THE HORMONAL CONTROL OF GLYCOGEN METABOLISM: THE AMINO ACID SEQUENCE AT THE PHOSPHORYLATION SITE OF PROTEIN PHOSPHATASE INHIBITOR-1

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1. Introduction

Glycogen metabolism in skeletal muscle is controlled by a regulatory network which involves three protein kinases and two protein phosphatases (fig.1 and ref. [1] for recent review). The conversion of phosphorylase *b* to *a* is catalysed by phosphorylase kinase while the activity of phosphorylase kinase is itself controlled by cyclic AMP-dependent protein kinase. This latter reaction is complex and involves phosphorylation at two sites on the enzyme. The rapid phosphorylation of one serine on the β-subunit of phosphorylase kinase converts it to a form which is 40-times more active. The subsequent, slower, phosphorylation of a further serine on the α-subunit greatly enhances the rate at which the β-subunit can be dephosphorylated and therefore determines the time at which the inactivation of phosphorylase kinase can be initiated [2,3].

Cyclic AMP-dependent protein kinase also catalyses the phosphorylation of glycogen synthetase resulting in its conversion to a less active form, termed glycogen synthetase b_1 [4] (fig.1). Glycogen synthetase is however also phosphorylated by glycogen synthetase kinase-2, which converts it to another less active, but kinetically distinct, form termed glycogen synthetase b_2 [4] (fig.1). Phosphorylation of glycogen synthetase *a* by both cyclic AMP-dependent protein kinase and glycogen synthetase kinase-2 yields an essentially inactive form termed glycogen synthetase $b_{1,2}$ [4] (not illustrated).

A single enzyme, termed protein phosphatase-III has been shown to catalyse the inactivation of phosphorylase, phosphorylase kinase (through the dephosphorylation of the β-subunit) and the reactivation of both glycogen synthetase b_1 and b_2 and therefore carriers out all four dephosphorylations which either inhibit glycogenolysis or activate glycogen synthesis [5,6]. A second activity, termed protein phosphatase-II, specifically dephosphorylates the α-subunit of phosphorylase kinase, and therefore opposes the action of protein phosphatase-III against the β-subunit of phosphorylase kinase [5–7].

Current interest in the regulation of the protein phosphatases of glycogen metabolism was stimulated

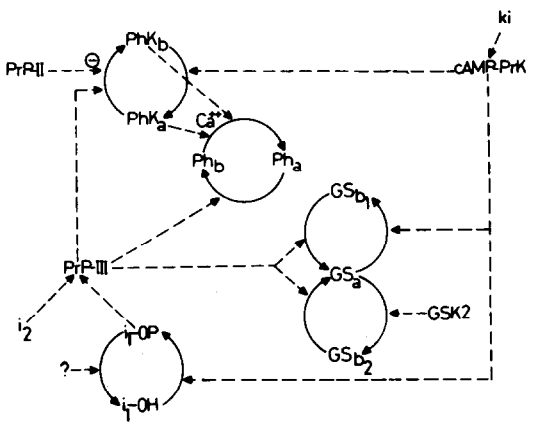

Fig.1. The regulatory pathway of glycogen metabolism in skeletal muscle [1]. *Abbreviations: a*, high activity form; *b*, low activity form; Ph, phosphorylase; PhK, phosphorylase kinase; cAMP-PrK, cyclic AMP-dependent protein kinase; GS, glycogen synthetase; GSK2, glycogen synthetase kinase-2; PrP-II, protein phosphatase-II; PrP-III, protein phosphatase III, i_1-OH and i_1-OP, the dephosphorylated and phosphorylated forms of inhibitor-1; i_2, inhibitor-2; *ki*, the heat stable protein inhibitor of cyclic AMP-dependent protein kinase.

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by the report that liver and muscle extracts contain a heat-stable, trypsin-labile protein(s) which is a potent inhibitor(s) of phosphorylase phosphatase [8,9]. This work was extended by Huang and Glinsmann who demonstrated the existence of two heat-stable protein inhibitors of phosphorylase phosphatase in skeletal muscle extracts, which they termed inhibitor-1 and inhibitor-2 [10,11]. The made the important discovery that inhibitor-1 only inhibited phosphorylase phosphatase when it was itself phosphorylated by cyclic AMP-dependent protein kinase (fig.1). Inhibitor-2 was effective without the need for prior phosphorylation and was distinct from both inhibitor-1 and from the heat-stable protein which specifically inhibits cyclic AMP-dependent protein kinase [11–13].

We recently showed that a heat-stable protein, purified 700-fold from skeletal muscle extracts, and which is probably identical to inhibitor-2, inhibited all the four activities of protein phosphatase III in parallel but it was over 200-times less effective in inhibiting protein phosphatase II [12,13].

While the physiological roles of inhibitors-1 and -2 are not yet known, inhibitor-1 could be of considerable importance in the hormonal control of glycogen metabolism. If the phosphorylation of this protein occurred in vivo, it might lead to the inactivation of protein phosphatase III as cyclic AMP-dependent protein kinase became activated, providing a further mechanism for amplifying the hormonal activation of glycogenolysis and for excercising control over several protein kinase–protein phosphatase cycles. Inhibitor-1 may therefore be a novel protein in metabolic regulation mediating the control of a protein phosphatase by a protein kinase. Knowledge of the structure at the phosphorylation site of this protein, given in this communication, may therefore provide valuable information about the specificity and mechanism of action of both interconverting enzymes.

2. Methods and results

Inhibitor-1 was purified to homogeneity. It showed a single protein-staining band when subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, corresponding to mol. wt 25 000. This is in close agreement to the value reported by Huang and Glinsmann for the partially purified protein [11]. Phosphorylation of inhibitor-1 by cyclic AMP-dependent protein kinase resulted in the incorporation of one molecule covalently bound phosphate/molecule protein (G. A. Nimmo and P. Cohen unpublished results).

The rate of phosphorylation of inhibitor-1 by cyclic AMP-dependent protein kinase was compared to the rate of phophorylation of two other physiological substrates for this enzyme, phosphorylase kinase and histone H-1 (fig.2). It was calculated from these results that the rate of phosphorylation of inhibitor-1 was nearly four-times faster than histone H-1 and it was 30% that of the β-subunit of phosphorylase kinase.

^{32}P-Labelled inhibitor-1, 5.0 mg (200 nmol), was incubated with 0.5 mg thermolysin in 5.0 ml 0.1 M ammonium bicarbonate for 2 h at 37°C. The reaction was terminated by lyophilisation and the material was then subjected to two-dimensional peptide mapping using pyridine/acetic acid/water, pH 3.6 (1:10:190)

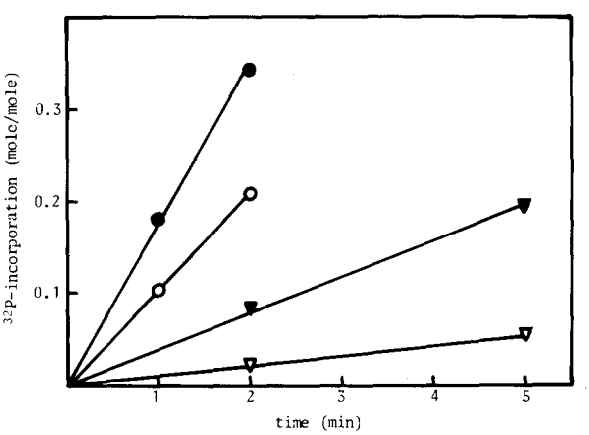

Fig.2. Relative rate of phosphorylation of inhibitor-1, phosphorylase kinase and histone H1 by cyclic AMP-dependent protein kinase. The final concentrations in the assays were, protein substrate 6 μM, sodium glycerophosphate 10 mM, EDTA 0.4 mM, EGTA 0.1 mM, cyclic AMP 0.01 mM magnesium chloride 2.0 mM and [γ-^{32}P]ATP 0.2 mM. (○–○) Phosphorylation of phosphorylase kinase by trace endogenous cyclic AMP-dependent protein kinase. (●–●) Phosphorylation of phosphorylase kinase by endogenous cyclic AMP-dependent protein kinase plus 0.01 units of added partially purified cyclic AMP-dependent protein kinase [13]. (▼–▼) Phosphorylation of inhibitor-1 by 0.02 units of cyclic AMP-dependent protein kinase. (▽–▽) Phosphorylation of histone H1 by 0.02 units of cyclic AMP-dependent protein kinase. The preparations of inhibitor-1 and histone H1 did not have any endogenous cyclic AMP-dependent protein kinase activity.

Table 1
Amino acid composition of the phosphopeptide obtained by digestion of ^{32}P-labelled inhibitor-1 with thermolysin

Amino acid	
Threonine	1.75 (2)
Glutamic acid	0.17
Proline	2.10 (2)
Glycine	0.16
Alanine	1.09 (1)
Isoleucine	1.00 (1)
Lysine	0.14
Arginine	3.94 (4)
^{32}P-Radioactivity	0.92 (1)
Total	10

Impurities below 0.1 residues are omitted.

for 75 min at 50 V/cm. Descending chromatography was carried out in the second-dimension using butanol/pyridine/acetic acid/water (90:60:18:72) for 40 h. The paper was autoradiographed and showed the presence of one major ^{32}P-labelled peptide which contained > 95% of the ^{32}P-radioactivity on the paper (not illustrated). This peptide had an electrophoretic migration of 0.6 relative to lysine, and a chromatographic mobility of 0.1 relative to phenol red. The ^{32}P-labelled peptide corresponded in position to a strongly staining fluorescamine positive spot. The peptide was eluted using 1.0 M acetic acid and 60 nmol peptide was obtained corresponding to an overall yield of 30%.

An aliquot of the purified peptide was hydrolysed in 6 N HCl and subjected to amino acid analysis. The amino acid composition (table 1) indicated that the peptide contained ten amino acids, four of which were arginine. The amino acid composition also surprisingly indicated that serine was absent from the peptide. In order to establish that the protein was phosphorylated on a threonine residue, an aliquot of the ^{32}P-labelled protein was subjected to partial acid hydrolysis, followed by electrophoresis at pH 1.9. The results confirmed that inhibitor-1 was phosphorylated on a threonine residue (fig.3). In contrast, ^{32}P-labelled phosphorylase kinase hydrolysed under similar conditions showed only the presence of phosphoserine and not phosphothreonine (fig.3), as expected from the known structure of the two phosphorylation sites on the enzyme [3].

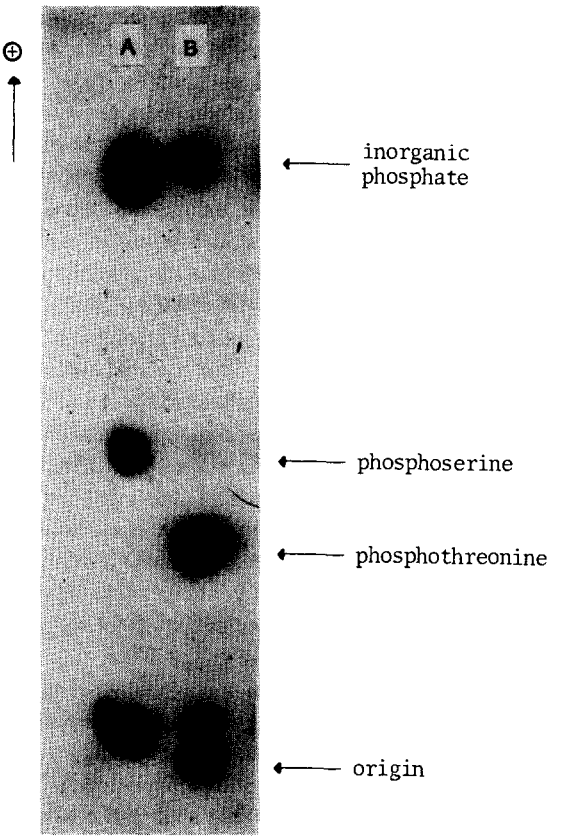

Fig.3. Identification of phosphothreonine in inhibitor-1. ^{32}P-Labelled phosphorylase kinase (A) and inhibitor-1 (B), prepared by maximal phosphorylation with cyclic AMP-dependent protein kinase, were hydrolysed with 6 N HCl at 110°C for 1.5 h and 4.0 h, respectively. The samples were dried and subjected to electrophoresis in acetic acid/formic acid/water (2:1:25) pH 1.9, at 50 V/cm for 1 h. The figure shows an autoradiograph of the electrophoretogram. The radioactive spots near the origin represent incompletely hydrolysed material.

The complete amino acid sequence of the peptide was established unambiguously by the micro dansyl-Edman procedure of Bruton and Hartley [14] and was entirely consistent with the amino acid composition (table 1). The amino acid sequence was shown to be:

Ile–Arg–Arg–Arg–Arg–Pro–Thr(P)–Pro–Ala–Thr

The identity of phosphothreonine as the seventh resi-

due was established by the simultaneous appearance of two dansyl derivatives, dansyl phosphothreonine, which migrates at a characteristic position below serine in the third solvent (ethyl acetate/methanol/acetic acid, 20:1:1) and dansyl threonine resulting from partial hydrolysis of the phosphorylated amino acid derivative. The C-terminal threonine residue was confirmed by digestion with carboxypeptidase A.

3. Discussion

The results presented in this paper show that cyclic AMP-dependent protein kinase phosphorylates just one site on inhibitor-1. The structure at the phosphorylation site is also very unusual since firstly it includes a sequence of four consecutive arginines and secondly the phosphorylation occurs on threonine and not on a serine residue.

A sequence of four arginines has, to our knowledge, only been observed in one other class of protein, namely the protamines which are complexed with nucleic acids in the mature sperm of some fish and birds. These proteins contain about 32 amino acids of which 70% are arginine residues, and they contain several sequences of four or five consecutive arginine residues [15]. The homology with protamines actually extends beyond this, since the pentapeptide arg–arg–arg–arg–pro occurs in all protamines, while the hexapeptide ile–arg–arg–arg–arg–pro is found in two protamines [15]. Even though the probability of obtaining two identical hexapeptide sequences by chance is exceedingly low, it is difficult to believe that inhibitor-1 and protamines are derived from a common ancestral protein, since inhibitor-1 contains 200 amino acids, it is not a basic protein and it contains only 15 arginine residues in the whole molecule (G. A. Nimmo and P. Cohen unpublished results). Nevertheless, it is intriguing that protamine is a potent inhibitor of phosphorylase phosphatase activity (A. Burchell and P. Cohen unpublished results). In the standard assay [6], the K_1 was 1–2 μM, and even allowing for the presence of 20 arginines/molecule of protamine, this is at least 1000-times more inhibitory than equivalent amounts of free arginine ($K_i = 5 \times 10^{-2}$ M), which has been known for 20 years to inhibit phosphorylase phosphatase [16]. The phosphorylated form of inhibitor protein-1 is however a much stronger inhibitor of protein phosphatase III than protamines.

The amino acid sequence at the sites on phosphorylase kinase, histones H1 and H2B and pyruvate kinase which are phosphorylated by cyclic AMP dependent protein kinase are given in table 2. These proteins represent the best substrates for cyclic AMP-dependent

Table 2
Amino acid sequences at the phosphorylation sites of substrates for cyclic AMP-dependent protein kinase

Substrate	Sequence	Reference
Phosphorylase kinase (β-subunit)	Ala–Arg–Thr–Lys–Arg–Ser–Gly–$\overset{100}{\text{Ser(P)}}$–$\overset{\text{Val}}{\text{Ile}}$–Tyr–Glu–Pro–Leu–Lys	[17,18]
Histone H2B	Lys–Lys–Arg–Lys–Arg–$\overset{8}{\text{Ser(P)}}$–Arg–Lys–Glu–$\overset{50}{\text{Ser(P)}}$–Tyr–Ser–Val–Tyr–Val–Tyr–Lys	[17,18]
Pyruvate kinase (rat liver)	Gly–Val–Leu–Arg–Arg–Ala–$\overset{35}{\text{Ser(P)}}$–Val–Ala–Glx–Leu	[18,19]
Inhibitor-1	Ile –Arg–Arg–Arg–Arg–Pro–$\overset{30}{\text{Thr(P)}}$–Pro–Ala–Thr	This paper
Phosphorylase kinase (α-subunit)	Phe–Arg–Arg–Leu–$\overset{20}{\text{Ser(P)}}$–Ile –Ser–Thr–Glu–Ser–Glx–Pro	[17,18]
Histone H1 (rat liver)	Ala–Lys–Arg–Lys–Ala–$\overset{8}{\text{Ser(P)}}$–Gly–Pro–Pro–Val–Ser	[20]

The numbers above the phosphorylated residues refer to the rates at which the sites are phosphorylated relative to the β-subunit of phosphorylase kinase (100%). The assay conditions are given in fig.2. Basic amino acids are underlined and broken lines indicate the phosphorylated residue.

protein kinase in vitro that have been identified. We have pointed out previously that a striking feature common to each of these sequences is the presence of two adjacent basic amino acids, at least one of which is arginine, just N-terminal to the phosphorylatable residue [17,18] and in this respect the structure of the phosphorylation site of inhibitor-1 can be said to conform to the basic specificity pattern that seems to be emerging.

The importance of two adjacent basic amino acids has been emphasized by the results of Zetterqvist et al. [21] and Kemp et al. [22] who have studied the phosphorylation of synthetic peptides corresponding to the phosphorylation site of pyruvate kinase (table 2). Zetterqvist et al. [21] found that the heptapeptide leu–arg–arg–ala–ser–val–ala– was phosphorylated with kinetic constants (K_m and V_{max}) comparable to those of the native protein. Furthermore, if either arginine of the pentapeptide arg–arg–ala–ser–val– was replaced by a leucine, the rate of phosphorylation of the peptide fell by more than 100-fold. However, they also reported that if the serine in the pentapeptide was replaced by a threonine, then the rate of phosphorylation of the peptide again fell by more than 100-fold. This suggested that the presence of threonine might not be compatible with rates of phosphorylation required in vivo, and was consistent with the finding that all physiological substrates to date were phosphorylated on serine residues (table 2). However, the present finding, that inhibitor protein-1 is phosphorylated on a threonine residue at a similar rate to other physiological substrates (table 2, figure 2) shows the molecular specificity of cyclic AMP-dependent protein kinase must be more complex and suggests the need to carry out more extensive specificity studies with synthetic peptides.

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