Phosphorus-31 Nuclear Magnetic Resonance of Phosphoenzymes of Sodium- and Potassium-Activated and of Calcium-Activated Adenosinetriphosphatase†

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ABSTRACT: Prior studies identified phosphoenzyme intermediates in the turnover of sodium- and potassium-activated adenosinetriphosphatase [(Na,K)ATPase] from several sources and of the calcium-activated adenosinetriphosphatase [(Ca)-ATPase] of skeletal muscle sarcoplasmic reticulum. In both cases, the transphosphorylation is to a β-aspartyl carboxyl group at the active site. We now report observation of a K^+-sensitive phosphorylated intermediate of purified (Na,-K)ATPase from the salt gland of the duck using high-field ^{31}P nuclear magnetic resonance. Addition of ATP to a suspension of this enzyme in the presence of Mg^{2+} and Na^+ produced a resonance at about +17 ppm relative to 85% phosphoric acid. Addition of inorganic phosphate and Mg^{2+} to (Na,K)ATPase also produced a resonance at about +17 ppm which was enhanced in the presence of a saturating concentration of the inhibitor, ouabain; again, addition of K^+ made this resonance disappear. These findings are consistent with earlier kinetic characterization of an acid-stable (Na,-K)ATPase phosphoenzyme intermediate by ^{32}P-labeled phosphate incorporation into a denatured precipitate of the enzyme. We attribute the +17-ppm resonance to formation of an acyl phosphate at an aspartyl residue of the catalytic site of (Na,K)ATPase. This is supported by our finding of a similar resonance at +17 ppm after phosphorylation of another membrane-bound cation transport enzyme, sarcoplasmic reticulum (Ca)ATPase, as well as by a similar resonance at about +17 ppm after phosphorylation of the model dipeptide L-seryl-L-aspartate.

Substantial evidence has accumulated indicating that the active transport of sodium and potassium across cell membranes involves transphosphorylation from ATP to the transport enzyme complex of sodium- and potassium-activated adenosinetriphosphatase [(Na,K)ATPase] at a β-aspartyl site (Post & Kume, 1973; Degani & Boyer, 1973; Nishigaki et al., 1974). Prior conclusions have been based on the ^{32}P content of enzyme preparations phosphorylated from radiolabeled ATP or inorganic phosphate followed by acid or detergent denaturation (Robinson & Flashner, 1979; Wallick et al., 1979; Hobbs & Albers, 1980). Observation of one or more phosphorylated intermediates in (Na,K)ATPase by ^{31}P nuclear magnetic resonance (NMR) would provide an opportunity for further characterization of intermediates through direct observation of active enzyme during catalysis.

Phosphorylated intermediates have previously been observed by ^{31}P NMR in phosphoglucomutase (Mildvan, 1973) and alkaline phosphatase (Brock & Sheard, 1975; Hull et al., 1976; Chlebowski et al., 1976). These are soluble enzymes, obtainable in high concentrations, and both the enzyme and its linkage to phosphate are relatively stable. Active (Na,K)-ATPase is a membrane-bound enzyme isolated with high phospholipid content (Goldman & Albers, 1973). Both the enzyme itself and its phosphorylated intermediate are unstable at 20 °C. Samples at a sufficient concentration of active sites for NMR study are viscous. The phosphorylation sites may be hindered by inclusion in the phospholipid matrix. These factors, together with the low concentration of phosphorylated sites obtainable and a broadened signal from the phosphate being partially immobilized by the intrinsic phospholipid, combine to make observation of phosphoenzyme intermediates in the catalytic process a task of considerable difficulty. In order to maximize our chance of observing intermediates, we worked at the highest fields available to us (63.5 kG and 85 kG) with the largest sample volumes feasible (10 mL) and used a highly purified (Na,K)ATPase preparation from the supraorbital salt secretory gland of the duck (Hopkins et al., 1976).

This report describes the observation by ^{31}P NMR of a resonance corresponding to a phosphorylated enzyme intermediate (Post & Kume, 1973; Nishigaki et al., 1974). A resonance arising from an intermediate of this type would be expected to exhibit properties with respect to its formation and discharge that are similar to those previously reported to be characteristic of the enzyme as studied by ^{32}P phosphorylation under similar conditions. Additionally, it has been shown that the phosphorylated intermediate may be formed either from ATP in the presence of Na^+ and Mg^{2+} or from inorganic phosphate in the presence of Mg^{2+} alone. Our criteria for the establishment of a resonance as representative of a phosphorylated intermediate of (Na,K)ATPase therefore include formation under these two sets of conditions. Finally, we report an analogous ^{31}P NMR resonance occurring with phosphorylation of the sarcoplasmic reticulum calcium transport enzyme (Ca)ATPase, further supporting the assignment of the resonance to a phosphoenzyme intermediate.

Experimental Procedures

Preparation and Characterization of (Na,K)ATPase. (Na,K)ATPase was prepared as previously described (Hopkins et al., 1976) with a modification of the density-gradient centrifugation step to increase the yield of enzyme obtained from each preparation. Ten milliliters of sodium dodecyl sulfate treated enzyme (1.4 mg/mL) was layered on a step gradient

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consisting of 20 mL of 29.4%, 12 mL of 15%, and 8 mL of 10% sucrose (w/v) containing 1 mM H_4ETDA and 20 mM Tris-HCl, pH 7.4, in 50-mL tubes for the Beckman 35 rotor. Centrifugation was at 35000 rpm for 5 h, and pellets were combined and assayed as previously described (Hopkins et al., 1976). Specific activity averaged 28 μmol of P_i (mg of protein)$^{-1}$ min^{-1}. The final pellets contained approximately 0.5 μmol of active sites (100 mg of protein) and were resuspended to 10 mL with ice-cold buffered media containing the ionic constituents described for individual experiments.

Aliquots of enzyme from a preparation with a specific activity of 17 μmol of P_i mg^{-1} min^{-1} were taken for ^{32}P phosphorylation (Bastide et al., 1973). Phosphorylation from ATP labeled with ^{32}P at the γ position produced 2.6 nmol of ^{32}P/mg of enzyme protein, and phosphorylation of the enzyme from 1 mM P_i and 5 mM Mg^{2+} produced about 40% of the level of phosphoenzyme obtained by phosphorylation from ATP. This level was increased 1.3–1.5-fold by addition of 0.1 or 1.0 mM ouabain.

(Ca)ATPase. Sarcoplasmic reticulum was prepared from rabbit white skeletal muscle by a modification of the method of Harigaya & Schwartz (1969). A pellet of this preparation formed by centrifugation for 30 min at 38000*g* was resuspended in 0.05 M imidazole hydrochloride (pH 6.8) at a concentration of approximately 30 mg/mL and dialyzed for 16 h against 100 volumes of the same buffer. This material was either used immediately or stored at −70 °C. The calcium-activated ATPase activity measured at 25 °C (Glynn & Chappell, 1964) at a protein concentration of 0.23 mg/mL was 0.35 ± 0.02 mol of Pi (mg of protein)$^{-1}$ min^{-1} (SE; n = 3). For determination of optimal conditions for phosphoprotein formation at the high protein concentrations and low ratios of Mg^{2+} to ATP required for detection of the +17.4-ppm signal and for determination of the extent of phosphorylation, [γ-^{32}P]ATP, prepared by the method of Glynn & Chappell (1964), unlabeled ATP were freed of monovalent cations by passage through Dowex 50 W-X8 (H^+) columns (Shigekawa et al., 1978). Phosphorylation reactions were carried out in a total volume of 50 μL at 0 °C in 0.02 M imidazole hydrochloride buffer (pH 6.8) and stopped by addition of 2.0 mL of 5% (w/v) trichloroacetic acid containing 1.0 mM ATP and 0.1 mM P_i (Shigekawa et al., 1978). The pellets formed by 15 min of centrifugation at 2000*g* were resuspended in 2.0 mL of 5% trichloroacetic acid containing 0.1 mM P_i. After three cycles of centrifugation and resuspension, the pellets were solubilized in 1.0 mL of 1% sodium dodecyl sulfate–0.03 M NaOH. Aliquots neutralized with 0.02 M H_3PO_4 were measured for ^{32}P content in a liquid scintillation spectrometer and assayed for protein by the method of Lowry et al. (1951), standardized against bovine serum albumin. Under the conditions used for NMR spectroscopy (10 mM ATP; 0.4 mM Mg^{2+}, 20 μM Ca^{2+}; 14 mg/mL protein), a typical preparation contained 1.39 nmol of ^{32}P/mg of protein after 30 s and 0.81 nmol/mg at 15 min.

NMR Methods. Preliminary ^{31}P NMR studies of the (Na,K)ATPase and model peptides were performed on a Bruker HX-270 Fourier transform spectrometer operating at 109.3 MHz. The spectrometer was interfaced with a Nicolet BNC 12 20K computer. Data were stored automatically on a Diablo moving head disc system. The spectrometer was modified to operate under a four-phase quadrature detection mode controlled by the Nicolet NTCFT program. A 10-mm probe was used. Ninety-degree pulse widths were 42 μs; 14-μs pulses were ordinarily employed with a recycle time of 0.54 s. T_1 measurements (spin–lattice relaxation times) were made by use of the standard 180–τ–90° inversion–recovery pulse sequence. The spectrometer was locked on D_2O in the sample and was maintained at 4 °C throughout the experiment. T_1 values of ATP and P_i under the same solution and temperature conditions used for observation of the (Na,K)ATPase phosphoprotein were as follows: α-ATP, 2.2 s; β-ATP, 4.3 s; γ-ATP, 4.4 s; P_i, 7.8 s. These values were the same in the case of the reaction solution containing (Ca)ATPase. Thus, the T_1 measurements were made under the exact conditions that were used for observations of the resonance of the intermediate.

All other ^{31}P NMR experiments were carried out on a Nicolet wide-bore NT-360 spectrometer operating at 145.75 MHz. This spectrometer was controlled by a Nicolet 1180 computer with an 80K memory and a Diablo dual moving head disc system. Spectral widths of 12 KHz were employed with 4K computer data points. Forty-five-degree radio-frequency pulses of 18 μs were used with a 20 mm OD probe and a recycle time of 0.45 s. The spectrometer was run unlocked and sample temperature was maintained at 0 °C. Under these conditions with use of the standard equation relating magnetization to T_1 and recycle time, the β and γ resonances of ATP were found to be 82% saturated, the α resonance was found to be 68% saturated, and the inorganic phosphate resonance was found to be saturated by about 90%. However, in a separate measurement the T_1 of the resonance of the phosphoenzyme intermediate was found to be less than 0.2 s and therefore was not saturated under the acquisition conditions. Chemical shifts are expressed relative to 85% phosphoric acid with positive values increasing at higher field. Both the (Na,K)ATPase and the (Ca)ATPase were stable at 0–4 °C for periods exceeding 12 h.

Preparation of Model Compounds. For investigation of the NMR spectrum of a model compound containing a β-aspartyl phosphate, the peptide L-seryl-L-aspartate (Vega-Fox Biochemicals) was phosphorylated at pH 5.0 by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide in 5-fold excess and inorganic phosphate in 2-fold excess. The pH was adjusted to 7.4, and the ^{31}P NMR spectrum of the mixture was determined directly without purification of the reaction mixture. Additionally, Cbz-L-aspartyl *O*-benzyl ester was phosphorylated specifically on the aspartyl side chain carboxyl group by reaction with the above carbodiimide derivative and inorganic phosphate. The product of this reaction was purified and hydrogenated over 10% Pd/charcoal according to the method of Post & Kume (1973).

Results and Discussion

Upon addition of 5mM ATP to a suspension of (Na,K)-ATPase in 2 mM $MgCl_2$ and 100 mM NaCl buffered at pH 7.4 with 2 mM Tris–phosphate, observation by ^{31}P NMR disclosed spectra that contained a resonance at +17.4 ppm (Figure 1A) in addition to the resonances of ATP, ADP, and P_i. This result was typical of five similar experiments, all of which yielded a resonance at +17.4 ppm appearing after the addition of ATP. An increase in the ratio of Mg^{2+} to ATP caused the β resonance of ATP to shift from +21 ppm toward +18 ppm as this ratio approached unity. Therefore, low ratios of Mg^{2+} to ATP facilitated resolution of the +17.4-ppm resonance. Figure 1A shows a typical spectrum following the addition of 5 mM ATP. Addition of 10 mM KCl, which is known to reduce markedly the level of phosphorylated intermediate (Robinson & Flashner, 1979; Wallick et al., 1979; Hobbs & Albers, 1980), dissipated the +17.4-ppm resonance. Accumulation of data during the 30 min immediately after addition of K^+ produced a spectrum in which no +17.4-ppm resonance could be discerned (Figure 1B). Spectra were ob-

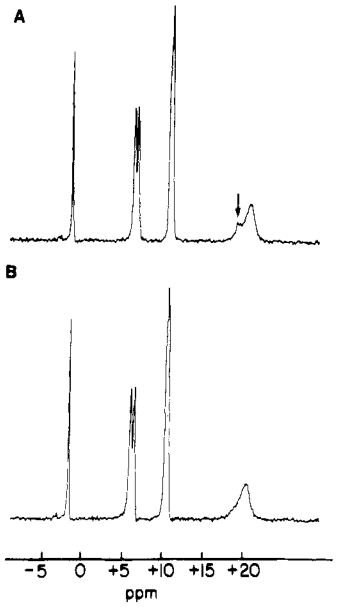

FIGURE 1: (A) 145.75-MHz ^{31}P spectrum of (Na,K)ATPase at 4 °C to which has been added 2 mM $MgCl_2$ and 100 mM NaCl buffered at pH 7.4 with 2 mM Tris–phosphate and 5 mM ATP. (B) Sample as in (A) to which 10 mM KCl has been added. Major resonances, from left to right, are as follows: P_i (−1.8 ppm); γ-ATP (+5.7 ppm); α-ATP (+10.3 ppm); β-ATP (+20.3 ppm). The resonance attributed to the phosphoenzyme appears at +17.4 ppm, just to the left of the β resonance of ATP (marked by the arrow), and is absent after addition of 10 mM KCl. Each spectrum results from 5000 scans with a 0.45-sec repetition rate. Spectral widths are 12 kHz. 18-μs (45°) radio-frequency pulses were used. A line broadening function equivalent to 8 Hz was applied to improve signal-to-noise ratios.

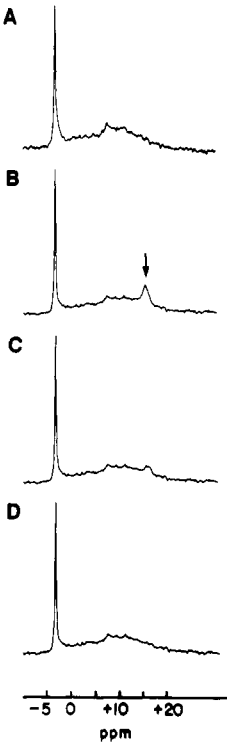

FIGURE 2: (A) 145.75-MHz ^{31}P spectrum of (Na,K)ATPase at 4 °C to which has been added Mg^{2+} and P_i. (B) Sample as in (A) to which was added 1 mM ouabain; the resonance attributed to the phosphoenzyme appears at +17.5 ppm and is marked by an arrow. (C) Sample as in (B) to which was added 10 mM KCl (0–30 min following addition). (D) Sample as in (C) (30–60 min following addition of KCl). Each spectrum results from the accumulation of 4000 scans with a 0.45-s repetition rate. Spectral widths of 12 kHz and 18-μs (45°) radio-frequency pulses were used. A line broadening function equivalent to 20 Hz was used to improve signal-to-noise ratios.

tained under conditions which resulted in saturation of the ATP signal, reducing the β and γ resonances of ATP by 82%. The resonance of the phosphoenzyme intermediate, however, having a much shorter relaxation time, was not at all saturated in the spectra in Figure 1. For quantitation of the amount of phosphoenzyme observed, spectrum 1B was subtracted from spectrum 1A. This resulted in an area at +17.4 ppm of approximately 6–7% of the area of the ATP resonance. As 10 mL of 5 mM ATP was present in the sample and the resonance area of the β-phosphate was reduced 82% by saturation, the 6–7% area in the difference spectrum represents about 0.6 μmol of phosphoenzyme in the sample, in reasonable agreement with a value of 0.4 μmol estimated from the known phosphorylation yield of the enzyme.

A phosphoenzyme intermediate is also formed after the addition of P_i and Mg^{2+} to (Na,K)ATPase in the absence of Na^+. This intermediate is stabilized by the addition of ouabain, thereby increasing the level of phosphorylation (Post et al., 1975). This was confirmed by phosphorylation from ^{32}P-labeled inorganic phosphate of an aliquot of the enzyme used in the NMR experiments, demonstrating that incorporation from ^{32}P-labeled P_i in the presence of Mg^{2+} was enhanced up to 1.5-fold by the addition of ouabain. In our ^{31}P NMR experiments, we were unable to identify with certainty any resonance peak except that of P_i in a mixture of P_i, Mg^{2+}, and enzyme, although a small peak was seen indistinctly at about +17 ppm. However, upon addition of 1 mM ouabain, a resonance appeared at +17.6 ppm (Figure 2B). This resonance, if due to a phosphorylated intermediate, should also be dissipated by the addition of K^+. However, the rate of dephosphorylation would be slower than that in the previous experiments due to stabilization by ouabain of the phosphoenzyme (Post et al., 1975). Accordingly, data accumulation was begun immediately subsequent to addition of 10 mM KCl. Data acquired during the initial 30 min after KCl addition contained the +17.6-ppm resonance; however, its magnitude was decreased by approximately 65% (Figure 2C). Data acquired between 30 and 60 min following K^+ addition contained no discernible resonance other than that of P_i (Figure 2D).

The concentration of P_i in the solution was 1 mM, and the volume is 10 mL. Since the P_i resonance was 90% saturated, comparison of the area of the +17.6-ppm resonance of the phosphoenzyme intermediate with the area of the P_i resonance results in a calculated value of about 0.5 μmol of phosphoenzyme present in this experiment, again in reasonable agreement with phosphorylation levels predicted from ^{32}P phosphorylation data.

Evidence has been presented (Bastide et al., 1973; Post & Kume, 1973; Nishigaki et al., 1974; Post & Orcutt, 1973) indicating that (Na,K)ATPase turnover involves transphosphorylation of the γ-phosphate from ATP to the β-carboxyl group of an aspartic acid residue to form a β-phosphoaspartyl residue. Chemical shift values for ^{31}P NMR spectra of acyl phosphates are not well documented in the literature. Acetyl phosphate appears at +2 ppm at neutral pH. For β-phosphoaspartic acid we found the ^{31}P resonance at +11.6 ppm. In this compound the β-carboxyl group of the side chain is known to be phosphorylated since the α-carboxyl was blocked during phosphorylation in our synthetic scheme. In any case, the chemical shift of about +17 ppm observed in (Na,K)ATPase is relatively high for an acyl phosphate. It was, therefore, desirable to investigate other model compounds. Phosphorylation of L-seryl-L-aspartate afforded two resonances, one at +17.4 ppm and the other at −3.7 ppm with approxi-

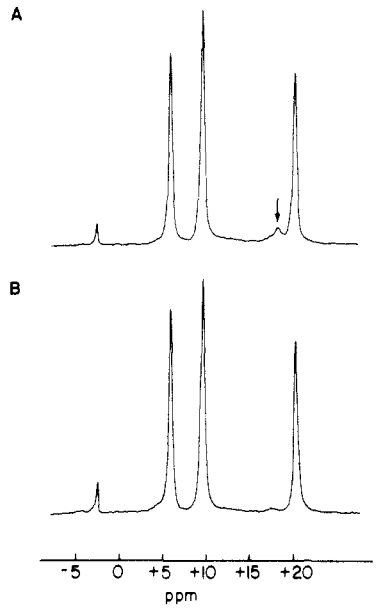

FIGURE 3: (A) 145.75-MHz ^{31}P spectrum of (Ca)ATPase to which was added 20 μM $CaCl_2$, 0.4 mM $MgCl_2$, and 10 mM ATP. The resonance attributed to the phosphoenzyme appears at +17.5 ppm and is indicated by an arrow. (B) Sample as in (A) to which was added hydroxylamine. Each spectrum results from the accumulation of 5000 scans with a repetition rate of 0.45 s. Spectral widths are 12 kHz, and 18-s (45°) radio-frequency pulses were used. A line broadening function equivalent to 20 Hz was used to improve signal-to-noise ratios.

mately equal intensities. The later is typical of phosphoserine, suggesting that the +17.4-ppm resonance arose from phosphoaspartate. This resonance was a poorly resolved triplet resulting from proton–phosphorus coupling with the adjacent methylene group.

No simple peptide can be expected to be an adequate model for the phosphoenzyme since the structure of the active center of the enzyme can modify the environment of the phosphate group and since it is known that a large number of factors can influence chemical shift values. These include dihedral angle (Gorenstein, 1975) and hydrophobicity and pH (Moon & Richards, 1973). Accordingly, we also investigated the phosphoenzyme of sarcoplasmic reticulum (Ca)ATPase from rabbit skeletal muscle. This enzyme shares features of active-site homology with (Na,K)ATPase in that the sequence serine-aspartate is also found at the transphosphorylation site (Allen & Green, 1976).

(Ca)ATPase from sarcoplasmic reticulum of rabbit skeletal muscle was phosphorylated in the presence of 20 μM $CaCl_2$, 0.4 mM $MgCl_2$, and 10 mM ATP. The ^{31}P NMR spectra of this mixture contained a resonance at +17.5 ppm (Figure 3A). As in the studies of the (Na,K)ATPase, the ratio of divalent cation to ATP was adjusted to prevent the β resonance of ATP from obscuring the new resonance at +17.5 ppm. When corrected for the known degree of saturation of the β resonance of ATP (82%), the relative magnitude of the +17.5-ppm resonances is similar to the value predicted on the basis of the phosphorylation yield as noted under Experimental Procedures. The result derived from the NMR measurement is 1.2 μmol of phosphoenzyme in the sample, whereas calculation based on phosphorylation from [γ-^{32}P]ATP would predict 1.1 μmol of phosphoenzyme.

Hydroxylamine reactivity was used to characterize further the nature of the +17.5-ppm resonance. Acyl phosphates are known to be split by hydroxylamine; however, this splitting may be slow in the case of native enzyme (Post & Kume, 1973). Following phosphorylation in the presence of Ca^{2+}, Mg and ATP, hydroxylamine was added to the mixture and the ^{31}P NMR spectrum was observed. No +17.5-ppm resonance was found (Figure 3B). Thus, in a second membrane-bound cation transport enzyme known to have a β-aspartyl phosphate intermediate, we observed a resonance, the appearance and disappearance of which coincided with conditions for formation and splitting of the acyl phosphate bond.

We conclude that the phosphoenzyme intermediate of both the (Na,K)ATPase of the duck salt gland and the (Ca)ATPase of skeletal muscle sarcoplasmic reticulum was detected by ^{31}P NMR at +17.4 to +17.5 ppm. While such a resonance could conceivably arise from nonspecific binding of ATP to the protein, this is unlikely since the conditions for appearance, enhancement, and disappearance of this resonance correspond closely to those characteristic of the intermediate as demonstrated by biochemical methods. These include (1) requirements for Na^+ and Mg^{2+} for formation of the (Na,K)ATPase intermediate in the presence of ATP, (2) rapid discharge of this intermediate by K^+, (3) formation of the intermediate in the presence of Mg^{2+} and P_i in the absence of ATP, (4) enhancement by ouabain of the steady-state levels of the intermediate formed in the presence of Mg^{2+} and P_i, (5) appropriately slow kinetics of discharge of the ouabain-stabilized intermediate by K^+, (6) requirements for Ca^{2+} and Mg^{2+} for formation of the (Ca)ATPase intermediate, and (7) sensitivity of the latter intermediate to hydroxylamine, as expected for an acyl phosphate linkage. The chemical shift of +17.4 to +17.5 ppm exceeds that found in acetyl phosphate or in aspartyl phosphate but agrees closely with the +17.4-ppm resonance observed after phosphorylation of serylaspartate.

These experiments demonstrate that existence of the phosphoenzyme intermediate of two cation transport ATPase's in the native enzymes. Further experiments using ^{31}P NMR may be useful in delineating changes in the energy levels of these active-site phosphate groups (Robinson & Flashner, 1979; de Mies & Vianna, 1979).

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Phosphorylase Kinase from Dogfish Skeletal Muscle. Purification and Properties†

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ABSTRACT: Phosphorylase kinase (EC 2.7.1.38) has been isolated from the white skeletal muscle of an early vertebrate, the Pacific dogfish (*Squalus acanthias*), by a combination of ammonium sulfate fractionation, batchwise adsorption on DE-52-cellulose, and gel filtration on Sepharose 4B. The final material is essentially homogeneous as judged by the criteria of ultracentrifuge analysis and polyacrylamide gel electrophoresis where it displays similar patterns as purified rabbit skeletal muscle phosphorylase kinase. The specific activity of the dogfish enzyme (2.8 μmol of phosphorylase *b* monomer converted to phosphorylase *a* per min per mg of kinase at pH 8.2, 30 °C) is of the same order of magnitude as that of the purified, phosphorylated rabbit enzyme maximally activated by Ca^{2+}. It can utilize both rabbit phosphorylase and dogfish phosphorylase as substrate with K_m's of 84 μM and 100 μM, respectively, at pH 8.2. It is devoid of cAMP-dependent protein kinase, phosphorylase, phosphorylase phosphatase, glycogen synthase, or ATPase activity. Similar to the rabbit enzyme, it can utilize ATP, dATP, and adenosine 5′-(3-thiotriphosphate) as phosphoryl donors; by contrast, it cannot use GTP or dogfish or rabbit muscle troponin I as substrate. Phosphorylase kinase from the dogfish displays the same molecular weight of 1.3×10^6 estimated by Sepharose 4B chromatography and sedimentation velocity and tends to aggregate upon standing. Likewise, it has an absolute requirement for Ca^{2+} ions as demonstrated by complete inhibition in the presence of EGTA or purified elements of the sarcoplasmic reticulum. Half-maximal activation occurs at $[Ca^{2+}] = 3 \times 10^{-7}$ M. On the other hand, the dogfish enzyme shows a major difference in its regulatory properties in that no phosphorylation could be demonstrated in the presence of different protein kinases or by autophosphorylation as seen with the mammalian enzyme, and its activity was unaffected by protein phosphatase from various sources.

Glycogen synthase and phosphorylase, the two enzymes directly involved in the synthesis and breakdown of glycogen, are regulated by both allosteric and covalent modifications. Interconversion by phosphorylation–dephosphorylation is brought about by a complex set of reactions in which a number of enzymes act successively on one another; in mammalian muscle, this cascade of enzymatic reactions can be initiated by hormone release or the nerve impulse that triggers contraction [for reviews, see Fischer et al. (1971), Soderling & Park (1974), Cohen (1974), Fischer et al. (1975), and Carlson et al. (1979)].

Phosphorylase kinase is one of the regulatory enzymes involved. Because it displays an absolute requirement for calcium ions (Krebs et al., 1959; Heilmeyer et al., 1970; Brostrom et al., 1971), it sits at the crossroad of glycogenolysis and muscle contraction (Ebashi et al., 1969; Fischer et al., 1975). In mammalian systems, it can be further activated by a phosphorylation reaction catalyzed either by the cAMP-dependent protein kinase (deLange et al., 1968; Walsh et al., 1971) or by itself, in an autocatalytic process (Walsh et al., 1970; Wang et al., 1976; Carlson & Graves, 1976).

Phosphorylase kinase has been studied in a number of tissues and species, including liver (Sutherland & Rall, 1960; Riley & Wahba, 1969; Shimazu & Amakawa, 1975; Khoo & Steinberg, 1975; Vandenheede et al., 1976; Sakai et al., 1979), brain (Drummond & Bellward, 1970; Ozawa, 1973), adipose tissue (Khoo et al., 1972, 1973; Khoo, 1976), heart (Hammermeister et al., 1965; Drummond & Duncan, 1966; Mayer et al., 1970; Daegelen-Proux et al., 1976; Cooper et al., 1980), platelets (Gear & Schneider, 1975; Chaiken et al., 1975), soleus muscle (Jennissen & Heilmeyer, 1974; Burchell et al., 1976; Sharma et al., 1980), smooth muscle (Namm, 1971), insect flight muscle (Sacktor et al., 1971), and *Neurospora crassa* (Gold et al., 1974). However, the rabbit skeletal muscle enzyme has been the most thoroughly characterized in terms of its physicochemical, enzymatic, and, more importantly, regulatory properties [for reviews, see Carlson et al. (1979), Krebs & Beavo (1979), and Cohen (1980)].

Because of the complexity of the regulation of glycogenolysis, it appeared of interest to investigate how it originally

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