly, the reaction mixture of the Na^+-ATPase in the absence of K^+ was analyzed by ^{31}P-NMR. The reaction was initiated by 1 mM ATP at 37°C, and after 1 min, 0.1 M CH_3NHOH was added and the reaction was continued for a further 5 min. After cooling to 4°C, ^{31}P-NMR spectra recording was initiated. The chemical shift corresponding to P_i was found at +3.198 ppm; it was increased by the externally added P_i. The unique chemical shift appeared at +6.608 ppm only when CH_3NHOH was added in the presence of 2 M NaCl (Fig. 4). We attributed this peak to the N-P compound; according to the literature the value of the ^{31}P-NMR chemical shift of phosphoramidate is +3.03 ppm relative to +0.13 ppm for P_i (*27*). We did not detect this resonance peak when the experiment was repeated at a lower ionic strength, 0.1 M NaCl (not shown).

Under these conditions, ATP was almost completely hydrolyzed before cooling, and only the α and β phosphate of ADP, and occasionally a small peak of β phosphate of the remaining ATP, were observed in the high magnetic field region. Neither the ATP solution nor the enzyme suspen-

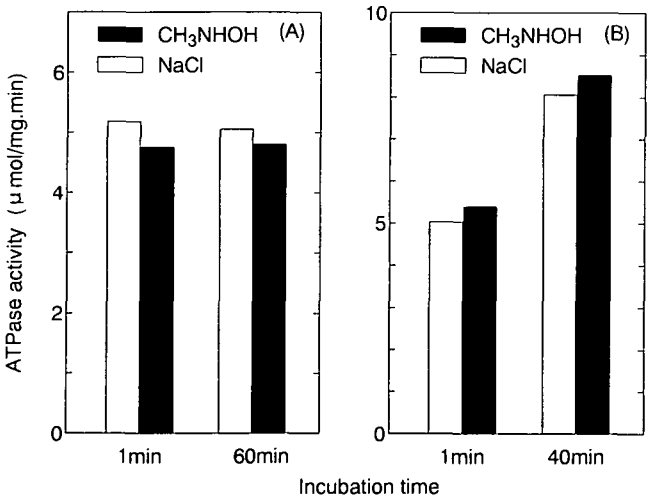

Fig. 2. **No inhibition of the (Na^+,K^+)-ATPase activity after cycling through phosphorylated intermediate in the presence of methylhydroxylamine.** Turnover of the reaction cycle of (Na^+, K^+)-ATPase (0.1 mg) was allowed at 37°C and pH 7.3 in 3 ml of solution containing 60 μmol histidine, 1.5 μmol EGTA, 12 μmol $MgCl_2$, 12 μmol ATP, and 6 mmol NaCl and 30 μmol KCl (A) or 300 μmol NaCl (B) in the presence of 300 μmol CH_3NHOH or 300 μmol NaCl. After 1, 40, or 60 min, the reaction mixtures were centrifuged, and the recovered enzyme was washed three times with a solution of 20 mM histidine, 0.5 mM EGTA, 4 mM $MgCl_2$, 100 mM NaCl, and 15 mM KCl, and resuspended in the same solution. In the same solution at 37°C and pH 7.3, the ATPase activity of CH_3NHOH-treated enzyme (closed column) or NaCl-treated enzyme (open column) was determined by the addition of the enzyme suspension to make a total volume of 1 ml. After 10 min, the reaction was terminated by the addition of 0.1 ml of 20% SDS, and the amount of P_i was determined (*19, 20*). Data are the averages of duplicate determinations. (The reason for the discrepancy between the specific activity at 1 min and that at 40 min in (B) is not clear. The maximum difference among 8 data sets in experiment (B) was 4.1 μmol/mg·min.)

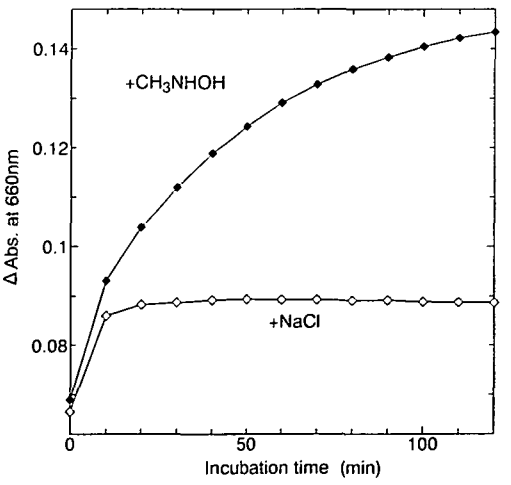

Fig. 3. **Formation of a labile N-P compound produced from (Na^+,K^+)-ATPase and methylhydroxylamine.** (Na^+,K^+)-ATPase (20 μg) was incubated with 0.1 mmol CH_3NHOH (◆) or with 0.1 mmol NaCl (◇) at 2°C and pH 7.3, in 1 ml of solution containing 20 μmol histidine, 0.5 μmol EGTA, 2 mmol NaCl, 0.2 μmol $MgCl_2$, and 0.2 μmol ATP. After 21 h, the reaction was terminated by adding 0.1 ml of 20% SDS, and the Fiske-SabbaRow procedure (*19, 20*) to detect P_i was initiated at room temperature. Absorbance was measured at 660 nm. The blank readings (0.06 at zero time and 0.13 at 120 min) were obtained in the absence of enzyme and subtracted from all data points.

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sion contained any contaminating phosphate compounds, whereas an unexpected resonance peak always appeared at +4.375 ppm, independent of the concentration of NaCl even in the absence of CH_3NHOH (Fig. 4). This resonance peak seemed to correspond to AMP, since the peak height increased by the externally added AMP (not shown). Even though this enzyme preparation is treated with SDS (*15, 16*), the possibility of contamination with soluble adenylate kinase cannot be ruled out. More likely, the preparation may contain membrane-bound ecto-ATPases (*28*), but this remains to be confined.

We also tried to separate the N-P compound by anion analysis chromatography using a TSKgelIC-Anion-SW column (Tosoh, Tokyo), similar to the method of Stelte and Witzel (*29*). They isolated *N*-phosphohydroxylamine, formed from the intermediate of nucleoside phosphotransferase of carrots and NH_2OH, using Dowex resin according to the method of Jencks *et al.* (*30*). In one experiment, a unique peak specific to the CH_3NHOH treatment was observed before that of P_i on the chromatogram, however, the observation was not consistent, probably because of poor separation of the N-P compound from P_i in our system.

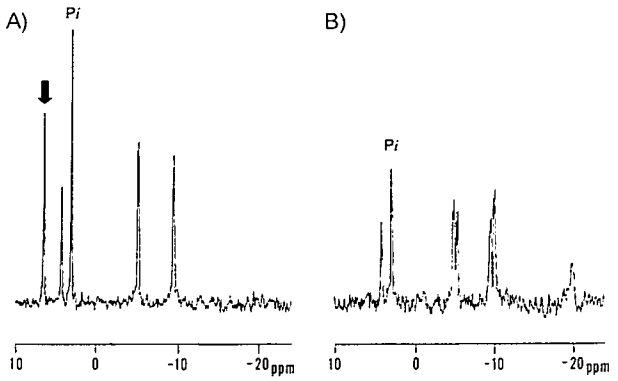

Fig. 4. **Detection of putative N-P compound by ^{31}P-NMR spectroscopy.** Phosphorylation of (Na^+,K^+)-ATPase (5 mg) was initiated by adding 5 μmol ATP to a 5 ml reaction mixture containing 100 μmol histidine, 2.5 μmol EGTA, 7.5 μmol $MgCl_2$, 10 mmol NaCl, at 37°C and pH 7.3. One minute after addition of ATP to the reaction mixture, 0.5 mmol CH_3NHOH (A) or 0.5 mmol NaCl (B) was added. The reaction was incubated for 5 min, then quenched by cooling to 4°C, and ^{31}P-NMR spectra were recorded at 161.7 MHz. Zero ppm corresponds to the chemical shift of 85% P_i determined in a separate measurement. The chemical shift that was specific to the incubation with CH_3NHOH is indicated by the arrow (A; ↓). The chemical shift of P_i in these conditions is indicated as P_i.

TABLE I. **Acetohydroxamic acid formation from acetylphosphate and hydroxylamine in a wide range of pH.** AcP (2 μmol) was incubated with (+) or without (−) 1 mmol NH_2OH at 37°C in 1 ml of a solution of either 0.5 mmol HCl, 0.1 mmol HCl, 20 μmol Gly-HCl (pH 3.0), or 20 μmol histidine-HCl (pH 7.0). The incubations without NH_2OH were for the correction of the decrease in AcP due to its hydrolysis. After 15 min, all solutions were adjusted to neutral pH (by adding NaOH), to a 2 mmol of NH_2OH (by readdition), and to a 2 ml total volume. The incubation was continued for a further 15 min at 37°C to complete acetohydroxamic acid formation (*10-12*). Then, 3 ml of 10% $FeCl_3$ dissolved in 1 M HCl was added, and the amount of Fe·acetohydroxamate complex was determined from the absorbance at 540 nm (*21*).

pH	The amount of hydroxamic acid formed (μmol)		Recovery as hydroxamic acid (relative, %)
	$+NH_2OH$	$-NH_2OH$	
0.3	1.35	1.32	102
1	1.57	1.54	102
3	1.78	1.73	103
7	1.86	1.81	103

Reaction of Acetylphosphate with Hydroxylamine—AcP has been regarded as a model compound for the acyl phosphate linkage of the phosphoenzyme of P-type ATPase, and it is generally accepted that AcP reacts with NH_2OH to produce hydroxamic acid, at least at neutral pH (*10-12*).

As CH_3NHOH treatment gave no indication of the formation of hydroxamic acid at the active site (Fig. 2), we ourselves examined whether the product of the reaction between AcP and NH_2OH is hydroxamic acid. Under various pH conditions from pH 0.3 to pH 7.0, which covers the pK_a values (1.2 and 4.8) of AcP, 2 mM AcP and 1 M NH_2OH were incubated at 37°C. To estimate the reduction in the amount of AcP due to hydrolysis, incubations without NH_2OH were carried out in parallel at each pH. After 15 min, all the samples including those without NH_2OH were adjusted to neutral pH, and the concentrations of NH_2OH

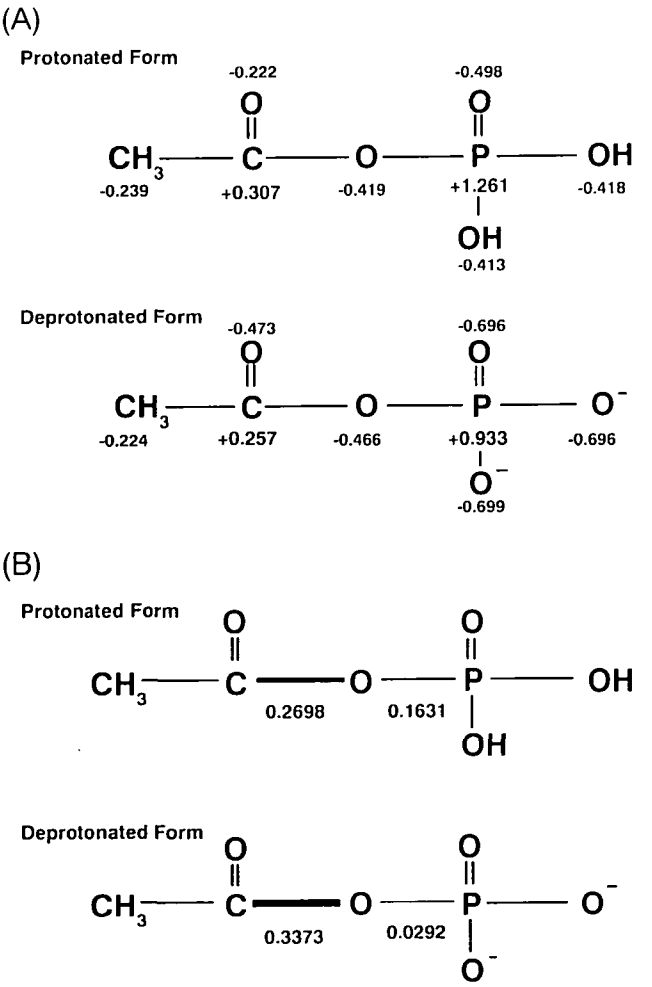

Fig. 5. **Electronic structure of acetylphosphate in the state of protonation or deprotonation.** (A) Net atomic charge. (B) Overlap population. This figure is reproduced from Ref. 22.

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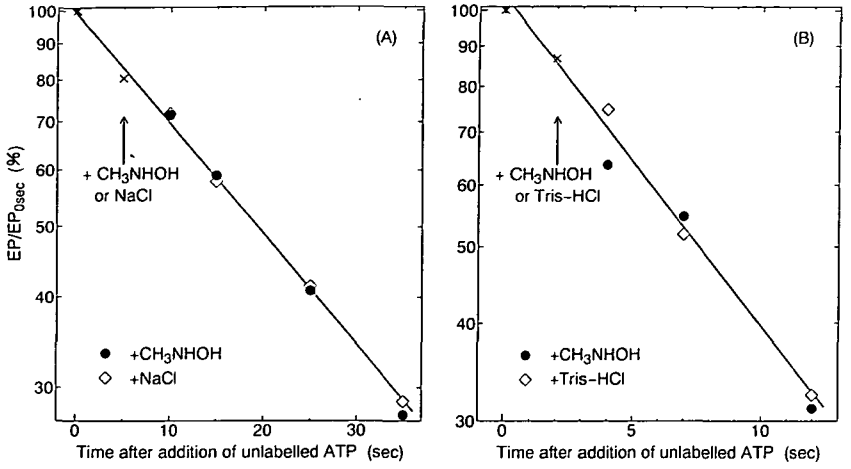

Fig. 8. **Non-susceptibility of the phosphoenzyme of (Ca^{2+})-ATPase to methylhydroxylamine.** (Ca^{2+})-ATPase (10 μg) of the sarcoplasmic reticulum from rabbit skeletal muscle was phosphorylated in a 0.95 ml solution as described in the legend to Fig. 1, with the omission of NaCl and the addition of 1.5 μmol $CaCl_2$ plus 0.1 mmol KCl (A) or the addition of 0.502 μmol $CaCl_2$ (B). The phosphorylation was started by adding 0.05 ml of 20 nmol [γ-^{32}P]ATP 60 s (A) or 40 s (B) before zero time. At zero time, further formation of [^{32}P]phosphoenzyme was interrupted by addition of 0.1 ml containing 1.1 μmol unlabeled ATP (×). At 5 s (A) or 2 s (B) as indicated by the arrows, 0.1 ml containing 2 mmol CH_3NHOH (●), 2 mmol NaCl (A; ◇), or 2 mmol Tris·HCl (B; ◇) was added. Data points are the averages of duplicate determinations.

Values for the overlapping electron population in the C-O and the O-P bonds became larger and smaller respectively after deprotonation of the phosphate group (Fig. 5B). In any case, the relative strength of the O-P chemical bond is weaker than that of the C-O bond. Again, cleavage of the O-P bond appeared to be more likely, as was the case in the native phosphoenzymes (Fig. 2), rather than cleavage of the C-O bond, as was the case in AcP in solution (Table I).

Thus the results of the molecular orbital calculations under vacuum strongly reflect the environment surrounding the acyl phosphate group in the native phosphoenzymes rather than the aqueous environment of AcP in solution.

Insensitivity of Ouabain-Inhibited Phosphoenzyme to Hydroxylamine—All the enzymatic reactivity is lost in the ouabain-inhibited E_2P, including the reaction with the enzymatic substrate H_2O. The extreme stability of ouabain-inhibited E_2P may be due to complete isolation of the active site from a substance access from the bulk phase. This idea was tested by adding 0.1 M CH_3NHOH to E_2P in the presence of 0.5 mM ouabain. To eliminate residual ouabain-free E_2P, 15 mM KCl was added to the reaction prior to CH_3NHOH (Fig. 6). Ouabain-inhibited E_2P was completely insensitive to CH_3NHOH, in contrast to the active state of E_2P (*cf.* Fig. 1B).

Ouabain may completely isolate the acyl phosphate group from the exterior of the enzyme molecule, however, non-susceptibility to CH_3NHOH was not specific to the inhibited phosphoenzyme but was also the case for the active form of the phosphoenzyme in the sarcoplasmic reticulum (Ca^{2+})-ATPase (see below "*Comparison with Sarcoplasmic Reticulum (Ca^{2+})-ATPase*" and Fig. 8).

Insensitivity of Divalent Cation-Free Phosphoenzyme to Hydroxylamine—Another inactive form of the phosphoenzyme is the form that is free of the bound divalent cation (*31*). To investigate its reactivity with NH_2OH, we phosphorylated the enzyme with ATP by replacing $MgCl_2$ with $CaCl_2$, then stabilized the phosphoenzyme by adding an excess amount of EGTA plus unlabeled ATP, according to the method of Fukushima and Post (*31*). The enzyme was phosphorylated in the presence of 2 M NaCl since calcium-free E_1P is easier to prepare than calcium-free E_2P (*32*). ADP was added to remove any residual active E_1P. About 80% of the phosphoenzyme was stabilized (Fig. 7).

Like the ouabain-inhibited phosphoenzyme, metal-free phosphoenzyme was entirely unreactive with NH_2OH. The reactivity with NH_2OH as well as with ADP was restored by adding $MgCl_2$ to reconstruct magnesium-bound E_1P (Fig. 7).

Comparison with Sarcoplasmic Reticulum (Ca^{2+})-ATPase—To generalize the chemical state of the acyl phosphate linkage in the phosphoenzyme among P-type ATPases, we studied the reactivity with CH_3NHOH of the (Ca^{2+})-ATPase phosphoenzyme from skeletal muscle sarcoplasmic reticulum.

Active phosphoenzymes of the (Ca^{2+})-ATPase, E_1P and E_2P, were formed under the same conditions, as for the (Na^+,K^+)-ATPase experiments, except that NaCl was omitted and 1.0 mM free Ca^{2+} with 0.1 M KCl or 7 μM free Ca^{2+} without KCl were included.

Contrary to our expectations, there was no acceleration of the hydrolysis of the active phosphoenzymes, either E_1P or E_2P, when 0.1 M CH_3NHOH was added after a chase with unlabeled ATP (Fig. 8). In contrast, (Na^+,K^+)-ATPase phosphoenzyme was unreactive with CH_3NHOH or NH_2OH only in its inhibited form (Figs. 6 and 7). This unreactivity of the active phosphoenzyme may be a unique feature of (Ca^{2+})-ATPase.

DISCUSSION

By using the nonphysiological nucleophile, hydroxylamine or its *N*-methyl derivative, these experiments revealed the chemical nature of the acyl phosphate linkage in the active site of (Na^+,K^+)-ATPase. With respect to the enzymatic E_2P hydrolysis of P-type ATPase, it has previously been shown, by means of the exchange reaction in the presence of [^{18}O]H_2O, that the nucleophilic center of the acyl phosphate linkage is the phosphorus atom (*33-35*).

Acyl Phosphate Linkage in the Active Enzyme versus the Model Compound or Denatured Enzyme—Initially, these experiments were designed to find different reaction modes of hydroxylamine between the two principal phosphoenzymes, before (E_1P) and after (E_2P) release the occluding sodium. If either of the two phosphoenzyme phosphate groups could accept covalent replacement with hydroxylamine, then Asp-369 could be specifically labeled with ^{15}N-enriched NH_2OH to allow NMR monitering of the behavior of the residue; however, these experiments

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demonstrated that it is not the carbon atom but rather the phosphorus atom of the acyl phosphate group of the phosphoenzyme that is attacked by hydroxylamine (Fig. 1) to produce free enzyme (Fig. 2) and a N-P compound (Figs. 3 and 4). The acyl phosphate linkage in solution (AcP) or in homogeneous suspension (denatured phosphoenzyme), on the other hand, always reacted with hydroxylamine at the carbon atom to form hydroxamic acid derivatives with release of P_i (Table I, and Refs. *12-14*).

These different reaction modes reflect the specific environment surrounding the acyl phosphate linkage in the active site of the enzyme. This environment is limited to the vicinity of the acyl phosphate linkage, and not the entire active site, since the present experiments do not examine the microenvironments at other subdomains which recognize the adenine or ribose moieties of ATP.

More than 20 years ago, Post and Kume (*24*) suggested that "hydroxylamine attacks preferentially the P-O bond, as water does in the native enzyme, and attacks the C-O bond after denaturation." They analyzed the reaction products of the $[^{32}P]E_1P$ form of *N*-ethylmaleimide-modified (Na^+,K^+)-ATPase and *N*-methylhydroxylamine by paper electrophoresis, and observed a faint radioactive spot moving faster than $[^{32}P]P_i$. The instability of the N-P bond (see "*Trial for Detection of N-P Product*" and Ref. *19*) results in most of the product decomposing during the analysis, hence only a faint radioactive spot was detected.

Microenvironment in the Vicinity of the Acyl Phosphate Linkage in the Active Site—The calculated electronic structures of AcP (Fig. 5) were compatible with the results for the native phosphoenzyme (Figs. 1 to 4) but were incompatible with the results for AcP in solution (Table I). As the calculations did not account for any specific interactions between AcP and H_2O molecules, these results suggest that the acyl phosphate linkage is analogous to a nonhydrated state in the active site of the phosphoenzyme. The environment in the vicinity of the acyl phosphate linkage in the active site is thought to be nonaqueous.

Preliminary molecular orbital calculations showed that bond order of the C-O was always greater than that of the O-P when the dielectric constant value was increased from 1.0 to 24.55, 32.63, 45.0 and even to the value of water, 78.3 (S. Goto and Y. Shinohara, unpublished results). One factor bringing about the dominant cleavage of the C-O bond in solution might be a specific interaction of H_2O with a specific atom in the acyl phosphate linkage, for example the =O in the C(=O)-O-P. There may not be such an interaction of the acyl phosphate group with H_2O or other amino acid residue within the active site of the native phosphoenzyme.

Bulk Phase Substance Acceptability into the Phosphate Site—The nucleophilic center was the phosphorus atom of the acyl phosphate linkage in both E_1P and E_2P for the reaction with hydroxylamine, although the reaction rate for E_2P appeared to be 10-fold faster than for E_1P (Fig. 1). This may reflect the state of E_2P as the enzymatic reaction intermediate. E_2P is a hydrolyzable intermediate with the H_2O molecule bound to a specific site of E_2P (*3-6*). Since the substrate H_2O could not be excluded in these experiments, it always occupied its site in E_2P and specifically activated the acyl phosphate linkage, and thereby O-P bond cleavage is innately stimulated by another nonspecific nucleophile. Although we could not detect the N-P product directly in the case of E_2P ("*Trial for Detection of the N-P Product*" in "RESULTS"), we prefer the explanation that hydroxylamine attacks the phosphorus atom of the acyl phosphate linkage in E_2P as well as that in E_1P, based on circumstantial evidence (Fig. 2, and Refs. *23* and *26*).

A prerequisite for the reaction of the phosphate group and hydroxylamine added to the bulk phase is an accessibility of hydroxylamine to the reaction center. Actually there was no acceleration of the cleavage of the hydrolizable E_2P in the (Ca^{2+})-ATPase (Fig. 8), so that the reactivity of the phosphoenzyme with hydroxylamine primarily depends on the substance accessibility from the bulk phase. The phosphate site of the E_2P may be more accessible than that of E_1P from the exterior of the enzyme molecule.

It was fortunate that we chose (Na^+,K^+)-ATPase to look at the state inside the active site, since (Ca^{2+})-ATPase did not accept hydroxylamine (Figs. 1 and 8). There appear to be minute differences in the active sites between these two enzymes (*17, 36, 37*).

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REFERENCES

1. Repke, K.R.H. (1996) Reinstatement of the ATP high energy paradigm. *Mol. Cell. Biochem.* **160/161**, 95-99
2. Repke, K.R.H. and Schön, R. (1992) Chemistry and energetics of transphosphorylations in the mechanism of Na^+/K^+-transporting ATPase: An attempt at a unifying model. *Biochim. Biophys. Acta* **1154**, 1-16
3. Skou, J.C. and Esmann, M. (1992) The Na,K-ATPase. *J. Bioenerg. Biomembr.* **24**, 249-261
4. Glynn, I.M. and Karlish, S.J.D. (1990) Occluded cations in active transport. *Annu. Rev. Biochem.* **59**, 171-205
5. Stein, W.D. (1990) *Channels, Carriers, and Pumps*, pp. 221-243, Academic Press, San Diego, California
6. Jørgensen, P.L. (1990) Structure and molecular mechanism of the Na,K-pump in *Monovalent Cations in Biological Systems* (Pasternak, C.A., ed.) pp. 117-154, CRC Press, Boca Raton, Florida
7. Hokin, L.E. and Dahl, J.L. (1972) The sodium-potassium adenosinetriphosphatase in *Metabolic Pathways* (Hokin, L.E., ed.) Vol. 6, pp. 269-315, Academic Press, New York
8. Chignell, C.F. and Titus, E. (1968) The effect of hydroxylamine and *N*-methylhydroxylamine on beef brain microsomal $(Na^+ + K^+)$-ATPase. *Biochim. Biophys. Acta* **159**, 345-351
9. Bader, H., Wilkes, A.B., and Jean, D.H. (1970) The effect of hydroxylamine, mercaptans, divalent metals on $(Na^+ + K^+)$-ATPase. A possible control mechanism. *Biochim. Biophys. Acta* **198**, 583-593
10. Di Sabato, G. and Jencks, W.P. (1961) Mechanism and catalysis of acyl phosphates. I. Nucleophilic reactions. *J. Am. Chem. Soc.* **83**, 4393-4400
11. Jencks, W.P. (1958) The reaction of hydroxylamine with activated acyl groups. I. Formation of *O*-acylhydroxylamine. *J. Am. Chem. Soc.* **80**, 4581-4585
12. Yamamoto, T., Yoda, A., and Tonomura, Y. (1971) Reaction mechanism of the Ca^{2+}-dependent ATPase of sarcoplasmic reticulum from skeletal muscle IV. Hydroxamate formation from a phosphorylated intermediate and 2-hydroxy-5-nitrobenzyl hydroxylamine. *J. Biochem.* **69**, 807-809
13. Schwartz, A., Lindenmayer, G.E., and Allen, J.C. (1972) The Na^+,K^+-ATPase membrane system: Importance in cellular system in *Current Topics in Membranes and Transport* (Bronner, F. and Kleizeller, A., eds.) Vol. 13, pp. 1-82, Academic Press, New

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York
14. Whittam, R. and Wheeler, K.P. (1970) Transport across membranes. *Annu. Rev. Physiol.* **32**, 21-60
15. Jørgensen, P.L. (1988) Purification of Na^+,K^+-ATPase: Enzyme sources, preparative problems, and preparation from mammalian kidney in *Methods in Enzymology* (Fleischer, S. and Fleischer, B., eds.) Vol. 156, pp. 29-43, Academic Press, San Diego, California
16. Hayashi, Y., Mimura, K., Matsui, H., and Takagi, T. (1989) Minimum enzyme unit for Na^+/K^+-ATPase in the $\alpha\beta$-protomer. Determination by low-angle laser light scattering photometry coupled with high-performance gel chromatography for substantially simultaneous measurement of ATPase activity and molecular weight. *Biochim. Biophys. Acta* **983**, 217-229
17. Fukushima, Y. (1987) Inhibition by amine bases or by sodium ions and protection by divalent cations in the hydrolysis of phosphoenzyme of (Na,K)-ATPase. *J. Biol. Chem.* **262**, 11000-11005
18. Meissner, G., Conner, G.E., and Fleischer, S. (1973) Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{2+}-pump and Ca^{2+}-binding proteins. *Biochim. Biophys. Acta* **298**, 246-269
19. Leloir, L.F. and Cardini, C.E. (1957) Characterization of phosphorus compounds by acid lability in *Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O., eds.) Vol. III, pp. 840-850, Academic Press, New York
20. Tashima, Y. (1975) Removal of protein interference in the Fiske-SubbaRow method by sodium dodecyl sulfate. *Anal. Biochem.* **69**, 410-414
21. Lipmann, F. and Tuttle, L.C. (1945) A specific micromethod for the determination of acyl phosphates. *J. Biol. Chem.* **159**, 21-28
22. Shinohara, Y. (1990) Approaches to the molecular mechanism of ATP hydrolysis catalyzed by ATPases from electronic structural analysis (in Japanese). Thesis, University of Tokushima, Tokushima
23. Fedosova, N.U., Cornelius, F., Forbush III, B., and Klodos, I. (1997) Diversity of E_2P phosphoforms of Na,K-ATPase in *Na/K ATPase* (Gadsby, D.G., ed.) in press, New York Academy of Science, New York
24. Post, R.L. and Kume, S. (1973) Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase. *J. Biol. Chem.* **248**, 6993-7000
25. Bornstein, P. and Balian, G. (1977) Cleavage at Asn-Gly bonds with hydroxylamine in *Methods in Enzymology* (Hirs, C.H.W. and Timasheff, S.N., eds.) Vol. 47, pp. 132-145, Academic Press, New York
26. Herschlag, D. and Jencks, W.P. (1987) The effect of divalent metal ions on the rate and transition-state structure of phosphoryl-transfer reactions. *J. Am. Chem. Soc.* **109**, 4665-4674
27. Lecroisey, A., Lascu, I., Bominaar, A., Veron, M., and Delepierre, M. (1995) Phosphorylation mechanism of nucleoside diphosphate kinase: ^{31}P-Nuclear magnetic resonance studies. *Biochemistry* **34**, 12445-12450
28. Sabolic, I., Culic, O., Lin, S.-H., and Brown, D. (1992) Localization of ecto-ATPase in rat kidney and isolated renal cortical membrane vesicles. *Am. J. Physiol.* **262**, F217-F228
29. Stelte, B. and Witzel, H. (1986) Formation of an aspartyl phosphate intermediate in the reactions of nucleoside phosphotransferase from carrots. *Eur. J. Biochem.* **155**, 121-124
30. Jencks, W.P. and Gilchrist, M. (1965) Reactions of nucleophilic reagents with phosphoramidate. *J. Am. Chem. Soc.* **87**, 3199-3209
31. Fukushima, Y. and Post, R.L. (1978) Binding of divalent cation to phosphoenzyme of sodium- and potassium-transport adenosine triphosphatase. *J. Biol. Chem.* **253**, 6853-6862
32. Fukushima, Y. and Nakao, M. (1980) Changes in affinity of Na^+- and K^+-transport ATPase for divalent cations during its reaction sequence. *J. Biol. Chem.* **255**, 7813-7819
33. Dahms, A.S., Kanazawa, T., and Boyer, P.D. (1973) Source of the oxygen in the C-O-P linkage of the acyl phosphate in transport adenosine triphosphatases. *J. Biol. Chem.* **248**, 6592-6595
34. Dahms, A.S. and Boyer, P.D. (1973) Occurrence and characteristics of ^{18}O exchange reactions catalyzed by sodium- and potassium-dependent adenosine triphosphatases. *J. Biol. Chem.* **248**, 3155-3162
35. Kanazawa, T. and Boyer, P.D. (1973) Occurrence and characteristics of a rapid exchange of phosphate oxygens catalyzed by sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **248**, 3163-3172
36. Fukushima, Y., Yamada, S., and Nakao, M. (1984) ATP inactivates hydrolysis of the K^+-sensitive phosphoenzyme of kidney Na^+,K^+-transport ATPase and activates that of muscle sarcoplasmic reticulum Ca^{2+}-transport ATPase. *J. Biochem.* **95**, 359-368
37. Fukushima, Y., Asano, S., and Takada, J. (1992) K^+-site-directed pyridine derivative, AU-1421, activates hydrolysis of the K^+-sensitive phosphoenzyme of sarcoplasmic reticulum Ca^{2+}-ATPase and inactivates that of K^+-transporting ATPases. *Biochim. Biophys. Acta* **1106**, 71-76

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