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Phosphorylase Kinase from Dogfish Skeletal Muscle. Purification and Properties†

Sitivad Pocinwong, Hubert Blum,‡ Dean Malencik, and Edmond H. Fischer*

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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arose and evolved with time. The Pacific dogfish (*Squalus acanthias*) was selected because (a) it represents an early vertebrate that has separated from the main line of evolution more than 400 million years ago, (b) it is already endowed with a well-developed endocrine system, and (c) phosphorylase, the primary substrate of the enzyme, is abundant in this species and has been well characterized (Cohen et al., 1971). This manuscript describes the purification and some physical, chemical, enzymatic, and regulatory properties of phosphorylase kinase from this same organism.

Materials and Methods

Sepharose 4B was obtained from Pharmacia, DEAE-cellulose (DE-52) from Whatman, adenosine 5′-(3-thiotriphosphate) (ATPγS)[1] from Boehringer, dATP from Sigma Chemical Co., and AMPPNP from International Nuclear. ATPγCH_3 was a generous gift from Dr. F. Eckstein. [γ-^{32}P]ATP (sp radioact approximately 300 mCi/mmol) was prepared according to Glynn & Chappell (1964) from carrier-free $^{32}P_i$ (International Nuclear).

Rabbit muscle phosphorylase *b* was prepared according to Fischer & Krebs (1958), phosphorylase kinase according to Cohen (1973), and cAMP-dependent protein kinase (DEAE-cellulose fraction II) according to Reimann et al. (1971). Dogfish muscle phosphorylase *b* was prepared according to Cohen et al. (1971) and sarcoplasmic reticulum (SR) according to the procedure of MacLennan (1970) carried out to the R_1 washed step. Phosphorylase kinase phosphatases, from both dogfish muscle and rabbit muscle, were partially purified according to Riley et al. (1968) and the final products were stored as lyophilized powder at −15 °C. A protein phosphatase, different from phosphorylase phosphatase, copurified with dogfish protein kinase (Blum et al., 1973) up to the Sephadex G-100 step but emerged early during elution. Dogfish and rabbit skeletal muscle structural proteins (TN-I, TN-T, TN-C, myosin, tropomyosin, and actin) were prepared according to Greaser & Gregely (1973) and Malencik et al. (1975). Antiserum against dogfish phosphorylase was obtained from rabbits immunized by subcutaneous injections of 5 mg of phosphorylase in Freund's complete adjuvant. Immunodiffusion experiments were performed on Ouchterlony plates layered with 1% agarose containing 100 mM sodium glycerophosphate, 150 mM NaCl, and 0.02% azide, pH 7.5.

Phosphorylase kinase was assayed by a modification of the procedure of Krebs (1966). The reaction mixture contained 60 mM Tris–60 mM sodium glycerophosphate, adjusted to pH 6.8 or 8.2 with HCl, 12 mM $MgCl_2$, 3.6 mM ATP, and 8 mg/mL phosphorylase *b* in a total of 0.1 mL. After 1-min preincubation at 30 °C, the reaction was started by addition of 0.01 mL of phosphorylase kinase diluted in 50 mM sodium glycerophosphate, pH 7.0, containing 10% sucrose and 7 mM 2-mercaptoethanol. The reaction was stopped at 0, 2.5, and 5 min by diluting 0.01-mL aliquots 20-fold in ice-cold 100 mM maleate buffer, pH 6.5, containing 0.1% bovine serum albumin, 2 mM EGTA, and 40 mM 2-mercaptoethanol. Phosphorylase *a* was assayed in the direction of glycogen synthesis according to Hedrick & Fischer (1965). One unit of phosphorylase kinase activity is defined as the number of micromoles of phosphorylase *b* monomer (M_r 97000) converted to phosphorylase *a* per minute. The specific activity of phosphorylase *a* in the absence of AMP was taken as 54 units/mg.

Protein kinase activity was measured by the filter paper technique of Corbin & Reimann (1974), glycogen synthase according to Thomas et al. (1968), and phosphorylase phosphatase either by method II of Haschke et al. (1970) or by loss of phosphorylase *a* activity (Gratecos et al., 1977).

ATPase activity was measured at 30 °C in 50 mM Tris-HCl, pH 7.5, containing 60 mM KCl, 0.5 mM $CaCl_2$, 5 mM $MgCl_2$, and 2 mM ATP. At given times, 0.1-mL aliquots were withdrawn and added to 0.1 mL of 10% trichloroacetic acid. The inorganic phosphate generated was determined according to Marsh (1959).

Protein was measured by the procedure of Lowry et al. (1951) or by absorbance at 280 nm, using a value $A_{280}^{1\%}$ of 11.6 based on the amino acid composition of the enzyme (Hsiu et al., 1964). Sedimentation velocity experiments were carried out at 48000 rpm and 5 °C in a Spinco Model E ultracentrifuge equipped with a double-sector cell, in the presence of 0.1 M sodium glycerophosphate, pH 7.0, containing 2 mM EDTA, 1 mM ATP, and 7 mM 2-mercaptoethanol.

Polyacrylamide gel electrophoresis was carried out as described by Davis et al. (1967) with 4% acrylamide gels containing 0.11% *N,N′*-methylenebis(acrylamide). Electrophoresis in the presence of sodium dodecyl sulfate was carried out according to Studier (1973). The polyacrylamide gels were stained with Coomassie Brilliant Blue R-250 and subsequently destained in acetic acid and methanol (Weber & Osborn, 1969) either electrophoretically or by diffusion.

Two buffers were routinely used: buffer A containing 0.1 M sodium glycerophosphate and 2 mM EDTA, pH 7.0, and buffer B consisting of 0.1 M sodium glycerophosphate, 2 mM EDTA, 10% sucrose, and 1 mM ATP, pH 7.0. Each solution was adjusted to the desired pH with HCl.

Results

Purification of Dogfish Muscle Phosphorylase Kinase. Dogfish were netted in the waters of Puget Sound; they were kept for no less than 3 days and no longer than 1 month in a 4000-gal, donut-shaped tank with circulating seawater generously provided by the Department of Fisheries. The fish were killed by a blow on the head; the back muscles, consisting of more than 95% white fibers, were removed and processed immediately. Purification of phosphorylase kinase was carried out at 4 °C.

Preparation of Crude Extract. Muscle (1 kg) was minced and stirred for 15 min in 2.5 L of 10 mM sodium pyrophosphate adjusted to pH 9.0 with acetic acid, containing 5 mM magnesium acetate and 0.2 mM phenylmethanesulfonyl fluoride; the pH drops immediately and was maintained at 7.0 with 6 M NH_4OH. The suspension was strained through two layers of cheesecloth and centrifuged at 12000*g* for 30 min; the clear supernatant underlying a thick lipid layer was collected by siphoning.

Ammonium Sulfate Precipitation. One volume of neutralized 3 M ammonium sulfate solution was added to 1 volume of extract. The solution was allowed to stand for 15 min, the precipitate was collected by centrifugation, resuspended in one-tenth the original volume of buffer A containing 10% sucrose, 0.25 M KCl, and 10 mM ATP, and recentrifuged at 80000*g* for 1 h. After removal by suction of a thin layer of remaining lipid, the translucent solution was collected and diluted with buffer A to approximately 1 mg of protein/mL.

Batchwise Absorption. The solution was passed through a Buchner funnel containing 300 mL of a packed suspension

[1] Abbreviations used: ATPγS, adenosine 5′-(3-thiotriphosphate); SR, sarcoplasmic reticulum; Tris, tris(hydroxymethyl)aminomethane; TN-I, inhibitory component of troponin; TN-T, tropomyosin-binding component of troponin; TN-C, Ca^{2+}-binding component of troponin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetate; EDTA, ethylenediaminetetraacetic acid; UDPG, uridine 5′-diphosphate α-D-glucose; cAMP, adenosine cyclic 3′,5′-phosphate.

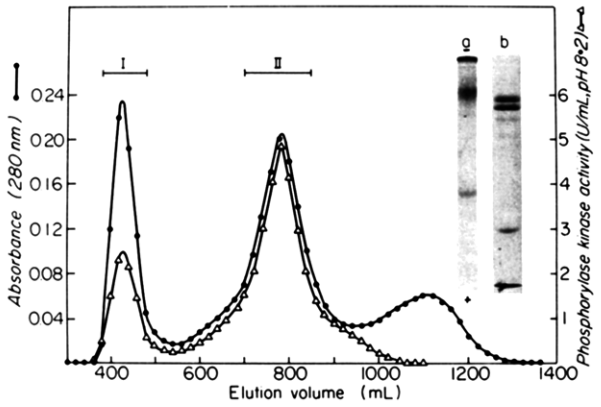

FIGURE 1: Fractionation of phosphorylase kinse (100 mg) on a Sepharose 4B column (4 × 90 cm) equilibrated in buffer B. Fractions (4.5 mL) were collected at a flow rate of 40 mL/h; those under bars were pooled. (Insert) gel electrophoresis in the absence (gel a) and presence (gel b) of dodecyl sulfate were carried out on ca. 30 and 20 μg of pooled material from peak II, respectively.

Table I: Purification of Dogfish Phosphorylase Kinase[a]

step	volume (mL)	total units[b]	total protein[c] (mg)	sp act. (units/mg)	yield (%)
crude extract	2300	5600	20200	0.03	100
ammonium sulfate precipitation	230	4680	1060	0.44	84
DE-52-cellulose eluate	620	2780	190	1.46	50
Sepharose 4B chromatography	150	504	18	2.80	9

[a] Starting from 1 kg of fresh dogfish skeletal muscle. [b] Assayed at pH 8.2 by using rabbit phosphorylase *b* as the substrate (see Materials and Methods). [c] Measured by the procedure of Lowry et al. (1951).

of DE-52-cellulose in buffer A, and the adsorbed enzyme was eluted with the same solution containing 0.4 M NaCl. At this stage, the enzyme can be lyophilized, and the dry material can be stored at −15 °C almost indefinitely with little loss of activity. For removal of salts, the lyophilized powder was suspended in cold distilled water (ca. 1 g/2.8 mL) and centrifuged at 27000*g* for 10 min. The protein pellet was then dissolved in 1 mL of buffer B (3–5 mg/mL) and clarified by centrifugation.

Sepharose 4B Chromatography. The concentrated enzyme solution was passed through a Sepharose 4B column in which it separated into two active fractions (Figure 1). Fraction I was turbid and contained the enzyme in an aggregated form with a molecular weight estimated to be in the millions by its elution behavior. Fraction II was clear and consisted mainly of a single protein species with a molecular weight of ca. 1.3×10^6. At this stage, the enzyme was more than 90% pure, and this fraction was used for all subsequent studies. Rechromatography of the clear fraction II, after concentration, resulted once more in a separation into turbid and clear fractions with considerable loss of activity. A typical purification scheme is summarized in Table I.

Criteria of Purity. Purified phosphorylase kinase displayed no protein kinase, phosphorylase, phosphorylase phosphatase, glycogen synthase, or ATPase activity. It showed no cross-reactivity with an antiserum against pure dogfish phosphorylase. Since it has a strong tendency to aggregate, some material always remained at the origin on polyacrylamide gel electrophoresis (see insert of Figure 1, gel a), as observed for rabbit phosphorylase kinase (Hayakawa et al., 1973a,b);

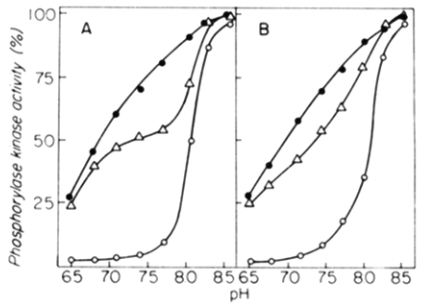

FIGURE 2: pH dependence of activity of purified dogfish and rabbit phosphorylase kinase when using dogfish (A) and rabbit (B) phosphorylase *b* as the substrate. Dogfish phosphorylase kinase, (△); rabbit phosphorylase kinase, nonphosphorylated (○) and phosphorylated (●). Assay conditions are described under Materials and Methods.

dissolution of the excluded material as well as the diffuse second band in dodecyl sulfate followed by dodecyl sulfate gel electrophoresis showed essentially the same pattern as that observed for the soluble enzyme (Figure 1, gel b). No enzymatic activity could be detected on gel slices under any condition. Often, a third component of 96000 daltons, tentatively ascribed to α-actinin, accompanied the enzyme.

Polyacrylamide gel electrophoresis in the presence of dodecyl sulfate (Figure 1, gel b) showed the typical subunit patterns previously described for rabbit muscle phosphorylase kinase including a calmodulin subunit (Hayakawa et al., 1973b; Cohen, 1973; Cohen et al., 1978). Characterization of the subunit structure of the enzyme will be described elsewhere (S. Pocinwong et al., unpublished experiments). The fourth component [δ subunit identified as calmodulin (Cohen et al., 1978; Cohen, 1980)] runs at the front and can be seen only on overloaded gels.

Stability. Dogfish phosphorylase kinase appears to be more labile at low ionic strengths, at low pH, and at temperatures above 20 °C than its rabbit counterpart. Even under the most favorable conditions (e.g., at 3 mg/mL in buffer B containing 0.3 M NaCl or in buffer A containing 1 mM dithiothreitol), the enzyme loses 50% activity within 10 min at 30 °C and within 1 week at −15 °C. By contrast, both the phosphorylated and nonphosphorylated forms of rabbit phosphorylase kinase were stable under the same conditions. Irreversible inactivation of the dogfish enzyme occurred rapidly below pH 6 or during desalting by gel filtration or dialysis at ionic strength below 0.1.

Attempts to stabilize the enzyme by addition of sucrose, glycerol, salts, sulfhydryl compounds, or reagents were unsuccessful. Likewise, no protection was afforded by effectors such as glycogen, glucose, Glc-1-P, Glc-6-P, mono-, di-, or trinucleotides, or divalent metal ions and protease inhibitors.

Molecular Weight. As estimated from Sepharose 4B chromatography (Figure 1), dogfish phosphorylase kinase has a molecular weight of 1.3×10^6, close to that of the rabbit enzyme (Hayakawa et al., 1973b; Cohen, 1973), which displays a similar elution profile. Sedimentation velocity centrifugation experiments showed essentially a single component ($s_{20,w}$ = 25 S) with some faster sedimenting aggregates (35–45 S) and much material accumulating at the bottom of the cell. Sedimentation equilibrium experiments never gave satisfactory results, again because of the tendency of the enzyme to aggregate.

Enzymatic Activity. The pH dependence of dogfish phosphorylase kinase activity is shown in Figure 2, with dogfish (Figure 2A) and rabbit (Figure 2B) phosphorylases *b* as substrates; results are compared to those obtained with rabbit phosphorylase kinase. The dogfish kinase displays an activity

Table II: Phosphoryl Donors and Acceptors

substrates	rel rates (%) of phosphorylase kinase: dogfish	rabbit
phosphoryl donors[a]		
ATP	100	100
dATP	54	96
ATPγS	10	10
GTP	0	4
phosphoryl acceptors[b]		
phosphorylase *b*[c]	100	100
TN-I[c]	0	10
TN-T[c]	0	2
myosin[c]	0	1
casein	0	<1

[a] Assays were carried out at pH 8.2 and 30 °C. [b] Phosphorylation of the protein substrates was carried out at pH 8.2 in 0.1 mL of reaction mixture at 30 °C containing 25 mM Tris, 25 mM sodium glycerophosphate, 0.05 mM $CaCl_2$, 50 mM NaF, 10 mM $MgCl_2$, 0.33 mM [γ-^{32}P]ATP, 2.5 mg of protein substrate, and 2 μg of phosphorylase kinase. The uptake of ^{32}P was determined by the filter paper method of Corbin & Reimann (1974). [c] Dogfish or rabbit.

curve that is somewhat intermediate between the nonactivated (i.e., nonphosphorylated) and activated species of rabbit phosphorylase kinase, as if the dogfish enzyme existed always in a partially activated form. The significance of this observation will be further detailed under regulation of enzyme activity. The reason for the biphasic nature of the curve, always observed when dogfish kinase acts on dogfish phosphorylase *b* (see Figure 2A), is not understood.

Substrate Specificity. Dogfish phosphorylase kinase appears to be more specific than its rabbit counterpart in terms of its nucleotide and protein substrates. By contrast to the rabbit kinase, only ATP, dATP, and ATPγS could serve as phosphoryl donors but not GTP (Table II). Neither enzyme could use ITP, CTP, UTP, creatine-P, phosphoenolpyruvate, adenosine 5′-(3-methyltriphosphate) ($ATP_\gamma CH_3$), or 5′-adenylyl imidodiphosphate (AMPPNP). Another major difference is that the dogfish kinase could not phosphorylate either dogfish or rabbit TN-I, TN-T, or myosin, all of which are involved in muscle contraction. Enzymes from both species were inactive on rabbit skeletal muscle, actin, tropomyosin, troponin, troponin-C, and parvalbumin. They were also inactive on yeast phosphorylase *b*, histone IIa, phosvitin, and protamine.

Kinetic Parameters. Difficulities were encountered in carrying out kinetic experiments on dogfish phosphorylase kinase. First, slight differences were obtained, whether dogfish or rabbit phosphorylase *b* was used as substrate. Second, K_m's were so large for the protein substrates that, for all practical purposes, assays could no be performed under saturating conditions. Some of the kinetic data are summarized in Table III. Rabbit phosphorylase *b* appears to be a better substrate (i.e., yielding a higher V_{max}) than the protein isolated from the same species; increase in activity at higher pH results from a more favorable K_m. When dogfish kinase acts on dogfish phosphorylase *b*, uncertainties of measurements at high substrate concentration did not allow for an umambiguous extrapolation for K_m and V_{max} determination, particularly at pH 6.8.

As observed for many kinases, optimum activity occurs at a divalent metal ion to ATP ratio equal to or greater than one. For the dogfish enzyme, an optimum Mg^{2+}/ATP ratio of 4 was obtained at pH 6.8 and of 2 at pH 8.2.

Effectors. To date, no metabolite tested was found to have any significant effect on the activity of dogfish phosphorylase

Table III: Kinetic Data for Dogfish Phosphorylase Kinase

substrate	pH	K_m (μM)	V_{max}[a]
phosphorylase *b* (dogfish)	6.8	50	5
	8.2	100	10
phosphorylase *b* (rabbit)	6.8	167	28
	8.2	84	28
ATP	6.8	2000	9
	8.2	2000	19
ATPγS	8.2	17200	19

[a] Expressed as micromoles of substrate converted per minute per milligram of enzyme at 30 °C.

kinase, whether they were preincubated with the enzyme or added to the assay mixture. These included 2% glycogen, 10 mM each of glucose, Glc-1-P, Glc-6-P, or UDPG, 1 mM AMP, or 50 mM P_i. Likewise, 1 mg each of purified dogfish myosin, actin, tropomyosin, troponin, parvalbumin, and partially purified rabbit glycogen synthase had no effect.

Regulation of Enzyme Activity. Two basic events are required to bring about the full activation of mammalian skeletal muscle phosphorylase kinase: (a) there is an absolute requirement for Ca^{2+} ions; (b) activity is further enhanced by phosphorylation of the enzyme. It was therefore of interest to determine whether either or both mechanisms were operative in a primative vertebrate.

Calcium Requirement. Even though 2 mM EDTA was added to all buffers used in the course of kinase purification, the enzyme obtained was in a form that did not require added calcium for activity. Of course, in the ca. 1000-fold dilution preceding activity measurements, there is usually enough spurious Ca^{2+} contamination to fully saturate the enzyme; therefore, Ca^{2+} requirements could only be demonstrated by adding EDTA or EGTA (>0.1 mM) directly to the dilution buffer, the assay system, or both. In this instance, readdition of Ca^{2+} to give a free metal ion concentration in the micromolar range brought about a ca. 80% restoration of activity. Direct measurements of Ca^{2+} binding to dogfish phosphorylase kinase by equilibrium dialysis or gel filtration were generally unsuccessful due to the instability of the Ca^{2+}-free enzyme. However, a binding constant for Ca^{2+} could be estimated by determining phosphorylase kinase activity in Ca–EGTA buffers in which the free Ca^{2+} ion concentrations were calculated from stability constants as reported by Kerrick & Donaldson (1972). From Figure 3, half-maximal activation occurred at a Ca^{2+} concentration of ca. 0.4 μM at pH 7.0; a similar value of ca. 0.6 μM was obtained in parallel experiments carried out on rabbit phosphorylase kinase. As observed with one purified preparation of rabbit skeletal muscle phosphorylase kinase, very little increase in activity (ca. 1.3-fold) was obtained by addition of excess Ca^{2+}–calmodulin. Relative to the activation by calcium taken as 100%, the following values were obtained for other cations: Sr^{2+}, 45; Ba^{2+}, 26; Mn^{2+}, 15; Co^{2+}, Li^+, Fe^{3+}, and Mg^{2+} <10. The enzyme was inactive in the presence of Fe^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Sn^{2+}, and Al^{3+}.

Phosphorylation. At no stage of its purification could dogfish phosphorylase kinase be further activated by incubation with protein kinase, with or without 3′,5′-cyclic nucleotides (0.01–1 mM) such as cAMP, cGMP, cUMP, or cCMP, in the presence or absence of theophylline, NaF, or both. As described above, the dogfish enzyme is extracted in an active state; its pH 6.8/pH 8.2 activity ratio is 0.3–0.5 as opposed to 0.01–0.02 for the rabbit enzyme. Neither activation nor inactivation occurred following incubation with protein kinase or a partially purified preparation of a protein phosphatase (Riley et al., 1968; Blum et al., 1973) obtained from either

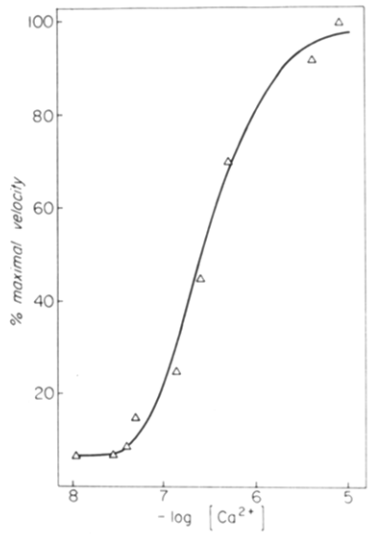

FIGURE 3: Activation of phosphorylase kinase by Ca^{2+}. Assays were carried out at 20 °C in an imidazole hydrochloride buffer, pH 7.0. The reaction contained 3 mM ATP, 10 mM $MgCl_2$, 0.8 mg of phosphorylase *b*, 0.15 μg of phosphorylase kinase, and varying amounts of K_2EGTA and $CaCl_2$ to provide the desired concentration of Ca^{2+} ions. The ionic strength was maintained at 0.1 by varying the concentration of imidazole hydrochloride.

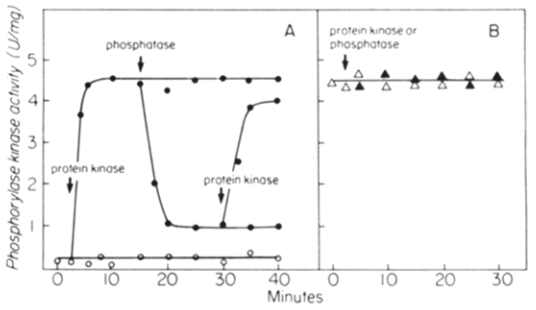

FIGURE 4: Effect of protein kinase and phosphatase on purified rabbit (A) and dogfish (B) phosphorylase kinase. Phosphorylase kinase (0.52 mg) and rabbit muscle cAMP-dependent protein kinase (0.1 mg) were preincubated for 2 min at pH 7.0 and 20 °C in 40 mM sodium glycerophosphate, 3.2 mM EGTA, and 0.8 μM cAMP. Activation of phosphorylase kinase was initiated by the addition of 2 mM Mg^{2+} and 0.6 mM ATP; the reverse reaction was carried out by adding an excess of a partially purified fraction of protein kinase inhibitor (0.4 mg), followed by 0.1 mg of protein phosphatase. In (A), a control without protein kinase was included (○); in (B), the reaction was carried out in the presence of protein kinase (▲) or protein phosphatase (△).

rabbit or dogfish skeletal muscle under conditions where the activity of the rabbit phosphorylase kinase was greatly affected (Figure 4). Likewise, incubation of a crude dogfish muscle homogenate for up to 1 h at 10 °C with large excesses (0.1 mM) of epinephrine, norepinephrine, glucagon, insulin, cortisone, cortisol, corticosterone, serotonin, or dopamine had no effect.

The rate of conversion of phosphorylase *b* to *a* catalyzed by nonactivated *rabbit* phosphorylase kinase was shown to increase with time in a manner typical of an autocatalytic reaction. This phenomenon was attributed to an autophosphorylation of the kinase occurring in the course of the reaction; the role of the substrate as an accelerator of the reaction was investigated (Carlson & Graves, 1976). No lag in phosphorylase *b* to *a* conversion by dogfish phosphorylase kinase was ever observed (Figure 5). Preincubation of the enzyme with calcium and [γ-^{32}P]ATP–Mg^{2+} with or without substrate showed no increase in activity with time and resulted in no incorporation or radioactivity.

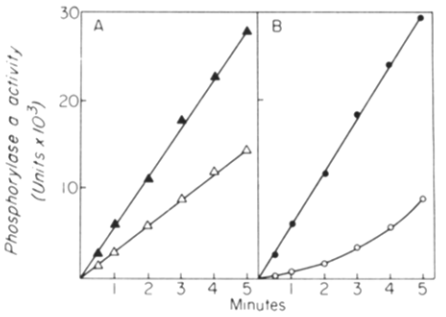

FIGURE 5: Conversion of phosphorylase *b* to *a* under the influence of purified dogfish (A) and rabbit (B) phosphorylase kinase. The reactions were carried out at pH 6.8 (open symbols) and 8.2 (closed symbols), respectively.

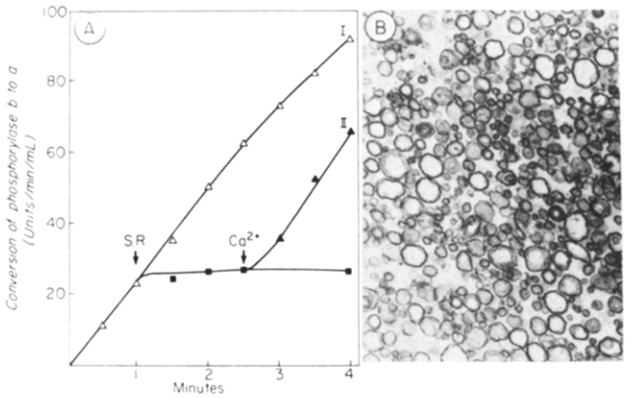

FIGURE 6: (A) Ca^{2+}-dependent reversal of the inhibition of phosphorylase kinase by purified elements of sarcoplasmic reticulum (SR). An initial reaction mixture at 30 °C was made up with 56 mM Tris-HCl, 56 mM sodium glycerophosphate, 1.9 mg of phosphorylase *b*, 3.2 mM ATP, 12 mM Mg^{2+}, and 0.15 μg of phosphorylase kinase, pH 7.1. After the last addition an aliquot was removed immediately and diluted 1:20 with 100 mM sodium maleate buffer (pH 6.5) containing 1% BSA for phosphorylase *a* assays. Aliquots were removed at various times and assayed as indicated. After 1 min the reaction mixture was divided into two portions. While one of these was left unchanged (control I), freshly prepared SR was added to the other. This second fraction was itself separated into two aliquots 90 s later, and excess Ca^{2+} was added to one of these (II), as indicated by arrow. (B) Electron micrograph of SR vesicles prepared by the procedure of MacLennan (1970) to the R_1 washed step. The sample was fixed in glutaraldehyde, thin sectioned, postfixed in 1% OsO_4, and counterstained with lead citrate and uranyl acetate. Magnification is ca. 7400×.

Removal of Calcium by Purified Preparations of Fragmented Sarcoplasmic Reticulum. Ebashi et al. (1969) and Brostrom et al. (1971) had shown that rabbit skeletal muscle phosphorylase kinase was inactivated by fragmented sarcoplasmic reticulum in the presence of Mg^{2+}–ATP needed for the calcium pump. Elements of the sarcoplasmic reticulum were prepared from fresh dogfish and rabbit striated muscle as described under Materials and Methods; they were shown to be homogeneous by electron microscopy kindly carried out by Dr. Mike Lowe. As shown in Figure 6, addition of this material to dogfish phosphorylase kinase resulted in an immediate blockage of kinase activity; this inhibition could be quantitatively reversed by the addition of excess calcium. Similar data were obtained when 0.25 mM EGTA was substituted for the sarcoplasmic reticulum.

Tissue Distribution. Tissue samples were removed from adult dogfish, homogenized in 2.5 volumes of 50 mM sodium glycerophosphate buffer, pH 7.0, containing 2 mM EDTA, and centrifuged for 10 min at 3000*g*. The supernatant was analyzed for phosphorylase kinase activity (pH 8.2), phosphorylase activity, and total protein content. Relative to the

kinase activity of white skeletal muscle taken as 1.0, the following values were obtained: brain, 0.15; heart and red back muscle, 0.1; kidney and liver, 0.05; lung and spleen, 0.01. Little variation was observed from animal to animal. Back muscle from the newborn fish (where it is difficult to distinguish between fiber types) contained between 50 and 60% of the kinase activity found in the adult. In all instances, phosphorylase activity closely paralleled that of phosphorylase kinase. Differential fractionation of skeletal muscle crude extracts indicated that better than 90% of the phosphorylase kinase (98% of phosphorylase) remained in the clear supernatant obtained after 90-min centrifugation at 105000*g*; these solutions were free of mitochondria, structural proteins, and elements of the sarcoplasmic reticulum.

Discussion

For 2–3 days after the fish are caught, one finds little phosphorylase kinase or glycogen synthase activity in muscle extracts, probably because of the injury sustained by the organisms upon netting. On the other hand, the fish cannot be kept for more than 1 month or 2 since they do not feed in captivity; this fasting state might explain the low occurrence of glycogen particles (Nag, 1972). In mammalian white muscle extracts, phosphorylase and other enzymes of glycogen metabolism are usually associated with these protein–glycogen complexes (Meyer et al., 1970). Therefore, the specimens under investigation are probably not under normal physiological conditions.

Dogfish phosphorylase kinase is far more labile than the rabbit enzyme at low pHs and higher temperatures. This latter instability is undoubtedly related to the cold environment (5–10 °C) in which the dogfish normally lives; it might also explain its sensitivity to low ionic strength ($\mu < 0.1$) which tends to destabilize hydrophobic interactions. Evolutionary adaptation of the dogfish to very restricted thermal environments must have led to the selection of proteins with rather unique physical and catalytic properties. Variability in primary structure has provided the basis for a natural selection of adaptive molecular properties. For instance, the thermostability of four enolases was shown to exhibit a positive correlation with the number of residues capable of forming side-chain hydrogen bonds and a negative correlation with the average hydrophobicity of the molecule (Barnes & Stellwagen, 1973). Dogfish phosphorylase kinase is also far more susceptible to proteolytic degradation than its rabbit counterpart; this, added to the fact that fish muscle generally contains potent proteases, might explain why purified preparations of the enzyme rapidly undergo autodigestion. A similar situation was observed during the isolation of troponin subunits from dogfish skeletal muscle (Heizmann et al., 1974; Malencik et al., 1975).

The absence of species specificity for the enzymes involved in the control of glycogen metabolism is further emphasized by the observation that dogfish phosphorylase kinase is at least as active on rabbit as on dogfish phosphorylase *b*. The biphasic nature of the pH-dependency curve illustrated in Figure 2 appears to result from a direct interaction between the two proteins since normal behavior is seen when the dogfish kinase acts on rabbit phosphorylase *b* or when rabbit kinase acts on dogfish phosphorylase *b*. One difference between the two substrates is that the active form of dogfish phosphorylase obtained by AMP or after *b* to *a* conversion does not undergo tetramerization (Cohen et al., 1971).

In contrast to the rabbit enzyme, dogfish phosphorylase kinase appears to be remarkably unaffected by metabolites. This is particularly noticeable in the case of glycogen which increases considerably the initial phase of the phosphorylase *b* to *a* conversion in the rabbit (Krebs et al., 1964). The acceleration could be ascribed, in part, to the fact that glycogen increases the rate of autophosphorylation of the rabbit kinase (Walsh et al., 1970); it also maintains phosphorylase *a* in the more active dimeric state (Wang & Graves, 1964). Lack of autophosphorylation of the dogfish kinase, and of tetramerization of dogfish phosphorylase, must also account for the absence of a lag during phosphorylase *b* to *a* conversion (see Figure 5).

Least expected was the finding that dogfish skeletal muscle phosphorylase kinase is not regulated by phosphorylation–dephosphorylation as observed for all mammalian enzymes. Regulation must, therefore, rely only on the availability of calcium ions released from the sarcoplasmic reticulum following nerve impulse. This is all the more surprising since (a) the enzyme has the same gross composition as the mammalian kinase and (b) dogfish muscle contains both cAMP-dependent and -independent protein kinases. No uptake of ^{32}P from γ-labeled ATP could be demonstrated with dogfish or rabbit protein kinases in the presence or absence of various cyclic nucleotides, with the pure catalytic subunit of beef heart cAMP-dependent protein kinase, or by direct autophosphorylation. In fact, no phosphorylation of dogfish glycogen synthase (Becker, unpublished results) or of the troponin subunits by this enzyme could be demonstrated either (Malencik et al., 1975). One might wonder whether glycogenolysis can be initiated by catecholamines under resting conditions when the concentration of free calcium is low.

Experiments involving the sarcoplasmic reticulum indicate that phosphorylase kinase must be in a calcium-free form in resting muscle and therefore inactive. Experiments on calcium fluxes between various muscle calcium-binding proteins and the sarcoplasmic reticulum have been described (Moeschler et al., 1979). The affinity of phosphorylase kinase for calcium is approximately identical with that of muscle parvalbumin and somewhat stronger than those of troponin C and calmodulin (Crouch & Klee, 1980). It should be emphasized that a real calcium-binding constant cannot be derived from activation curves, as given in Figure 3, since there are several classes of calcium-binding sites on the enzyme (Kilimann & Heilmeyer, 1977). Obviously the δ subunit (calmodulin) must be involved in calcium binding, but with an undetermined stoichiometry. Whether it is the sole calcium-binding subunit in this enzyme remains to be determined.

As expected for an enzyme involved in the regulation of glycogenolysis, much more of it is found in anaerobic, fast-twitch muscle than in red or cardiac tissue (Peter et al., 1972; Burchell et al., 1976; Cooper et al., 1980). The differences observed are even more prominent in the dogfish; indeed, contrary to mammalian skeletal muscle which is usually made up of a mixture of aerobic and anaerobic fibers, white and red muscles in the dogfish are sharply delineated. The dorsal muscle consists exclusively of white, fast-twitch glycolytic fibers surrounded by a 3–10 mm thick layer of red fibers amounting to less than 5% of the total muscle mass.

The data presented herein indicate that if hormonal control of glycogenolysis exists in a primitive vertebrate such as the dogfish, this control cannot be exercised by a direct interaction between the cAMP-dependent protein kinase and phosphorylase kinase, as has been shown in mammalian systems. Since dogfish phosphorylase kinase activity seems to rely solely upon the availability of calcium ions, the possibility remains that calcium release from the sarcoplasmic reticulum is itself under hormonal control. While this could be achieved by modulation of one of the sarcoplasmic reticulum components through a

phosphorylation–dephosphorylation reaction (Kirchberger et al., 1974; Tada et al., 1974; Schwartz et al., 1976; Hörl et al., 1978; Hörl & Heilmeyer, 1978; Jennissen & Lahr, 1980), no evidence has been obtained as yet that such a mechanism occurs in the dogfish.

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Kinetic Mechanism of Beef Pancreatic L-Asparagine Synthetase†

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ABSTRACT: The kinetic mechanism of bovine pancreatic asparagine synthetase was deduced from initial velocity studies and product inhibition studies of both the glutamine-dependent and ammonia-dependent reactions. For the glutamine-dependent pathway, parallel lines were observed in the double reciprocal plot of $1/V$ vs. 1/[glutamine] at varied aspartate concentrations, and in the plot of $1/V$ vs. 1/[ATP] at varied aspartate concentrations. Intersecting lines were found for the plot of $1/V$ vs. 1/[ATP] at varied glutamine concentrations. Product inhibition patterns, including dual inhibitor studies for measuring the synergistic effects of multiproduct inhibition, were used to support an ordered bi-uni-uni-ter ping-pong mechanism. Glutamine and ATP sequentially bind, followed by the release of glutamate and the addition of aspartate. Pyrophosphate, AMP, and asparagine are then sequentially released. When the ammonia-dependent reaction was studied, it was found that the mechanism was significantly different. NH_3 bound first followed by a random addition of ATP and aspartate. Pyrophosphate, AMP, and asparagine were then sequentially released as in the glutamine-utilizing mechanism. From these studies, a comprehensive mechanism has been proposed through which either glutamine or NH_3 can provide nitrogen for asparagine production from aspartate.

Asparagine, although one of the first amino acids to be discovered, is one of the last amino acids to have its metabolism and biosynthesis carefully examined. The metabolic importance of asparagine is becoming more apparent. Asparagine has been shown along with glutamine to be able to cross the blood–brain barriers (Tower et al., 1963); it is also required by the developing embryo for normal brain development (Newburg, 1976), and several different tumor lines have been shown to require exogenous asparagine for growth (Broome, 1968).

Ravel et al. (1962) were the first to demonstrate the presence of asparagine synthetase. This enzyme, derived from *Lactobacillus arabinosus*, was shown to produce asparagine from aspartate in the presence of ammonia, ATP, and magnesium. Later studies demonstrated the presence of asparagine synthetase in mammalian systems (Patterson & Orr, 1967). The mammalian enzyme requires the presence of ATP and Mg^{2+}; however, it utilizes glutamine in addition to ammonia to transform aspartate to asparagine (Levintow, 1957).

Recent reports from our laboratory have described the existence of three different molecular forms of asparagine synthetase from rat liver, all differing in molecular weight. These three forms have been shown to interconvert possibly as the result of some type of control system. The appearance of these three forms of asparagine synthetase has also been shown to be dependent upon dietary and circulatory asparagine levels (Markin & Schuster, 1979; R. S. Markin and S. M. Schuster, unpublished experiments).

Cedar & Schwartz (1969) have described the kinetic mechanism of asparagine synthetase derived from *Escherichia coli* to be bi-uni-uni-bi ping-pong. Recently, Milman et al. (1980) reported a uni-uni-bi-ter ping-pong Theorell–Chance kinetic mechanism for the glutamine-dependent reaction of mouse pancreatic asparagine synthetase. The data presented here elucidate the kinetic mechanism of bovine pancreatic asparagine synthetase in the presence of either NH_3 or glutamine.

Materials and Methods

Materials

DL-[4-^{14}C]Aspartate and all other scintillation supplies were obtained from Research Products International (Elk Grove Village, IL). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO) in the highest quality available. The radioactive aspartate was diluted in 0.4 M aspartate to

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