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Conservation, evolution, and specificity in cellular control by protein phosphorylation

H. W. Hofer

University of Konstanz, Faculty of Biology, P.O. Box 5560, M648, D-78434 Konstanz (Germany), Fax +49 7531 882903

Abstract. The glycolytic control enzyme phosphofructokinase from the parasitic nematode *Ascaris lumbricoides* is regulated by reversible phosphorylation. The enzyme is phosphorylated by an atypical cyclic adenosine monophosphate (cAMP)-dependent protein kinase whose substrate specificity deviates from that of the mammalian protein kinase. This variation is explained by structural peculiarities on the surface part of the catalytic groove of the protein kinase. Also, the protein phosphatases responsible for the reversal of phosphorylation appear to act specifically in glycolysis and are different from those participating in regulation of glycogenolysis.
Key words. Protein kinase (cAMP-dependent); protein phosphatases; phosphofructokinase; *Ascaris lumbricoides*; evolution of proteins; substrate specificity; regulation of glycolysis.

Phosphorylation: a universal mechanism for intercellular coordination

Phosphorylation and dephosphorylation of proteins is the most important mechanism regulating cellular function in eukaryotic cells and integrating multicellular systems. Chemical messages exchanged between neighbouring or distant cells are recorded by specific receptors located either in the cell membrane or, as for some lipophilic signalling molecules, in the cytosol and in the nucleus. The recognition of signals by receptors on the cell surface is directly or indirectly coupled to the activation of protein kinases, which finally elicit the intracellular responses. The classical mechanism of signal transduction involves the generation of second messengers, like cAMP[23] or inositol trisphosphate and diacylglycerol[1], and employs heterotrimeric G proteins for information transfer and amplification. Cyclic AMP and diacylglycerol are immediate and specific activators of protein kinase A (PkA) or protein kinase C (PkC), respectively, both specific for the hydroxyl groups of serine and threonine. Other responses to receptor stimulation can involve activation or inhibition of ion channels (often regulated by phosphorylation-dependent mechanisms) or the activation of tyrosine-specific protein kinases. Some of these tyrosine kinases are fused to the receptor and form its intracellular moiety. Examples of receptors comprising a built-in tyrosine kinase include receptors of growth factors (e.g. epidermal growth factor, platelet-derived growth factor) and insulin, whereas receptors for interleukins and antigens in lymphoid cells recruit soluble tyrosine protein kinases in order to transmit extracellular signals into the interior of the cells. In many cases, the first event in protein tyrosine kinase-mediated signal transduction is autophosphorylation, which triggers two different mechanisms of signal propagation: First, phosphorylation of soluble protein tyrosine kinases activates the protein kinase activity in a self-catalytic manner and may lead to phosphorylation of substrates [e.g. phospholipase Cγ, p85 subunit of phosphatidyl inositol-3 kinase (PI-3-K), insulin receptor substrate 1 (IRS-1) and others]. It is likely that these proteins are intrinsic to regulatory processes, but their exact role is presently not fully understood. Second, in addition to the augmentation of kinase activity, phosphorylated tyrosines are also used as tags for SH2-domain binding. These are phosphotyrosine binding sites found in protein tyrosine kinases and phosphatases, some enzymes and other proteins which act as linkers between the components of signal-transducing systems. Prominent examples are the coupling of growth factor receptors to c-Ras via Grb2, Shc and Sos, or of the linker protein IRS-1, phosphorylated by the tyrosine kinase of the insulin receptor, to PI-3-K. These initial events used to transfer the message of the occupation of growth factor or other receptors by an appropriate signal across the cell membrane to an intracellular recipient molecule subsequently elicit the activation of complex hierarchical protein phosphorylation cascades. The functional elements in these cascades are serine/threonine or 'bifunctional' threonine/tyrosine kinases whose activities are regulated by phosphorylation of specific hydroxyamino acids, as in c-Raf, MAPK/ERK kinase, mitogen-activated protein kinases (MAPK/ERK), ribosomal S6-protein kinase, and insulin-sensitive protein kinase.

Protein kinases: similarity and specificity

All known protein kinases belong to a single large family of proteins irrespective of whether they transfer phosphate to the hydroxyl groups of serine or threonine, or of tyrosine. The amino sequences within their catalytic domains are among the sequences best

conserved in evolution[11]. This is significant because the genome of vertebrates contains several hundred genes encoding protein kinases and, therefore, closely related proteins. Structural variation and formation of subfamilies is mainly created by combination of the catalytic part with regulatory centres leading to several subfamilies (e.g. cAMP- or Ca^{2+}/phospholipid-dependent protein kinases). A cell may contain several members of a given subfamily and, moreover, several isoforms of a given species of a protein kinase (e.g. α, β and γ isoforms of the catalytic subunits of cAMP-dependent protein kinase). Interestingly, the three-dimensional (3D) structures of the catalytic domains appear to be very similar even when members of different subfamilies are compared, as has been shown for serine- or threonine-specific cAMP-dependent protein kinase[18] and cyclin-dependent p34cdc2 [8], or tyrosine-specific insulin receptor kinase[16]. Apparently, nature allowed less flexibility in spatial structures of enzymes than in variations of amino acid sequences.

The close structural relationship between the catalytic centres inevitably leads to similar substrate specificities, which in many cases cannot be discriminated by in vitro assays. Moreover, isoenzymes, such as the isoforms of the catalytic C subunits of PkA, cannot even be separated in native form by the biochemical procedures presently available. With regard to the functional specificity of a given protein kinase, the structural similarity among protein kinases raises the problem of overlapping recognition of cellular substrates by enzyme proteins regulating sensitive cellular functions. In some instances cells may circumvent the specificity problem by restricting availability either by tissue-selective expression of a given protein kinase or by targeting a protein kinase and its intended substrate at a specific intracellular location.

On the other hand, the question may be raised whether small structural differences evolved gradually and concomitantly in protein kinases as well as in their physiological substrates. Instead of abrupt changes in the structure and function of the protein kinase, specificity may have developed by subtle steps of co-evolution leading to minor structural variations in the recognition sites of both partners that guarantee the specificity of signal transduction by phosphorylation. Rather limited structural variations may result in modest modulation of the functional properties (like quantitative changes in K_m or in the catalytic constant) of the protein kinase that are too small to be detected by in vitro techniques.

The topic is illustrated by a protein kinase and by protein phosphatases which were identified as interconverting enzymes of phosphofructokinase (PFK) in the parasitic nematode *Ascaris suum* (var. *suum*). Because of separate evolution, these enzymes appear sufficiently distant from their mammalian counterparts to allow some patterns of generation of specificity to be unveiled by biochemical methods.

Ascaris suum: parasitic lifestyle allows simplicity of metabolic organization

The metabolic requirements of the parasite are mainly characterized by the necessity of adenosine triphosphate (ATP) generation via anaerobic glycolysis and the accumulation of glycogen stores. ATP is continuously required for muscle contractions that antagonize peristaltic movement of bowels and keep the worm in its intestinal position. The energy metabolism of the adult parasites completely depends on carbohydrates. Glucose is either converted into glycogen or enters the anaerobic glycolytic pathway to phosphoenolpyruvate, which is metabolized into short-chain branched fatty acids (reviewed in ref. 26).

The periods allowing for glycogen synthesis are short and occur only when the host is in a post-prandial situation. This situation, however, is also characterized by intensive intestinal peristalsis. The channelling of fuels into anabolic and catabolic metabolism is regulated at the levels of glycogen synthase and phosphorylase for glycogen synthesis and degradation and at the level of PFK for glycolysis.

Glycolysis is regulated in a cAMP-dependent manner in *Ascaris* muscle

Unlike the situation in vertebrates, non-phosphorylated PFK from *Ascaris* is barely active, but its activity is dramatically increased by phosphorylation[13,6]. The phenomenon has been studied in some detail in our laboratory, and similar behaviour of PFK was observed in molluscs[2] and annelids. Like mammalian PFK, *Ascaris* PFK is inhibited by ATP, and the substrate saturation curve for the second substrate, fructose 6-phosphate, is sigmoidal in the presence of neutral pH and physiological, that is, millimolar, ATP concentrations. The enzyme is activated by fructose 2,6-bisphosphate and 5′-AMP. Figure 1 shows substrate saturation curves of PFK from *Ascaris* for fructose 6-phosphate. As is illustrated by the comparison of curve A (non-phosphorylated PFK) and curve C (phosphorylated PFK), the enzyme is dramatically activated following phosphorylation of one serine residue per subunit. Phosphorylation also increases the V_{max} in the presence of positive effectors. Phosphorylation of *Ascaris* PFK also occurs in vivo. Partially phosphorylated PFK is isolated from *Ascaris* muscle in the presence of fluoride (which is an inhibitor of protein phosphatases), whereas the absence of F^- during the purification procedure leads to a less active dephosphorylated enzyme[13]. The phosphate content of PFK increased following treatment of muscle strips with serotonin[12]. Serotonin is known as a trans-

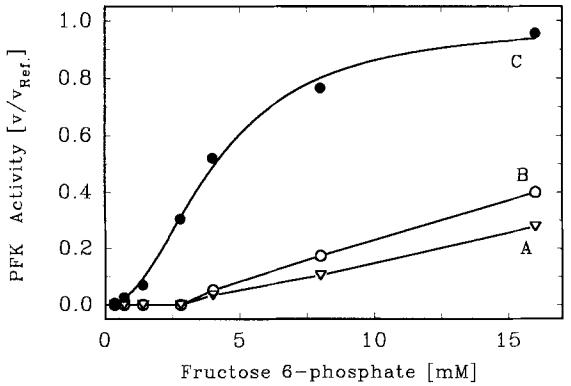

Figure 1. Effect of phosphorylation on the saturation of *Ascaris* PFK with fructose 6-phosphate. Curve *A*: non-phosphorylated PFK; curve *B*: PFK pre-incubated with the C subunit of bovine PkA (280 pkatal); curve *C*: PFK pre-incubated with *Ascaris* PkA (90 pkatal). The phosphate content after pre-incubation with *Ascaris* PkA was 1 phosphate per subunit of the PFK tetramer, whereas only ≈0.5 phosphate as incorporated by incubation with the bovine protein kinase.

mitter substance of modulatory neurons in nematodes[21]. It has also been shown that serotonin effects the increase of cAMP levels[9].

PFK is phosphorylated by cAMP-dependent protein kinase

The PFK-phosphorylating protein kinase was identified as the catalytic C subunit of cAMP-dependent protein kinase (PkA, ref. 24). The criterion initially used for characterization was inhibition of protein kinase activity by the heat-stable protein kinase inhibitor from rabbit muscle and by the regulatory R subunit isolated from bovine heart. This inhibition was relieved by cAMP. The purified PFK also exhibited weak cross-reaction with an antiserum raised against the C subunit from bovine heart. Although the molecular weight of the purified enzyme from *Ascaris* was similar to that of the mammalian enzyme, the observed properties were somewhat unexpected, since the protein kinase was almost inactive when extracted from the tissue and also after chromatography on a (diethylamino)ethyl (DEAE)-cellulose column, but was resistant to activation by cAMP. A cAMP-binding protein, later identified as R subunit[25], was not associated with the C subunit of the protein kinase. This puzzling discrepancy was explained by the isolation of an inhibitor protein of M_r 27,000[14], not related to the inhibitor from rabbit muscle, that is present in sufficient concentrations in *Ascaris* muscle to inhibit the C subunit quantitatively. The inhibitory action of this protein was antagonized by heparin. The transfer of the parasite from the pigs' intestines to the laboratory probably initiated a scenario of stress-dependent increase of intracellular cAMP concentration elicited by the liberation of serotonin. The resulting activation of the protein kinase was subsequently terminated by binding of the C subunit to the inhibitor, forming the complex in which the protein kinase finally was present in the extracts.

Protein kinase from *Ascaris* phosphorylates PFK more efficiently than does mammalian protein kinase

The C subunit of the *Ascaris* protein kinase has a 10-fold lower K_m for PFK than does mammalian protein kinase. Interestingly, this specificity is lost when the efficiency of both enzymes to phosphorylate a peptide containing the site of phosphorylation of *Ascaris* PFK is compared. The peptide contains the sequence Ala-Lys-Gly-Arg-Ser-Asp-Ser*-Ile-Val-Pro-Thr (ref. 20; the asterisk denotes the phosphorylated serine residue). The K_m of this peptide is 55 µM for both protein kinases. This value corresponds to the K_m of *Ascaris* PFK for the mammalian protein kinase but indicates a 10-fold lower affinity of the peptide for the *Ascaris* protein kinase compared with complete PFK, thus suggesting that portions of the protein substrate outside of the phosphorylated site contribute to binding of the substrate to the protein kinase.

Protein kinase from *Ascaris* belongs to a new subfamily within the PkA family

The primary structure of the C subunit of PkA from *Ascaris* was determined by cDNA cloning and sequencing[17]. The protein is not closely related to the PkA of a second nematode, *Caenorhabditis elegans*. The evolutionary distance between the nematodes *C. elegans* and *A. suum* is about $7 \cdot 10^7$ years[22]; the distance between the protein kinases is much greater. The amino acid sequences of the PkAs from *C. elegans* and mammals are 85% identical, whereas only 50% identical amino acids are found in PkA from *A. suum*. The relationship between PkA from various sources is shown in comparison with PkC and cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PkG) in figure 2. A group of closely related enzymes of vertebrates and invertebrates forms a 'conventional' PkA branch, and the amino acid identity among these enzymes exceeds 80%. The group comprises all known PkA forms from vertebrates but also PkA from unlike *Drosophila*, *Aplysia*, and the nematode *C. elegans* and the hookworm *Ancylostoma caninum*. Another separate branch is made up of the protein kinases from *Saccharomyces cerevisiae*. Between these two branches several 'non-conventional' PkA forms are found which show about the same structural distances from conventional and yeast PkA as from PkG. Among these enzymes are the PkA from *Ascaris* and the slime mold *Dictyostelium discoideum*. The amazing difference between the protein kinases from the nematodes *C. elegans* and *Ascaris* suggests that these enzymes were derived from different progeni-

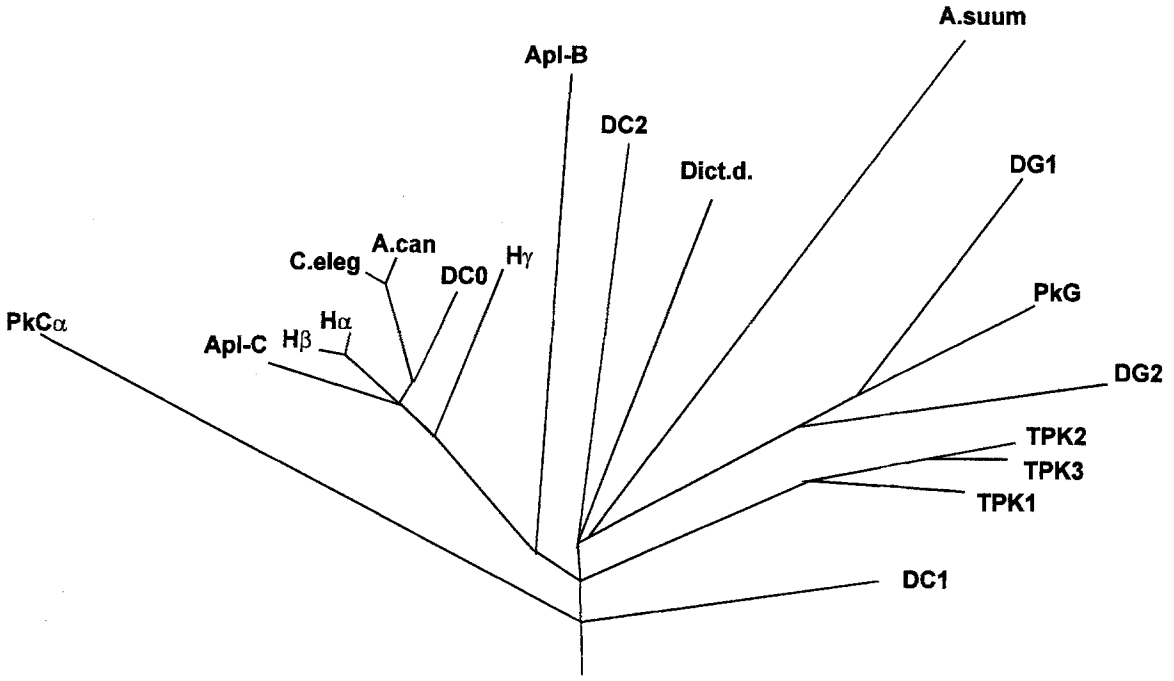

Figure 2. Phylogenetic tree indicating the relationship of the catalytic subunits of cAMP-dependent protein kinases. Protein kinase Cα (PkCα) and cGMP-dependent protein kinase (PkG) are included for comparison. Abbreviations: Apl-C, Apl-B: *Aplysia* C and B; Hα, Hβ, Hγ: Human alpha, beta and gamma; C.eleg.: *Caenorhabditis elegans*; A.can: *Ancylostoma caninum*; DC0, DC1, DC2: *Drosophila* (clones 0, 1, 2); Dict.d.: *Dictyostelium discoideum*; A. suum: *Ascaris lumbricoides* var. *suum*, DG1, DG2: *Drosophila* cGMP-dependent protein kinase 1 and 2; TPK1, TPK2, TPK3: *S. cerevisiae* protein kinase 1, 2 and 3.

tors with a common ancestor, which must have been a contemporary of dinosaurs. Apparently, descendants of single but different PkA branches survived evolution in these organisms, unlike *Drosophila*, in which three different kinds of PkA are found.

Structure of the PkA molecule from *Ascaris*

Molecular modelling of the 3D structure revealed that differences from mammalian PkA are mainly due to variations on the surface of the protein, whereas the catalytic region and the core backbone of the protein molecules are almost identical (see fig. 3). Importantly, the bottom of the catalytic cleft, which comprises the binding site for ATP-Mg^{2+} and the catalytic base, is entirely built of conserved amino acids. Therefore, small peptide substrates interacting only with the kinase in the area of the catalytic cleft exhibit identical affinity to *Ascaris* and mammalian protein kinase[27]. Elucidation of the structure of the ternary complex of mammalian PkA[19] revealed that two basic amino acids at the N-terminal side of the phosphorylated hydroxyamino acid form salt bridges with glutamates of the protein kinase and that the binding of the peptide substrate is additionally stabilized by hydrophobic interactions of an amino acid at the C-terminal side of the phosphorylation site. The amino acids involved in these interactions are conserved in mammalian and *Ascaris* PkA. The heat-stable inhibitor from rabbit muscle and the mammalian R subunit contain pseudo-substrate sites in which the

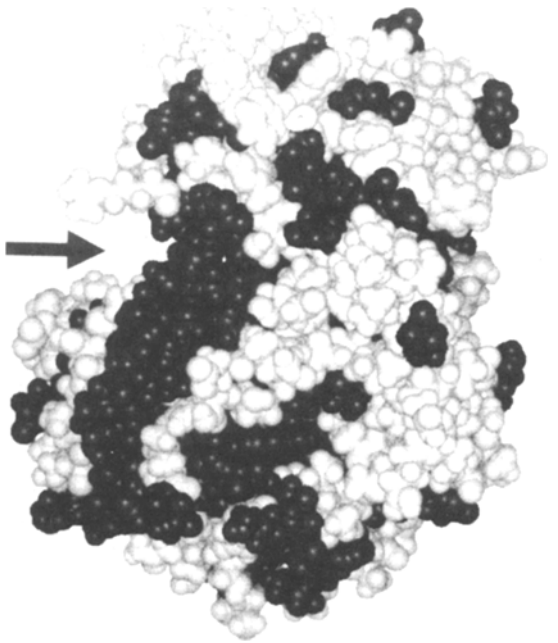

Figure 3. Conserved and non-conserved parts on the surface of *Ascaris* protein kinase. Atoms belonging to amino acids conserved in *Ascaris* and mouse protein kinase are printed in black; those belonging to non-conserved amino acids are printed in white. The arrow is pointing towards the catalytic cleft. The non-conserved atoms located at the 'small lobe' (shown above the arrow) belong to serine-67 and methionine-68; those shown below the arrow are parts of threonine-228, leucine-229 and aspartate-230.

phosphorylated serine is replaced by an alanine that allows ideal interaction with the catalytic domain of the kinase. Taking into account that the 3D structure within the catalytic cleft of the C subunit from *Ascaris* is the same as in the mammalian protein kinase, the cross-reaction of these inhibitor proteins with the *Ascaris* C subunit is well explained.

A protein like PFK (the M_r of the native form is about 360,000) presumably also binds to amino acids on the surface of the protein kinase molecule (M_r about 39,000), which may contribute to substrate affinity in addition to the interactions with the primary binding site within the catalytic centre.

Structural differences at the surface of protein kinases explain variations in affinities for protein substrates

Comparison between the C subunits of *Ascaris* and mammalian protein kinase demonstrates that extended amino acid sequence differences can nevertheless lead to high conservation of 3D structure. The ostensible similarity, however, has limitations at the outer rim of the catalytic groove, which in the mammalian (mouse) enzyme is formed in part by the amino acids corresponding to positions 81–83 and 242–244. The context of amino acid sequences of the protein kinases from mouse, *C. elegans*, *Ascaris* and yeast (*S. cerevisiae* TPK1) is as follows:

	80 90
Mouse α	V***KLK***QIEHTLN
C. elegans	----------
Ascaris	-***SMR***-T--VHS
TPK1	-R-------ND

	240 250
Mouse α	AD***QPI***QIYEKI
C. elegans	----------
Ascaris	GK***TLD***E-----
TPK1	DS***NTM***KT----

The amino acids contributing to the surface structure at the entrance to the catalytic cleft are printed in bold italics. While there is complete homology between the protein kinases from mouse and *C. elegans* at the indicated positions, non-conserved substitutions are present partly in the *Ascaris* enzyme and partly in the yeast enzyme, which also exhibits some variations in the acceptance of substrates when compared with the mammalian enzyme[10].

Although positions 81–83 and 242–244 do not contribute to the binding of small peptides, as shown by the crystallographic structure of the ternary complex between mouse protein kinase[18,19], ATP-Mg^{2+}, and the peptide comprising positions 5–24 of the rabbit muscle protein kinase inhibitor, the parts of the protein kinase surface formed by these amino acids inevitably are in contact with a protein substrate.

Different protein phosphatases regulate glycolysis and glycogenolysis in *Ascaris*

In contrast to protein kinases, which all belong to a single protein family, the protein phosphatases hydrolyzing the phosphate either from aliphatic amino acids or from tyrosine form two separate families. The structures of the individual members are well conserved within each family. The original classification of serine- or threonine-specific protein phosphatases included a very narrow range of proteins, but additional forms have been identified more recently[5,15]. The catalytic subunits of protein phosphatases PP1, PP2A and most of the 'new' forms belong to the same family of proteins. The phosphatase calcineurin (PP2B) is loosely related to this family. The structural relationship of the Mg^{2+}-dependent PP2C with other serine or threonine protein phosphatases is only marginal (reviewed in ref. 4). Protein tyrosine phosphatases are not related at all and, therefore, do not interfere with the activity of serine or threonine phosphatases. While this fact helps to clarify the substrate specificity of protein phosphatases, the picture is obscured by overlapping substrate specificities of PP1, PP2A and PP2C (and potentially some of the phosphatases more recently detected by molecular cloning) and by the fact that metal ions (Mg^{2+}, Mn^{2+}) and other effectors (e.g. polyamines) modulate the substrate specificity (see refs. 3 and 4 for reviews).

Four forms of PP2A capable of dephosphorylating PFK are present in *A. suum*[7]. They have apparent native molecular weights of 174,000 and 126,000 and are dissociated into subunits of M_r 33,000 and 63,000 by SDS. Their kinetic properties are in agreement with classification as PP2A: They are not inhibited by the inhibitors of PP1 (I-1 and I-2), prefer the α subunit of phosphorylase kinase before the β subunit, and are inhibited by subnanomolar concentrations of okadaic acid. PFK from *Ascaris* is dephosphorylated in both the absence the presence of Mn^{2+}. An important difference with respect to substrate specificity, however, occurs with phosphorylase as substrate. Rabbit muscle phosphorylase *a* is a good substrate of mammalian PP2A and also *Ascaris* PP2A in the presence of Mn^{2+} (two forms were also active with rabbit muscle phosphorylase *a* in the absence of Mn^{2+}). When phosphorylase *a* from *Ascaris* is used as substrate, however, dephosphorylating activity is very low, even in the presence of Mn^{2+} ions, and virtually no dephosphorylation occurs in the absence of divalent ions, suggesting that phosphorylase is not a substrate for PP2A in *Ascaris*, while this phosphatase type is responsible for the inactivation of the glycolytic enzyme, PFK. On the other hand, there is evidence that phosphorylase in *Ascaris* is dephosphorylated by a PP1-like enzyme, since 85% of the dephosphorylating activity in crude extracts is inhibited by mammalian I-2, and only 11.5% by 1 nM okadaic acid, which inhibits 50% of PFK dephosphorylation. These data indicate separate functions of protein phosphatases

of the types PP1 and PP2A in the metabolic regulation of *Ascaris*.

Conclusion

Adaptation of the metabolism of *Ascaris* to a parasitic lifestyle and restriction of metabolic pathways to a simple scheme may have been accompanied by simplification of the isoenzyme pattern of regulatory protein kinases. The presence of a single PkA, which is apparently a species lost during evolution of other organisms, facilitates quantitative studies to detect specificity which is obscured in more complex systems. The structures of protein kinases (possibly also of protein phosphatases) and their substrates – as shown for PFK and PkA in *Ascaris* – apparently evolved interdependently, resulting in low K_m and improved specificity of the kinase for its protein substrate. Such an optimization strategy, involving the kinase as well as its substrate (or substrates) and relying on quantitative rather than qualitative preferences, could nevertheless create considerable specificity of phosphorylation. Beyond the molecular level specificity also appears to be inherent in the organization of regulation of metabolic pathways. The regulation of glycogen metabolism and glycolysis is exerted by different protein kinases and phosphatases. Phosphorylase kinase and PP1 are responsible for glycogen metabolism, while glycolysis is regulated by PkA and PP2A. Use of protein-modifying enzymes with different regulatory backgrounds separates the regulation of interconnected metabolic fluxes and enlarges the adaptive power of an organism to complex environmental situations.

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