Activation by phosphorylation of phosphofructokinase from the annelid *Lumbricus terrestris* and comparison of phosphorylated sites in invertebrate phosphofructokinases

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Purified phosphofructokinase from the earthworm *Lumbricus terrestris* was phosphorylated *in vitro* by the catalytic subunit of cAMP-dependent protein kinase from the same organism to an extent of approx. 0.5 mol/mol of subunit. Activation of the enzyme occurred in parallel to the incorporation of covalently bound phosphate and was reversed by the action of the catalytic subunit of protein phosphatase 2A. Phosphorylation decreased the co-operativity of fructose 6-phosphate saturation in the presence of inhibitory concentrations of ATP, and increased the

apparent $V_{\max}$ obtained with saturating concentrations of the activators 5'-AMP and fructose 2,6-bisphosphate. The phosphorylated sites of phosphofructokinase from *L. terrestris* and from two molluscs (*Helix pomatia* and *Mytilus edulis*) were sequenced and shown to exhibit distinct similarity to sequences located near to the N-terminus of nematode phosphofructokinases [Klein, Olson, Favreau, Wintertowd, Hatzenbuehler, Shea, Nulf and Geary (1991) Mol. Biochem. Parasitol. 48, 17–26].

INTRODUCTION

The phosphofructokinase reaction is generally regarded as a key regulatory step in glycolysis. Allosteric inhibition of the enzyme by physiological concentrations of ATP and its relief by positive allosteric effectors provide an efficient kinetic basis for the control of enzyme activity. Fructose 2,6-bisphosphate has been found to be the most effective activator of phosphofructokinases from animal and plant tissues, and from yeast (see [1] for a review and literature). The cellular levels of this allosteric regulator of phosphofructokinase are controlled by a specific kinase and phosphatase that reside on a bifunctional protein in mammalian liver (see [2] for a review).

In addition to allosteric control, phosphofructokinase may also be regulated by reversible phosphorylation. Activation of phosphofructokinase by phosphorylation at a single serine residue was first described in *Ascaris suum* [3,4] and later confirmed for the enzyme from other helminths [5,6]. An activating cAMP-dependent protein kinase from *Ascaris suum* [4,7,8] and type 2A protein phosphatases from the nematode [4,9] that inactivate phosphofructokinase have been purified to homogeneity and characterized. There is biochemical [10] and functional [11] evidence that phosphorylation is also involved in the regulation of mollusc phosphofructokinase. In contrast to the situation in invertebrates, the functional significance of the regulation of phosphofructokinase from vertebrates by reversible phosphorylation has remained controversial (see [12] for a review).

The present investigation was undertaken in order to assess the occurrence and role of the phosphorylation of phosphofructokinase in annelids. Phosphorylation of the glycolytic enzyme from the muscle of the earthworm, *Lumbricus terrestris*, was effected by a cAMP-dependent protein kinase from the same tissue. We also compared the structure of the phosphorylated site

in phosphofructokinase from *Lumbricus* with the phosphorylation sites of mollusc phosphofructokinases.

MATERIALS AND METHODS

Materials

Chemicals and biochemicals were obtained from Merck (Darmstadt, Germany), Boehringer (Mannheim, Germany) or Sigma. [32 P]Phosphate was from Amersham and was used for the synthesis of [γ - 32 P]ATP as described previously [7]. Fructose 2,6-bisphosphate and pyrophosphate-dependent phosphofructokinase from potato tubers were prepared and assayed as described in [13]. Trypsin (bovine pancreas) was obtained from Boehringer Mannheim and treated with Tos-Phe-CH₂Cl ('TPCK') (Serva) before use.

Assays

Phosphofructokinase assays under 'regulatory conditions' were performed at pH 6.8 (30 °C) in a Gilford 250-S spectrophotometer at 340 nm. Unless otherwise indicated, the assay mixture contained 50 mM imidazole/HCl buffer, 5.0 mM MgCl₂, 0.15 mM NADH, 1.5 mM ATP, 1.0 mM fructose 6-phosphate, aldolase (0.01 mg/ml), triosephosphate isomerase (0.001 mg/ml) and glycerol-1-phosphate dehydrogenase (0.005 mg/ml). The reactions were initiated by the addition of phosphofructokinase. Reference activities were determined under 'non-regulatory conditions' at pH 8.0 in triethanolamine/HCl buffer in the presence of 1.5 mM ATP and 3.0 mM fructose 6-phosphate.

Protein determination

This was done by the method of Bradford [14] using purified rabbit skeletal muscle phosphofructokinase as standard.

Abbreviation used: PTH, phenylthiohydantoin.

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Tissue preparation and extraction

The purification procedure employed for phosphofructokinase from *Lumbricus* was similar to that previously described for the enzyme from *Ascaris suum* [15]. Briefly, the animals were anaesthetized by immersion into 10% ethanol and the muscular layer was dissected out, removing adhering parts of other organs. The tissue was then homogenized in a 6-fold volume (w/v) of buffer A [10 mM Tris/phosphate, pH 7.6, 1 mM EDTA, 5% glycerol, 10 units of the kallikrein inhibitor aprotinin (Trasylol, Bayer Leverkusen, Germany) and 1 μ M pepstatin A] containing 50 mM NH_4Cl . The homogenate was centrifuged at 30 000 *g* for 30 min and the supernatant was filtered through glass wool.

Purification of phosphofructokinase

In order to remove protein kinase activity from the purified phosphofructokinase, cAMP was added to the extract to a final concentration of 0.2 mM. After a 5 min incubation, an equivalent of 1 g of DEAE-cellulose (DE-52; Whatman) per g of tissue was added and the mixture was stirred manually for 10 min. The mixture was then washed with buffer A containing 0.02 mM cAMP and 80 mM NH_4Cl on a Buchner funnel until the difference in absorbance at 280 nm was less than 0.1 cm^{-1} . Then the ion-exchange material on the funnel was eluted with buffer A containing 150 mM NH_4Cl , and 50 ml fractions were collected.

An equivalent of 0.2 g of damp phosphocellulose (Sigma; equilibrated in buffer A) per g of tissue was added to the phosphofructokinase-containing fractions. About 60% of phosphofructokinase bound to the phosphocellulose. The cellulose was washed with buffer A plus 80 mM NH_4Cl on a Buchner funnel until the difference in A_{280} was less than 0.01 cm^{-1} . Then the material was dispersed in buffer A, packed into a chromatography column and the phosphofructokinase was eluted by buffer A containing 50 mM NH_4Cl and 5 mM ATP.

The phosphofructokinase-containing fractions were dialysed against buffer A for 2 h and subsequently chromatographed on a Mono Q FPLC column (Pharmacia) equilibrated in buffer A containing 50 mM NH_4Cl . Phosphofructokinase was eluted as a separate peak by a linear gradient from 50 to 500 mM NaCl in buffer A.

Phosphofructokinases from the molluscs *Mytilus edulis* and *Helix pomatia* were purified as previously described [10].

Purification of protein kinase

For the purification of the catalytic subunit of cAMP-dependent protein kinase, the tissue extract of *Lumbricus* was loaded on to a DE-52 column in the absence of cAMP. The column was extensively washed with buffer A containing 55 mM NaCl until the A_{280} of the eluate was equal to that of the buffer. Elution of the catalytic subunit of the protein kinase was accomplished using buffer A containing 45 mM NaCl and 0.1 mM cAMP. The protein kinase-containing fractions were dialysed against buffer A and applied to a chromatographic column containing histone immobilized on Sepharose. The column was washed with 20 vol. of buffer A containing 50 μ M cAMP and the protein kinase was subsequently eluted with a linear gradient from 0 to 300 mM NaCl in buffer A. BSA (0.2 mg/ml) was added to the pooled protein kinase-containing fractions, which were dialysed for 2 h against saturated ammonium sulphate adjusted to pH 7.0. The resulting precipitate was collected by centrifugation, dissolved in buffer A and applied to a Superose 12 column (Pharmacia 16/50). The protein kinase-containing fractions were concentrated by dialysis against glycerol, frozen in liquid N_2 and stored at -70°C .

Phosphorylation of phosphofructokinase

Phosphofructokinase was phosphorylated *in vitro* in buffer A containing 5 mM MgCl_2 , 0.1 mM ATP (specific radioactivity of $[\gamma\text{-}^{32}\text{P}]\text{ATP} = 10\text{--}20 \text{ MBq}/\mu\text{mol}$), 5 nM Microcystin-LR (Sigma) and the catalytic subunit of cAMP-dependent protein kinase from *Lumbricus* [final activity $0.12 \text{ nmol}\cdot\text{min}^{-1}\cdot\text{ml}^{-1}$ as determined with histone II-S (Sigma) as substrate]. The phosphorylation reaction was initiated by adding phosphofructokinase. The incorporation of phosphate was monitored as described previously [4]. To facilitate separation of phosphofructokinase from unreacted ATP, fructose 6-phosphate (final concentration 0.5 mM) was added after a 30 min incubation at 30°C . The mixture was chromatographed on Sephadex G-50 (medium grade; Pharmacia) equilibrated in buffer A in a Pasteur pipette column at 4°C . The enzyme was eluted in fractions of 100 μ l by 20 mM ammonium formate, pH 8.0, containing 15 mM 2-mercaptoethanol, 1 mM *p*-nitrophenyl phosphate (Merck) and 5 nM Microcystin-LR. Radioactivity was determined by Cerenkov counting.

Proteolytic cleavage

For limited tryptic cleavage, phosphorylated phosphofructokinase was added to Tos-Phe- CH_2Cl -treated trypsin dissolved in 15 mM ammonium formate containing 15 mM 2-mercaptoethanol. The ratio of proteinase to phosphofructokinase was 1:100 (w/w). The incubation was carried out at 30°C . The release of radioactive peptides was monitored by determining the amount of protein-bound ^{32}P . The reactions were terminated after 30 min by adding a 5-fold excess of soybean trypsin inhibitor (Sigma). Undigested protein was separated from low- M_r peptides by centrifugation (18 000 *g*) after 5 min of incubation at 96°C . The supernatant containing the radioactive peptides was concentrated to a volume of about 10 μ l.

Purification of the phosphopeptides

The aqueous peptide solution was diluted with 90 μ l of 10% acetonitrile containing 0.05% trifluoroacetic acid and applied on to a C_{18} -Spherisorb OPSII 4.6 mm $\times$ 250 mm reverse-phase HPLC column. Elution was carried out with a gradient of 10–80% acetonitrile in 0.05% trifluoroacetic acid. Peptides were detected by absorbance at 226 nm. Fractions of 0.5 ml were collected and counted for radioactivity (Cerenkov method). Fractions containing radioactivity were pooled, desiccated, diluted in water and re-chromatographed two or three times on a C_{18} reverse-phase HPLC column (Aquapore RP-300; 1 mm $\times$ 250 mm) using the Applied Biosystems 130A Separation System of the Welmet Laboratory at the University of Edinburgh. Elution was accomplished by a linear gradient of 0–70% acetonitrile in 1% trifluoroacetic acid at a flow rate of 0.1 ml/min during 65 min. Fractions were collected manually on the basis of the absorbance recording and counted for radioactivity.

Sequence analysis

Sequencing of purified phosphopeptides was done with an automatic Applied Biosystems 477A Protein Sequencer coupled to a 120A phenylthiohydantoin (PTH)-amino acid analyser, as described previously [16].

Curve fitting procedure

Experimental data describing the dependence of phosphofructokinase activity on substrate and activator concentrations were

fitted to the Hill equation, $v = (VS^n)/(S_{0.5}^h + S^n)$, by a non-linear least-squares method. The program was written in PASCAL for a personal computer following the approximation procedure outlined by Johnson [17].

RESULTS

Purification and properties of phosphofructokinase from *L. terrestris*: activation of the enzyme by phosphorylation

Phosphofructokinase from the muscle of the earthworm, *L. terrestris*, was purified 320-fold with 15% yield as summarized in Table 1. The preparations exhibited a single band on SDS/PAGE with an estimated M_r of 80000 (Figure 1, lane a). The protein was phosphorylated by cAMP-dependent protein kinase from earthworm muscle and also by the catalytic subunit of mammalian protein kinase in the presence of [γ - 32 P]ATP (Figure 1, lanes d and e). Maximum incorporation occurred to a stoichiometry of 0.42 phosphate per phosphofructokinase subunit (mol/mol). The phosphate content of the purified enzyme was less than 0.1 phosphate per subunit before phosphorylation due to the presence of protein phosphatases during the first steps of the preparation procedure. Although proteinase inhibitors were included during all steps of purification and the pattern of protein staining on polyacrylamide gels after electrophoresis in the presence of SDS did not indicate partial degradation of the enzyme, no firm conclusion can be reached as to whether the

Table 1 Purification of phosphofructokinase from *Lumbricus terrestris*

Step	Volume (ml)	Protein (mg)	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	Purification (-fold)	Yield (%)
Extract	310	2760	0.38	1	100
DE-52	80	152	3.8	10	55
P-Cellulose	22	5.80	42	110	23
Mono-Q	1.8	1.25	120	320	15

Figure 1 SDS/PAGE of purified phosphofructokinase from *Lumbricus terrestris*

Lanes a–e, gels stained with Coomassie Blue: a, marker proteins; b, phosphofructokinase from *L. terrestris*; c, phosphofructokinase plus dilute catalytic subunit of protein kinase A from *L. terrestris*; d, phosphofructokinase from *L. terrestris* plus concentrated catalytic subunit of protein kinase A from bovine heart; e, protein kinase A from bovine heart. Samples in lanes c–e were incubated with [γ - 32 P]ATP (0.1 mM). Lanes f–h, autoradiographs of lanes c–e respectively. Numbers to the left and right are $10^{-3} \times M_r$.

Figure 2 Time course of the incorporation of radioactive phosphate into phosphofructokinase from *Lumbricus terrestris* and of catalytic activity

Activity determinations (●) were done at pH 6.8 in the presence of 10 mM fructose 6-phosphate and 1.5 mM ATP, as described in the Materials and methods section. Incorporation of phosphate (○) is given as phosphate (P) per subunit (mol/mol).

Table 2 Reversibility of phosphorylation and activation of phosphofructokinase from *Lumbricus terrestris*

Purified phosphofructokinase was used. Treatment 1 consisted of incubation with protein kinase and [32 P]ATP for 10 min, followed by removal of ATP. Treatment 2 consisted of incubation with protein phosphatase for 10 min. n.d., not determined.

Treatment	Activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$)	Phosphate content (mol/mol)
None	0.08	n.d.
1	1.10	0.39
2	0.40	0.15

maximum extent of phosphorylation obtained in our experiments reflects half-of-the-site phosphorylation or partial loss of the phosphorylation site during enzyme purification. Phosphorylation of the enzyme protein led to a concomitant increase in phosphofructokinase activity (Figure 2). The increase in activity was reversed by the addition of partially purified protein phosphatase 2A_c (Table 2).

Effect of phosphorylation on the allosteric properties of phosphofructokinase

The effect of phosphorylation on the allosteric properties of phosphofructokinase is demonstrated in Figure 3. In the presence of an inhibitory, i.e. physiological, concentration of ATP (1.5 mM) and at pH 6.8, the saturation curve of the *L. terrestris* phosphofructokinase for fructose 6-phosphate is sigmoidal ($h = 2.8$). Incorporation of 0.4 phosphate/subunit (mol/mol) did not affect the sigmoidicity, but decreased $S_{0.5}$ from 18.5 to 7.8 mM fructose 6-phosphate (cf. Figure 3, top panel). The dotted curve represents the activity ratios of the phosphorylated and non-phosphorylated enzymes. While the maximum absolute increase in activity occurred in the presence of 11 mM fructose 6-

Figure 3 Saturation curves of phosphofructokinase from *Lumbricus* for fructose 6-phosphate (top), and activation by 5'-AMP (middle) and fructose 2,6-bisphosphate (bottom)

Phosphofructokinase (0.5 unit/ml) was preincubated for 10 min with 1.5 mM ATP-Mg²⁺ either in the absence (○) or in the presence (●) of the catalytic subunit of cAMP-dependent protein kinase from bovine heart (0.18 m-unit/ml). The assays were performed at pH 6.8 in the presence of 1.5 mM ATP and 5.0 mM Mg²⁺, and 1.0 mM fructose 6-phosphate for the activation experiments. The data points were fitted to the Hill equation. The ratios between the activities of phosphorylated (v_p) and non-phosphorylated (v_0) phosphofructokinase are indicated by the dotted curves (right-hand ordinate). The dashed curves show the difference $v_p - v_0$ (left-hand ordinate). Top panel: saturation by fructose 6-phosphate. The parameters describing the curves are $V = 0.49$, $S_{0.5} = 7.86$ mM, $h = 2.82$ for the experiment performed in the presence of protein kinase, and $V = 0.47$, $S_{0.5} = 18.49$ mM, $h = 2.87$ for that done in the absence of protein kinase. Middle panel: activation by 5'-AMP. The curves were drawn for $V = 0.83$, $A_{0.5}$

phosphate under the conditions of the experiments (indicated by the dashed curve), the relative increase in phosphofructokinase activity caused by phosphorylation was highest in the presence of low substrate concentrations. The activities of the samples measured under reference conditions, i.e. in the absence of ATP inhibition, were almost identical.

The phosphorylated and unphosphorylated forms of phosphofructokinase also behaved differently in the presence of the allosteric activators 5'-AMP and fructose 2,6-bisphosphate (Figure 3, middle and bottom panels). In both cases the saturation curves changed from sigmoidal to hyperbolic when phosphofructokinase had incorporated 0.4 phosphate/subunit. In the presence of 1 mM fructose 6-phosphate and 1.5 mM ATP, high concentrations of fructose 2,6-bisphosphate as well as of 5'-AMP were able to activate non-phosphorylated phosphofructokinase to about 50% of the activity obtained in the presence of saturating fructose 6-phosphate concentrations. The phosphorylated phosphofructokinase, however, reached the same activity as in the presence of saturating fructose 6-phosphate. Because of the conversion of the sigmoidal saturation curves into hyperbolic ones, the relative acceleration of the reaction rate by the phosphorylation of phosphofructokinase was greatest in the presence of low effector concentrations. The absolute increase in activity caused by the conversion of the dephospho- form into the phospho- form in the presence of increasing effector concentrations, however, followed a hyperbolic saturation function. Therefore the effect of phosphorylation was not much dependent on effector concentrations above 0.3 μ M fructose 2,6-bisphosphate and 50 μ M 5'-AMP.

Phosphopeptide analysis

Phosphopeptides from *Lumbricus* phosphofructokinase were obtained by limited tryptic digestion and reverse-phase HPLC. In addition to *Lumbricus*, phosphopeptides were also obtained and analysed from the phosphofructokinases of the molluscs *Helix pomatia* and *Mytilus edulis*. These enzymes have been shown previously to be activated by phosphorylation [10]. In a first series of reverse-phase chromatographies, most of the peptide-bound radioactive phosphate was eluted in single peaks (Figure 4, left-hand panels). These were subjected to further purification on a high-resolution reverse-phase liquid chromatography system (Figure 4, right-hand panels) and to sequencing. Assignment and yields of PTH derivatives of amino acids recovered during automatic sequencing by Edman degradation are listed in Table 3. In all cases, the third sequencing cycle was empty except for relatively small amounts of PTH-serine. Since PTH-phosphoamino acids are retained by the sample supports during gas phase sequencing, and partial hydrolysis of the phospho group may have taken place, the results suggest that phosphoserine was present at these positions of the peptides.

DISCUSSION

The importance of the regulatory role of phosphofructokinase in glycolytic metabolism is reflected by the conservation of its structure and regulatory properties during evolution (reviewed

= 86.3 μ M (where $A_{0.5}$ is the concentration giving 50% of maximum activation), $h = 0.99$ for the phosphorylated enzyme, and $V = 0.38$, $A_{0.5} = 80.3$ μ M, $h = 2.45$ for the dephosphorylated enzyme. Bottom panel: activation by fructose 2,6-bisphosphate. The parameters of the curves are $V = 0.77$, $A_{0.5} = 0.47$ μ M, $h = 0.95$, and $V = 0.34$, $A_{0.5} = 0.50$ μ M, $h = 1.82$ for the phosphorylated and dephosphorylated enzyme respectively.

Figure 4 Separation and purification of the phosphopeptides obtained by tryptic digestion of the phosphofructokinases from *Mytilus edulis*, *Helix pomatia* and *Lumbricus terrestris*

Left-hand panels: separation of crude digests on a C_{18} -Spherisorb OPS II 4.6 mm $\times$ 250 mm column, as recorded at 226 nm. The trace was not corrected for the absorption of the eluant, which was responsible for the high peak in the middle of the chromatograms. The vertical bars indicate the relative activity recovered in individual fractions. The fractions indicated by horizontal bars were taken for further purification. Right-hand panels: separation of peptides on an Aquapore RP-300 1 mm $\times$ 250 mm column. Values on the right indicate the acetonitrile gradient (% v/v). The peak fractions were concentrated and sequenced.

in [18]). Co-operative saturation for fructose 6-phosphate in the presence of physiological concentrations of ATP (or phosphoenol-pyruvate in some bacteria) and allosteric activation by positive effectors such as fructose 2,6-bisphosphate and 5'-AMP have been described for a majority of the phosphofructokinases so far studied, and have consistently been found for the enzyme isolated from animals. The phosphorylation of serine residues was first observed with phosphofructokinases from mammalian and helminthic tissues (reviewed in [12]), and was also observed in other vertebrates [19], molluscs [10] and yeast [20]. The influence of phosphorylation of the enzyme on its kinetic properties, however, was found to be variable. The properties of phosphofructokinase from mammals are not distinctly affected by phosphorylation (see [12] for discussion).

Similar observations were made with phosphofructokinase from trout and *Xenopus* muscle [19] and also with yeast [20]. On the other hand, the kinetic properties of phosphofructokinase from the nematode *Ascaris suum* correlated with the amount of phosphate contained in the enzyme after purification [3], and were reversibly altered by phosphorylation and dephosphorylation *in vitro* [4]. Similar observations with phosphofructokinases from the nematode *Dirofilaria immitis* [6] and the trematode *Fasciola hepatica* [5] suggest that the phenomenon is not specific to *Ascaris* but is common to nematodes.

The decreased activity of phosphofructokinase obtained from the foot muscle of bivalves kept under anoxic conditions [11] raised the possibility of underlying covalent modification. Biethinger et al. [10] had independently demonstrated that

Table 3 Sequence analyses of [32 P]phosphopeptides from tryptic digests of phosphofructokinases from *Lumbricus terrestris*, *Mytilus edulis* and *Helix pomatia*

Samples (200–500 pmol) were applied and the background-corrected recoveries of PTH-amino acids during gas phase sequencing are listed. *In cycle 3, the highest yield was obtained for serine.

Cycle	<i>Lumbricus</i>		<i>Mytilus</i>		<i>Helix</i>	
	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)	Amino acid	Yield (pmol)
1	S	108	K	154	K	104
2	Q	222	D	326	S	85
3	*(S)	*(16)	*(S)	*(50)	*(S)	*(25)
4	I	253	I	382	V	166
5	W	40	W	46	Y	126
6	I	141	I	277	A	108
7	T	76	Q	124	E	62
8	P	127	T	70	L	63
9	G	87	G	102	G	48
10	K	38	R	15	K	4
11	E	51	E	2.2		
12	G	63				
13	K	14				

... ↓

Lumbricus terrestris: K/R S Q S* I W I T P G K E G K

Mytilus edulis: K/R K D S* I W I Q T G R E

Helix pomatia: K/R K S S* V Y A E L G K

Ascaris lumbricoides: A K G R S D S* I V P T

Haemonchus contortus: R R L D S I V P T A G R E G S

Fasciola hepatica: R S T* M M I P - G M E G K

Figure 5 Proposed primary structures around the phosphorylation site of phosphofructokinases from various invertebrates

The asterisks indicate the putative positions of the phosphorylated amino acid. The alignment of the sequence from *Haemonchus contortus* is based on similarity, since phosphorylation of the protein has not been examined. The phosphofructokinase sequences from *Ascaris* [21], *Haemonchus* [23] and *Fasciola* [22] were taken from the literature. Circles above the sequences denote the conserved block of hydrophobic residues, and the arrow indicates the invariant glycine (see the text). The hyphen denotes an arbitrary gap introduced to align the sequence of the phosphopeptide from *Fasciola* with the other phosphopeptides.

phosphorylation of phosphofructokinase from the muscle of *Mytilus edulis* and of another mollusc, *Helix pomatia*, by protein kinase A led to activation of the enzyme. The present study shows a similar phenomenon with phosphofructokinase obtained from an annelid and the results, therefore, suggest that regulation of phosphofructokinase by covalent modification is a widespread phenomenon among invertebrates.

The sequences around phosphorylation sites of phosphofructokinases from *Lumbricus terrestris*, *Mytilus edulis* and *Helix pomatia* are listed in Figure 5. Phosphoserine is retained by the support during sequencing and, therefore, leads to an 'empty' cycle during sequencing. Since small amounts of PTH-serine were traced in these cycles which may have been generated by hydrolysis of the serine phosphate, we assume that the empty cycles correspond to positions of phosphoserine. Moreover, the peptides were obtained by tryptic cleavage, and a reasonable assumption based on this technique is an N-terminal extension of

the determined sequence by either arginine or lysine. The validity of this assumption is supported by the requirement for a basic amino acid at these positions in a site phosphorylated by cAMP-dependent protein kinase. The sequences of the phosphorylation sites of phosphofructokinases from *Ascaris suum* [21] and *Fasciola hepatica* [22] are given in Figure 5 for comparison. We also include the cDNA-derived sequence surrounding Ser-12 of the phosphofructokinase of *Haemonchus contortus* [23], which is a potential substrate site for protein kinase A.

The amino acid sequences in the neighbourhood of the putative phosphorylation sites clearly show the characteristics of recognition sites for protein kinase A, i.e. basic amino acids at the P–2 or P–3 position (P indicates the relative position of the phosphorylated hydroxyamino acid) and a hydrophobic residue at the P + 1 position. These mandatory prerequisites necessarily decrease the possible variability within the peptides sequenced. However, there are similarities between the peptides in addition to those reflecting the functional property of being a substrate of the protein kinase. The phosphorylated serine is followed by three hydrophobic amino acids in the phosphofructokinases from *Mytilus*, *Helix* and *Lumbricus*. Except in the *Fasciola* phosphofructokinase, this hydrophobic block is followed two residues later by a glycine residue that is common to all sequences shown in Figure 5, next to a basic amino acid (not taking into account the incomplete sequence of the *Ascaris* phosphopeptide in this area). Also, the sequence reported for the phosphorylated peptide in *Fasciola* phosphofructokinase shares these features to some extent. Since the structure of phosphofructokinase is well conserved (see [18] for a review) the similarities of the phosphopeptides are unlikely to be random. Therefore it is sensible to assume that the phosphorylation sites are located within homologous regions of the phosphofructokinases. Since the putative site in *Haemonchus* phosphofructokinase is near the N-terminus, such a location is also probable for other phosphofructokinases whose allosteric properties are affected by phosphorylation and dephosphorylation. The fact that small phosphorylated peptides were readily released from the proteins by partial enzymic hydrolysis is consistent with their location close to one of the termini.

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