Nonactivated Phosphorylase Kinase Is a Phosphoprotein: Differentiation of Two Classes of Endogenous Phosphoserine Residues by Phosphorus-31 Nuclear Magnetic Resonance Spectroscopy and Phosphatase Sensitivity†

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ABSTRACT: A standard preparation of phosphorylase kinase from rabbit skeletal muscle contains 2 mol of phosphoserine/mol of $\alpha\beta\gamma\delta$. This basal stoichiometry is not influenced by application of propranolol and insulin in vivo; these serine phosphates could not be hydrolyzed by phosphatases of the muscle extract or by alkaline phosphatases. When the enzyme is purified in the presence of the protein phosphatase inhibitor sodium fluoride, it contains either 1 or 3 additional mol of phosphoserine/mol of $\alpha\beta\gamma\delta$, termed phosphatase-sensitive phosphates. Both classes of phosphates yield in formic acid one single ^{31}P NMR signal of a narrow line width (~3 Hz) very similar in chemical shift to free phosphoserine. Phosphoserine is also identified by its chemical shift when dissolved in 8 M guanidinium chloride and by its electrophoretic mobility after acid hydrolysis. By self-phosphorylation of phosphorylase kinase, 14 additional mol of phosphate/mol of $\alpha\beta\gamma\delta$ was incorporated, and all were identified as phosphoserine by ^{31}P NMR spectroscopy. In native phosphorylase kinase, the ^{31}P NMR signals of both the basal and the phosphatase-sensitive phosphates are substantially broadened and reduced in intensity, indicating strong interactions of the phosphate groups with the protein. The basal and phosphatase-sensitive phosphates give in 8 M guanidinium chloride a homogeneous NMR signal above pH 6; it splits into a doublet below pH 6 and into a triplet below pH 5. When phosphorylase kinase is denaturated in sodium dodecyl sulfate, the resonance signal of the basal phosphates is at ~4.4 ppm, and that of the phosphatase-sensitive phosphates is at ~4.0 ppm at pH 7.0. The basal phosphates show an apparent pK_a of 6.4 and a Hill coefficient (h) of 1.9; the phosphatase-sensitive phosphates have an apparent pK_a of 6.6 and a Hill coefficient of 1.0. Both pK_a values are substantially enhanced compared to that of free phosphoserine (pK_a = 5.7).

Phosphorylation of proteins has been shown to be the main regulatory mechanism by which eucaryotic cells can convert neuronal or hormonal stimuli into intracellular responses. Its involvement in the control of glycogen metabolism is particularly well studied [for reviews, see Krebs & Beavo (1979) and Heilmeyer et al. (1980)]. In most instances, phosphate is bound as phosphoserine or phosphothreonine, while the phosphorylation of tyrosine may be involved in the mechanism of action of retroviral oncogenes and epithelial growth factor [for a review, see Hunter et al. (1981)]. Almost all work on protein phosphorylation has been carried out by studying the incorporation of radioactively labeled phosphate either in vitro or in response to a provoked stimulus (hormone, growth factor, virus transformation, etc.) in vivo. Little is known about endogenous phosphates in proteins in the absence of particular stimuli in vivo. Still, phosphorylation of many proteins may occur under such conditions as a regulatory or a nonregulatory, irreversible posttranslational modification. Endogenous phosphate is known, for example, to be present in the "nutritive" proteins casein, phosvitin, and ovalbumin and presumably serves a structural rather than a regulatory function [for ^{31}P NMR studies on these proteins, see Ho et al. (1969) and Vogel & Bridger (1982)]. Finally, an enzyme may contain both regulatory and structural phosphate groups. The catalytic subunit of the cAMP-dependent protein kinase contains one phosphoserine and one phosphothreonine residue for which structural and regulatory functions are discussed (Shoji et al., 1979, 1981).

Phosphorylase kinase, a tetramer of subunit composition $(\alpha\beta\gamma\delta)_4$, activates glycogen degradation in skeletal muscle during contraction or upon β-adrenergic stimulation [for reviews, see Fischer et al. (1971) and Cohen (1978)]. Nervous excitation results in an enhancement of the sarcoplasmic Ca^{2+} concentration which in turn probably activates a high-affinity Ca^{2+}-dependent activity, A_1 (Heilmeyer et al., 1980; Kilimann & Heilmeyer, 1982a,b). Adrenalin stimulates phosphorylation, catalyzed by the cAMP-dependent protein kinase, of one distinct serine residue on each of the enzyme's α and β subunits (Yeaman & Cohen, 1975). This induces a low-affinity Ca^{2+}-dependent activity, A_2 (Heilmeyer et al., 1980; Kilimann & Heilmeyer, 1982a,b). A cGMP-dependent protein kinase can phosphorylate the same serine residues in vitro (Cohen, 1980a). Self-phosphorylation can result in the incorporation of up to 20 mol of phosphate/mol of $\alpha\beta\gamma\delta$ (Walsh et al., 1971; Hörl et al., 1975; Kilimann & Heilmeyer, 1982b), which stimulates pronouncedly both partial activities, A_1 and A_2 (Kilimann & Heilmeyer, 1982a,b).

In addition to these ways of phosphorylation, it has been reported that the partially purified enzyme isolated from resting muscle without prior application of β-adrenergic agonists contains already ca. 1 mol of alkali-labile phosphate per 100 000 g of protein (Mayer & Krebs, 1970). This latter observation raises the question of whether the so-called nonactivated phosphorylase kinase, as conventionally purified from rabbit skeletal muscle, may already be a phosphoprotein containing phosphate groups of structural or regulatory function.

The present study shows that phosphorylase kinase contains 2 mol of phosphate/mol of $\alpha\beta\gamma\delta$ as a basal value. When the

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enzyme is isolated in the presence of the protein phosphatase inhibitor NaF, the enzyme contains additionally either 1 or 3 mol of phosphate/mol of $\alpha\beta\gamma\delta$. ^{31}P NMR spectroscopy identifies all of these phosphates as phosphoserine, allows us to differentiate the basal phosphates from the additional phosphatase-sensitive phosphates, and indicates that all these phosphate groups are engaged in intramolecular interactions.

Materials and Methods

Phosphorylase kinase was purified according to Cohen (1973) with an additional ion-exchange chromatography step as in Jennissen & Heilmeyer (1975). In a number of preparations, all solutions used contained 25 mM NaF.

Application of Drugs. Four to five milligrams of DL-propranolol per kilogram body weight was injected into the marginal ear vein of rabbits during 3 min, immediately followed by 90 units of insulin/kg. The animals were killed and processed 40 min after the application of insulin.

Treatment with Alkaline Phosphatases. Approximately 12 mg of phosphorylase kinase was incubated at 30 °C either in 300 mM tris(hydroxymethyl)aminomethane (Tris),[1] 10 mM imidazole, 100 mM NH_4Cl, and 1 mM dithioerythritol, pH 9.0, with 4.5 units of alkaline phosphatase from *Escherichia coli* (Sigma, 25 units/mg) or in 100 mM glycine, 10 mM imidazole, 1 mM DTE, 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$, pH 9.0, with 350 units of alkaline phosphatase from calf intestine (Sigma or Boehringer, ca. 500 units/mg). Several samples of ca. 1.5 mg of protein were withdrawn within 50 min and precipitated with trichloroacetic acid. In control experiments under these conditions, *p*-nitrophenyl phosphate was quantitatively hydrolyzed within seconds.

Protein-bound phosphate was determined according to Bartlett (1959). Each sample (ca. 1.5 mg of phosphorylase kinase) was precipitated 3 times with 7.5% trichloroacetic acid and dissolved in 98% formic acid. Successively, 0.3 mL of 5 M H_2SO_4 and two additions of 0.2 mL of 30% H_2O_2 were added, and each time the sample was incubated at 160 °C for at least 2 h. Phosphate was stained by the addition of 600 μL of water, 200 μL of 5% ammonium heptamolybdate, and 50 μL of 0.25% 1-amino-2-hydroxynaphthalenesulfonic acid, 15% $NaHSO_3$, and 0.5% Na_2SO_3 at 100 °C for 30 min. Optical density was measured at 830 nm.

Protein causes background staining; therefore, the optical densities of phosphate-free protein standards were subtracted from those of phosphorylase kinase samples. Routinely, phosphorylase *b* was used as the protein standard; bovine serum albumin and hemoglobin yielded identical results. If possible, all samples, processed in parallel, contained the same protein quantities.

For the identification of phospho amino acids, 30–50 mg of phosphorylase kinase was precipitated by HCl or trichloroacetic acid and redissolved in 30–50 mL of 6 N HCl. The material was hydrolyzed at 110 °C for 3–4 h under N_2, evaporated, dried in vacuo over KOH, and dissolved in ca. 50 μL of water. Electrophoretic separation of the hydrolysate was carried out on thick paper (Macherey & Nagel, MN 2827) in 7.8% acetic acid and 2.2% formic acid, pH 1.85, at 2–3 kV. The paper area around the origin retained substantial quantities of the only ninhydrin-positive material not migrating toward the cathode. This material was eluted with water, and rehydrolyzed for 4 h at 110 °C in 8 mL of 6 N HCl, dried, and electrophoresed as above, and stained with 0.01% ninhydrin.

Table I: Protein-Bound Phosphate in Nonactivated Phosphorylase Kinase[a]

preparation mode	mol of phosphate/mol of $\alpha\beta\gamma\delta$
without NaF	1.96 ± 0.33 (11)[b]
25 mM NaF	2.92 ± 0.20 (6)
	4.85 ± 0.51 (4)
without NaF after application of propranolol and insulin	2.12 ± 0.55 (3)

[a] Up to five independent determinations were carried out in separate experiments for every preparation, and three to four samples were analyzed simultaneously in each of these determinations. The molecular weight of the $\alpha\beta\gamma\delta$ unit is taken as 320 000 (Cohen, 1973). For other experimental details, see Materials and Methods. [b] Values in parentheses indicate the number of enzyme preparations.

Phosphorylase kinase activity was determined according to Jennissen & Heilmeyer (1974) and the activity of phosphorylases *b* and *a* according to Haschke & Heilmeyer (1972). Protein was determined on an autoanalyzer according to Lowry et al. (1951); $NaDodSO_4$–polyacrylamide gel electrophoresis was performed as described in Weber & Osborn (1969).

For the determination of inorganic phosphate and ATP contents in a muscle homogenate, samples were withdrawn within seconds after the homogenization was terminated and mixed with the same volume of 1.2 M perchloric acid; 1 M K_2CO_3 was added to neutralize the sample, and after 5 min on ice, the sample was centrifuged, phosphate was measured according to Haschke & Heilmeyer (1972) on an autoanalyzer, and ATP content was determined according to Lamprecht & Trautschold (1974).

^{31}P NMR Spectroscopy. Native phosphorylase kinase was transferred from the usual glycerol 2-phosphate buffer into imidazole–fluoride buffer (75 mM NH_4Cl, 25 mM NaF, 1 mM dithioerythritol, 1 mM EGTA, and 20 mM imidazole, pH 6.8) by gel filtration. The enzyme was precipitated by 1.25 M ammonium sulfate, pH 6.8, and redissolved in 12 mL of the same buffer. The final pH of samples was adjusted with 1 M HCl and NH_3. After this procedure, low molecular weight phosphorus compounds cannot be detected in the sample. Fourier-transformed ^{31}P NMR measurements were performed on a bruker WH 180 wide-bore superconducting spectrometer at 72.86 MHz. They were carried out at 2 °C with the native protein and at 20 °C with the denaturated protein. Sample volumes of 12 mL in 20-mm diameter tubes were used. A concentric 5-mm NMR tube containing ^2H_2O was employed as field/frequency lock. The spectra represent 4096 data points at a spectral width of 1200 or 2200 Hz. The pulse width was 25 μs. The recorded spectra were proton decoupled (0.4 W). Exponential line broadening applied prior to Fourier transformation was 5–10 Hz. All line-width data have been corrected for this line-broadening effect. Chemical shifts were referenced to 85% H_3PO_4. Upfield shifts are defined as positive.

Results

Phosphorylase kinase contains about 2 mol of phosphate/mol of $\alpha\beta\gamma\delta$ (Table I). This basal amount of phosphate is also present when the animal is pretreated with high doses of propranolol and insulin (Table I). When the enzyme is purified in the presence of 25 mM NaF, significantly higher amounts of protein-bound phosphate are found. Six enzyme

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid.

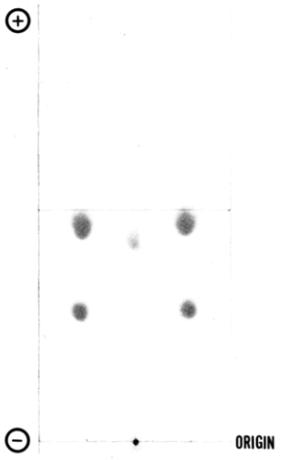

FIGURE 1: Electrophoretic identification of phosphoserine in acid hydrolysates of nonactivated phosphorylase kinase. Side lanes, 20 μg each of free phosphoserine (top) and phosphothreonine (bottom). Central lane, 30 mg of phosphorylase kinase which was hydrolyzed twice and resolved as described under Materials and Methods.

preparations cluster around three mol of phosphate/mol and four batches around 5 mol of phosphate/mol (Table I).

Apparently, the overall increase in the degree of phosphorylation is achieved by the inhibition of phosphoprotein phosphatase activity. It is unknown, however, which factors determine a stoichiometry of either 3 or 5 mol of phosphate. It can be excluded that the enzyme incorporates phosphate during homogenization or purification. When [γ-^{32}P]ATP was added to the muscle before homogenization, only 5.4 mmol of radioactive phosphate was found in the isolated enzyme compared to 4.6 mol of total phosphate/mol of $\alpha\beta\gamma\delta$.

All of the phosphates are identified as phosphoserine. Repeated acid hydrolysis (see Materials and Methods) and electrophoretic separation yield phosphoserine as the main product. The major ninhydrin-positive material migrates slightly slower than the phosphoserine employed as an external standard (Figure 1) but comigrates with phosphoserine when added to the hydrolysate as an inner standard (not shown). Two minor spots are also observed, one migrating faster and the other moving slower than phosphoserine. The same pattern is found when phosphorylase *a* is hydrolyzed and employed for comparison (not shown).

The basal 2 mol of phosphate/mol of $\alpha\beta\gamma\delta$ that is stable against the protein phosphatase activities in the muscle extract could not be split off by alkaline phosphatases (see Materials and Methods), which was attempted at various free Ca^{2+} (1 nM or 80 μM) or Mg^{2+} (0 or 12.5 mM) concentrations.

In ^{31}P NMR spectroscopy, phosphorylase kinase containing only these two basal phosphates shows one signal with a narrow line width of ca. 3 Hz when the protein is solubilized in formic acid (Figure 2). This resonance signal is very similar in chemical shift and identical in line width with the signal of free phosphoserine (δ 0.49) recorded in the same solvent, in agreement with the chemical identification as phosphoserine. No other signal is observed, especially near 0 ppm which would reveal contaminating nucleic acids [for comparison, see Koppitz et al. (1978)]. Enzyme which was purified in the presence of NaF and which contains the additional 1–3 mol of phosphate/mol of $\alpha\beta\gamma\delta$ yields the same single sharp ^{31}P NMR signal with the same chemical shift as the basal phosphate (not shown), identifying also the phosphatase-sensitive phosphate groups as phosphoserine. Fourteen moles of phosphate per mole of $\alpha\beta\gamma\delta$ was incorporated into phosphorylase kinase by self-phosphorylation. This form of the enzyme also yields one sharp ^{31}P NMR signal which coincides with

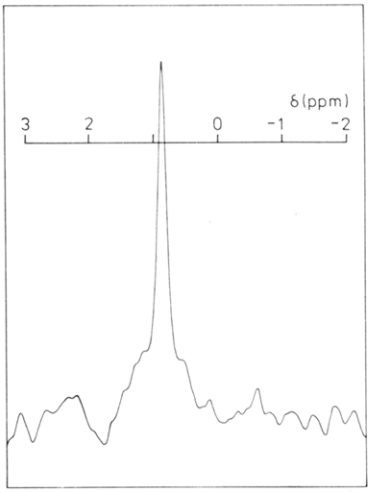

FIGURE 2: ^{31}P NMR spectrum of phosphorylase kinase denatured in formic acid. Phosphorylase kinase (105 mg, purified without NaF) containing 2.0 mol of phosphate/mol of $\alpha\beta\gamma\delta$ was reprecipitated 3 times with 5% trichloroacetic acid and dissolved in 98% formic acid. The spectrum represents 27 735 scans at a repetition time of 0.93 s.

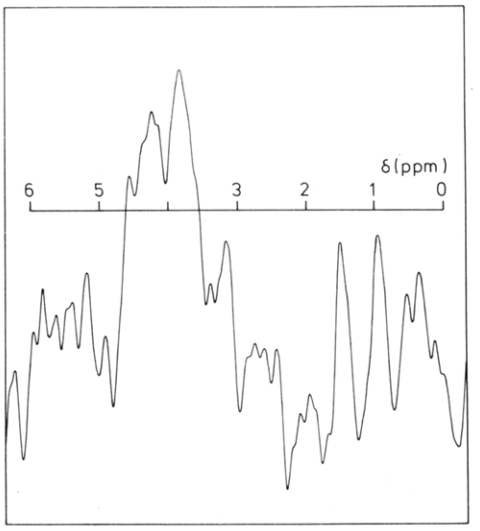

FIGURE 3: ^{31}P NMR spectrum of native phosphorylase kinase. Phosphorylase kinase (60 mg, purified in the presence of 25 mM NaF) containing 4.7 mol of phosphate/mol of $\alpha\beta\gamma\delta$ was dissolved in imidazole–fluoride buffer (cf. Materials and Methods) at pH 6.67. The spectrum represents 37 782 scans at a repetition time of 1.7 s.

the signal of the endogenous phosphate, demonstrating that self-phosphorylation occurs on serine residues only (not shown).

Native phosphorylase kinase containing either 2 or 3–5 mol of phosphate/mol of $\alpha\beta\gamma\delta$ shows a pronouncedly broadened ^{31}P NMR signal; line widths of 20–50 Hz are estimated. Therefore, resolution is poor, but it seems that a single signal is obtained with enzyme purified without NaF (not shown) and a doublet after purification in 25 mM NaF (Figure 3). It is concluded that in the native state, the phosphate groups interact with amino acid side chains in the protein, resulting in immobilization which causes the line broadening. Formic acid interrupts these interactions of the phosphate groups; therefore, a signal like that of free phosphoserine is found.

When phosphorylase kinase (purified in 25 mM NaF; 3.1 mol of phosphate/mol of $\alpha\beta\gamma\delta$) is denatured in 8 M guanidinium chloride, the chemical shift of the endogenous phosphate NMR signal is found to be pH dependent (Figure 4A). The titration curve obtained is closely parallel to that of free phosphoserine, and both curves coincide above pH 6, i.e., where phosphoserine is in its dianionic form. This confirms that all

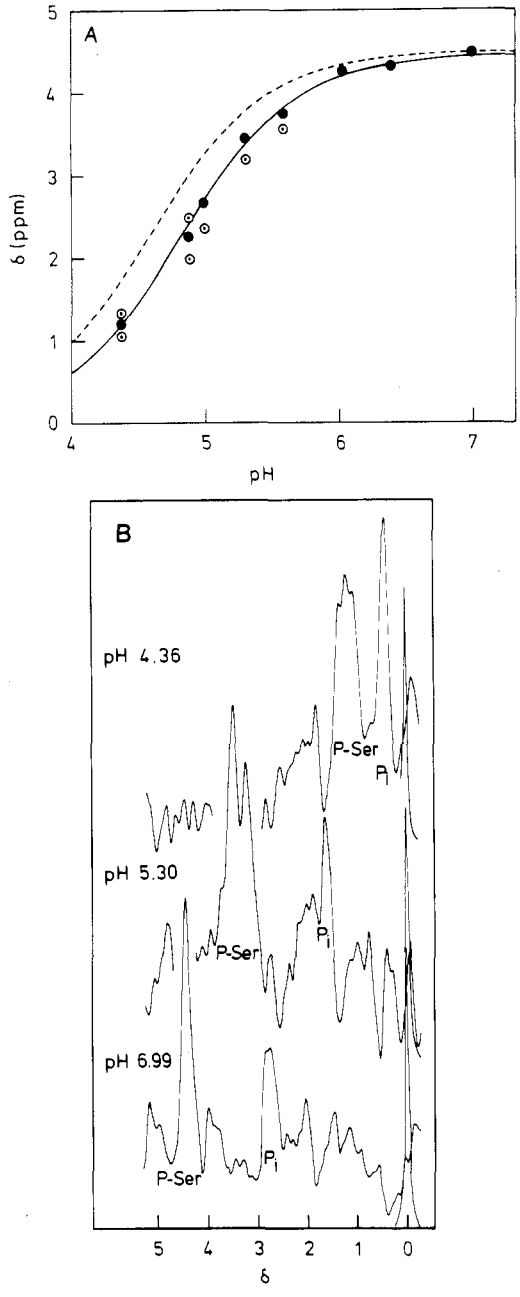

FIGURE 4: pH dependence of the ^{31}P NMR signals of phosphorylase kinase, denatured in guanidinium chloride. (A) (Titration curve) Phosphorylase kinase (70 mg, purified in the presence of 25 mM NaF, 3.1 mol of phosphate/mol of $\alpha\beta\gamma\delta$) was dissolved in imidazole–fluoride buffer plus 8 M guanidinium chloride and 10 mM EDTA: (●) main signal; (⊙) side signal(s); (—) curve fit to the main signal (Hill coefficient $h = 1$); (---) free phosphoserine standard in the same buffer. (B) Sample spectra demonstrating singlet (pH 6.99, 62 266 scans), doublet (pH 5.30, 85 167 scans), and triplet (pH 4.36, 144 461 scans) signals as a function of pH (repetition time 1.7 s).

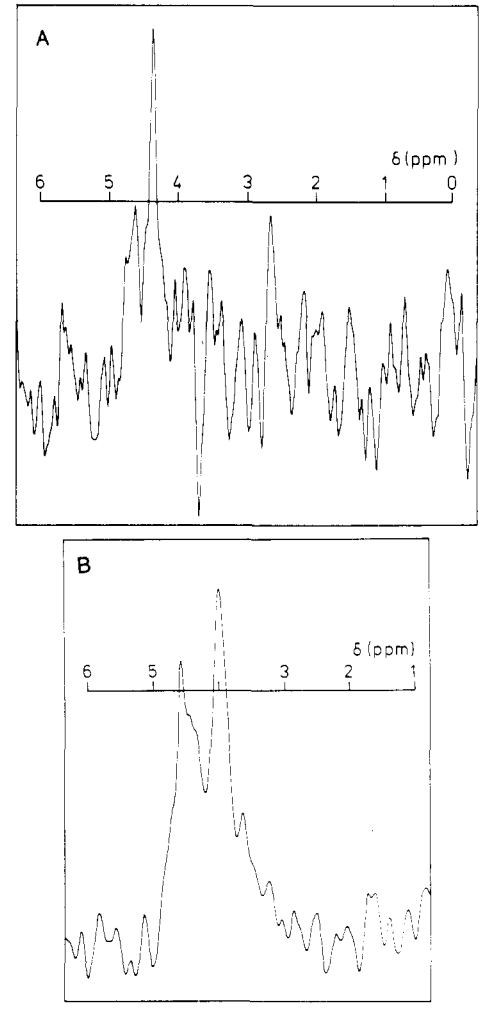

FIGURE 5: ^{31}P NMR spectra of $NaDodSO_4$-denatured phosphorylase kinase. (A) Phosphorylase kinase (90 mg, purified without NaF) containing 2.0 mol of phosphate/mol of $\alpha\beta\gamma\delta$ was dissolved in imidazole–fluoride buffer plus 5% $NaDodSO_4$, pH 7.0. The spectrum represents 91 734 scans at a repetition time of 1.7 s. (B) Phosphorylase kinase (100 mg, purified in the presence of 25 mM NaF) containing 4.5 mol of phosphate/mol of $\alpha\beta\gamma\delta$ was dissolved in imidazole–fluoride buffer plus 6% $NaDodSO_4$, pH 7.0. The spectrum represents 137 864 scans at a repetition time of 1.7 s.

the endogenous phosphate is present as a serine monoester rather than, potentially, a diester from which phosphoserine monoester might be liberated by acid hydrolysis in HCl or HCOOH.

Compared to free phosphoserine, the pK_a is shifted toward alkalinity by 0.2 pH unit, i.e., the protonated form is stabilized. This may be caused by anionic amino acid side chains in their vicinity, as found in other phosphoproteins [cf. Vogel & Bridger (1982)]. Moreover, it is found that the NMR signal, which is a sharp singlet above pH 6, splits into a doublet below pH 6 and into a triplet below pH 5 (Figure 4B). It suggests that there are three species of phosphoserine differing in their environment and consequently having slightly different pK_a values, which correlates well with the stoichiometry of 3.1 mol/mol of $\alpha\beta\gamma\delta$. The singlet peak appearing downfield is identified as inorganic phosphate; the inset peak is the phosphoric acid reference signal.

When denatured in $NaDodSO_4$, one NMR signal is obtained (δ 4.4 at pH 7.0) for enzyme purified without NaF (2.0 mol of phosphate/mol of $\alpha\beta\gamma\delta$) (Figure 5A). If purified in 25 mM NaF (phosphate stoichiometry of 4.5 mol/mol of $\alpha\beta\gamma\delta$), an additional signal appears at δ 4.0 (pH 7.0) (Figure 5B). Thus, basal and phosphatase-sensitive phosphates give separate NMR signals. Their pH titration curves differ substantially from that of free phosphoserine ($pK_a = 5.7$) in the same buffer, being shifted to the alkaline by almost 1 pH unit (Figure 6). Moreover, computer fitting shows that the titration curve of the phosphatase-sensitive phosphates ($pK_a = 6.6$) exhibits normal steepness of unity at the inflection point, while that of the basal phosphate ($pK_a = 6.4$) has a Hill coefficient of $h = 1.9$. Consequently, both curves intersect at pH 6.0. These effects either may reflect interactions within a residual native conformation that is not disrupted by $NaDodSO_4$ or may be produced by the association of $NaDodSO_4$. Even in the latter case, however, this association must occur

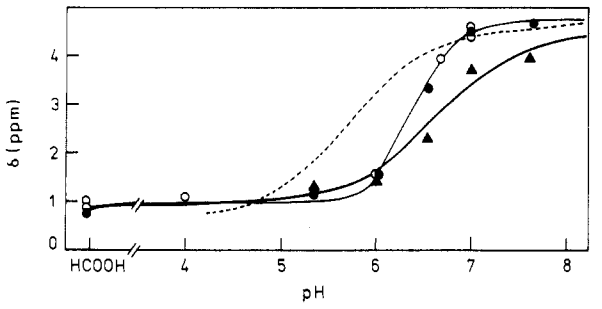

FIGURE 6: pH dependence of the ^{31}P NMR signals of phosphorylase kinase, denatured in $NaDodSO_4$. (○) Enzyme sample as in Figure 5A (basal phosphates); (●, ▲) enzyme sample as in Figure 5B; (●) basal phosphates; (▲) phosphatase-sensitive phosphates; (---) free phosphoserine standard dissolved in the same buffer. The curves were fitted with the program FIT as described by Jahnke & Heilmeyer (1980).

in an ordered way, reflecting distinct properties of the phosphorylation sites and their vicinity, as is shown by the clear resolution of the signals for the basal and the phosphatase-sensitive phosphates.

Discussion

Nonactivated phosphorylase kinase usually contains 2 mol of phosphate/mol of $\alpha\beta\gamma\delta$ when isolated without NaF. On rare occasions, the enzyme was obtained with higher phosphate contents (up to 2.8 mol/mol of $\alpha\beta\gamma\delta$). In such a case, the ^{31}P NMR spectrum revealed minor signals at 0.29 and −0.45 ppm not usually observable in purified phosphorylase kinase, identifying an additional 0.8 mol of phosphate/mol of $\alpha\beta\gamma\delta$ (by relative peak area) as contaminants; the latter signal coincides with that of phosphothreonine (data not shown).

Thus, ^{31}P NMR spectroscopy is a very sensitive and specific method to check the purity of a phosphoprotein with respect to other phosphate-containing materials; ~90% of our phosphorylase kinase preparations are ^{31}P NMR spectroscopically homogeneous.

The purification of phosphorylase kinase after addition of radioactively labeled ATP demonstrates that neither the basal phosphate nor the phosphatase-labile phosphates are incorporated after the onset of homogenization of the minced muscle. Apparently, chelation of Mg^{2+} by EDTA effectively inhibits the protein kinases of the homogenate. Signs of phosphatase action could not be found during storage or the late phases of purification. There remains the possibility of a postmortal change in the degree of phosphorylation during the slaughter of the animal and the chilling of the cadaver and the excised muscle on ice, and especially after mincing of the muscle.

Which protein kinase or kinases are responsible for the incorporation of the endogenous phosphate is at present unknown. The basal phosphate was not influenced by the application of propranolol in vivo. This argues against the involvement of a cAMP-dependent protein kinase in response to a β-adrenergic stimulus. Likewise, the participation of an insulin-dependent mechanism seems unlikely for the incorporation of the basal phosphates. Self-phosphorylation may be considered as a source for the basal as well as the phosphatase-sensitive phosphate, as it can be catalyzed by the Ca^{2+}-independent activity (A_0) of phosphorylase kinase and may therefore be expected to occur in resting muscle (Kilimann & Heilmeyer, 1982b).

The two basal phosphates are removed neither by protein phosphatases in the muscle extract nor by alkaline phosphatases. This stability may suggest that they play a structural rather than a regulatory role. They both seem to be located in similar environments since they yield a single NMR signal in the native as well as in the $NaDodSO_4$-solubilized form of the enzyme. However, they can apparently be resolved in guanidium chloride below pH 5 (Figure 4), resulting in a triplet for an enzyme preparation containing 1 additional mol of phosphatase-sensitive phosphate. The phosphatase-sensitive phosphates yield a ^{31}P NMR signal that differs from that of the basal phosphate when the enzyme is denatured in $NaDodSO_4$ (Figures 5 and 6) and apparently also in the native state (Figure 3). Again, these phosphates seem to be present in similar environments since they yield one resonance band which does not split during pH changes.

It remains to be investigated in detail whether and how the endogenous phosphates may modify the enzymatic properties of phosphorylase kinase. In particular, the lability of the phosphatase-sensitive phosphates would suggest a regulatory function for them. We could not observe significant differences between batches with 2, 3, or 5 mol of phosphate/mol of $\alpha\beta\gamma\delta$ in the standard activity assays at pH 6.8 or 8.2. A more discriminate investigation of the activities A_0, A_1, and A_2 is in progress. At present, it seems particularly difficult to look into the role of the basal phosphate as no means are known to prepare phosphorylase kinase with less than 2 mol of endogenous phosphate. Instead of directly influencing one of the enzyme's catalytic activities, the endogenous phosphate may modulate other regulatory mechanisms like a further phosphorylation or dephosphorylation or influence the interaction of phosphorylase kinase with exogenous calmodulin (δ'), troponin C (Cohen, 1980b), other proteins (Carlson & Graves, 1976), or glycogen (Krebs et al., 1964; Steiner & Mashall, 1982).

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Registry No. Phosphorylase kinase, 9001-88-1; L-phosphoserine, 407-41-0.

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Iminoglycine Transport System in Synaptosomes and Its Interaction with Enkephalins†

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ABSTRACT: Evidence is presented which suggests that proline, pipecolic acid, and glycine are accumulated by a common transport system in rat brain cortical synaptosomes and synaptosomal plasma membrane vesicles. This system is Na^+ dependent and appears to be similar to the iminoglycine transport system present in renal tubules and in renal brush border membranes. The opioid pentapeptides Leu- and Met-enkephalin specifically inhibit the uptake of these three imino/amino acids, presumably by interaction with a nonopioid receptor, since the inhibition is not affected by the opiate antagonist naloxone and occurs with des-tyrosyl enkephalins as well as with the intact pentapeptides.

Proline, hydroxyproline, and glycine have been shown to be transported via a common transport system in the renal tubule. Evidence for the presence of this system, commonly referred to as the iminoglycine transport system, stems from in vitro studies as well as from clinical studies on familial iminoglycinuria, an autosomal recessive inborn error of membrane transport characterized by an exaggerated renal clearance of proline, hydroxyproline, and glycine (Scriver et al., 1961; Scriver & Wilson, 1964; Rosenberg et al., 1968; Scriver, 1983). Iminoglycinuria appears to be the result of inactivation or defect in the membrane transport protein. From their studies on proline transport by isolated brain capillaries, Hwang et al. (1983) suggested that inherited abnormalities of renal transport such as iminoglycinuria may have a cerebral counterpart and that genetic regulation in the two tissues may be related. Other evidence suggests that pipecolic acid, a six-membered analogue of proline, shares a common transport system with proline in mouse brain synaptosomes (Nomura et al., 1980) and in rabbit brush border membrane vesicles (Ganapathy et al., 1983) where it was suggested that pipecolic acid is accumulated via the iminoglycine transport system.

We have recently shown (Rhoads et al., 1983c) that synaptosomal uptake of proline is strongly inhibited by micromolar concentrations of Leu- and Met-enkephalins and that this inhibition cannot be reversed by naloxone. We have also shown that enkephalins have no effect on several other putative or established neurotransmitters such as glutamic acid, aspartic acid, taurine, and γ-aminobutyric acid. In the present study, evidence is presented that in both synaptosomes and synaptosomal plasma membrane vesicles, proline, pipecolic acid, and glycine are accumulated via a common iminoglycine transport system and that Leu- and Met-enkephalins inhibit specifically this system presumably by interaction with a nonopioid receptor.

Experimental Procedures

Materials. L-[U-^{14}C]Proline and L-[U-^{14}C]glycine were purchased from New England Nuclear Corp. (Boston, MA) and had the following specific activities (millicuries per millimole): proline, 293; glycine, 104. DL-[^{3}H]Pipecolic acid (2.98

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