Isolation and characterisation of active fragments of protein phosphatase inhibitor-1 from rabbit skeletal muscle

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Protein phosphatase-1 *Inhibitor-1* *Cyclic AMP Proteinase* *Protein phosphorylation* *Primary structure*

1. INTRODUCTION

Inhibitor-1 is a small heat-stable protein first described by Huang and Glinsmann [1] that is a specific inhibitor of protein phosphatase-1 [2,3]. This enzyme is the major protein phosphatase involved in the regulation of glycogen metabolism in skeletal muscle [2–5], but its broad tissue distribution and ability to dephosphorylate enzymes involved in the regulation of glycolysis, fatty acid synthesis, cholesterol synthesis and protein synthesis suggests a wider role in metabolic regulation [3–6].

Inhibitor-1 is only active after phosphorylation by cyclic AMP-dependent protein kinase [1] on a threonine [7] 35 residues form the N-terminus [8]. It is therefore a novel protein in metabolic regulation that can mediate the control of a protein phosphatase by a protein kinase. The phosphorylation of inhibitor-1 in mammalian skeletal muscle increases in response to adrenalin [9] and decreases in response to insulin [10]. The latter is due to suppression of the effects of low concentrations of the β-adrenergic agonist isoproterenol [11]. These observations suggest that inhibitor-1 may play an important role in the hormonal control of glycogen metabolism and other cellular processes in which protein phosphatase-1 participates.

The phosphorylated form of inhibitor-1 is a non-competitive inhibitor [12] that is effective at nM levels, while the dephosphorylated form is completely inactive [12]. The physico-chemical properties of inhibitor-1 are unusual. It has a very low content of hydrophobic amino acids [8], possesses little ordered structure [13] and its activity is extremely stable to heat, exposure to low pH, organic solvents and detergents [1,8,9]. These observations suggest that the activity of inhibitor-1 might be determined by some feature of the primary structure, rather than the tertiary structure of the protein, a view supported by the isolation of a 65-residue CNBr peptide, containing threonine-35, that retained full activity [14]. However, a decapeptide, also containing threonine-35, was inactive [7].

We have determined the complete primary structure of inhibitor-1 and shown that it comprises 165 residues [8]. During the course of this analysis a large number of peptides containing threonine-35 were generated and tested for their ability to inhibit protein phosphatase-1. These results demonstrate that a hydrophobic region containing residues 9–22 is essential for the activity of inhibitor-1, in addition to the phosphorylation of threonine-35. A region between residues 42–54 may also be essential for activity.

2. MATERIALS AND METHODS

2.1. *Protein preparations*

^{32}P-Labelled inhibitor-1 [8], ^{32}P-labelled phosphorylase *a* [15] and protein phosphatase-1 [15] were isolated by published procedures. Proteinases and other materials were obtained from the sources given in [8].

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Table 1

Amino acid composition of ^{32}P-labelled subfragments of inhibitor-1 containing phosphothreonine-35

Amino acid	L1c	L1cA	L1b	L1bA	B1	Ch1
Aspartic acid asparagine	5.3(5)	3.9(4)	3.3(4)	2.3(3)	1.6(2)	2.4(1)
Threonine	3.8(4)	2.4(3)	3.4(4)	3.1(3)		3.2(2–3)
Serine	3.3(3)	3.0(3)	4.4(3)	2.6(3)		1.9(0)
Glutamic acid glutamine	9.1(8)	6.4(6)	8.1(8)	5.8(6)		4.9(4)
Proline	8.2(8)	5.7(6)	5.2(6)	4.2(4)	2.2(2)	5.1(4–5)
Glycine						2.4(0)
Alanine	3.0(3)	3.1(3)	3.3(3)	3.7(3)	1.2(1)	3.7(3)
Valine	2.7(3)	2.1(2)	2.8(3)	2.1(2)		1.7(0–2)
Isoleucine	2.5(3)	2.2(2)	1.5(2)	1.1(1)	1.0(1)	0.74(1)
Leucine	7.4(7)	4.1(4)	4.3(5)	2.4(2)	1.2(1)	3.6(3–5)
Phenylalanine	0.93(1)		0.88(1)			
Histidine	1.1(1)		0.97(1)			0.34(1)
Lysine	1.1(1)		0.94(1)			
Arginine	4.8(5)	4.5(5)	5.0(5)	4.7(5)	4.1(4)	3.8(4)
Total	52	38	46	32	11	23–29
Position in sequence	9–60	23–60	9–54	23–54	29–39	13/17-39/41

Peptides L1b and L1c obtained from inhibitor-1 by digestion with the lysine-specific proteinase, were cleaved to peptides L1bA and L1cA, respectively, by incubation at pH 2.9. Peptide B1 was obtained by subtilisin digestion of L1b/L1c and peptide Ch1 by chymotrypsin digestion of peptide CB-1 (see section 2). The numbers in parenthesis indicate residues present in the sequence. Serine and glycine in peptide Ch1 were contaminating amino acids from thin-layer peptide mapping. The first glycine residue in inhibitor-1 is at residue-93

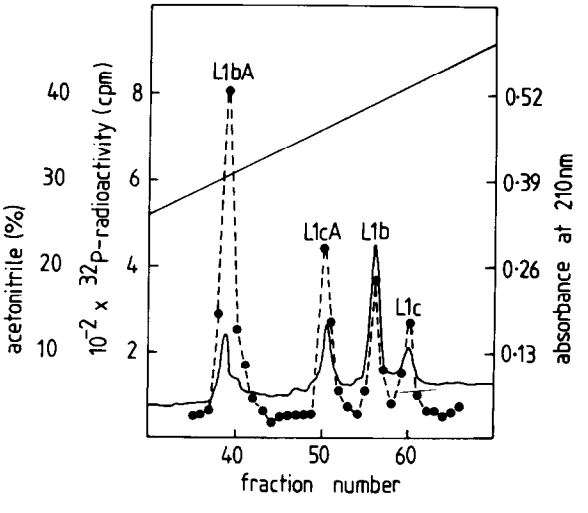

Fig.1. A fraction containing peptide L1b (40 nmol) and L1c (10 nmol) was incubated at pH 2.9 for 48 h as in section 2 and chromatographed on an Altex model 334 HPLC system using a Waters μbondapak C_{18} reverse-phase HPLC column (30 cm × 3.9 mm). A linear gradient of 0–55% acetonitrile/water containing 0.1% trifluoroacetic acid was employed at 1.0 ml/min. Fractions of 0.5 ml were collected: (•--•) ^{32}P-radioactivity (Cerenkov counting); (——) absorbance at 210 nm. The acetonitrile gradient is illustrated by the diagonal line.

2.2. *Isolation of ^{32}P-labelled peptides from inhibitor-1*

Digestion of inhibitor-1 with the proteinase from the fungus *Armillaria mellea* that cleaves specifically on the N-terminal side of lysine residues, termed 'lysine-specific proteinase', generates

a major [^{32}P]peptide L1c comprising residues 9–60 and two minor [^{32}P]peptides L1b and L1d comprising residues 9–54 and 33–60, respectively. The latter peptides arise from anomalous cleavage of Arg–Ile and Arg–Arg bonds, respectively [8]. A fraction containing peptide L1b (40 nmol) and L1c (10 nmol) was incubated for 48 h at 40°C in 1.0 M acetic acid, adjusted to pH 2.9 with pyridine. This dilute acid treatment produced ~70% cleavage of the Asp^{22}–Pro^{23} bond of inhibitor-1 [8]. The cleavage products and undigested peptides were purified by HPLC (fig.1) and their compositions are given in table 1. Peptides L1b/L1c (80 nmol) were also digested with subtilisin for 1 h at 37°C in 0.1 M *N*-ethyl morpholine acetate buffer (pH 8.5) at a weight ratio of proteinase: substrate of 1:100. The ^{32}P-labelled fragment (peptide B1) was purified by HPLC as in fig.1, and its composition is also given in table 1.

The active CNBr peptide CB-1 (residues 2–66, 2 mg) was digested with chymotrypsin for 1 h at 37°C in 0.05 M ammonium bicarbonate (pH 7.8) at a weight ratio of proteinase:substrate of 1:50. The ^{32}P-labelled peptide (termed Ch1) was isolated by gel filtration on Sephadex G-50 superfine and two dimensional peptide mapping. Automated sequencer analysis indicated that peptide Ch1 contained equal amounts of 2 peptides: one commencing at residue 13; the other at residue 17 of inhibitor-1 (not shown). The composition of the peptide (table 1) indicated that it terminated at residue(s) 39–41.

The isolation of other [^{32}P]peptides from inhibitor-1 are given in [8].

2.3. *Assay of inhibitor-1 and its subfragments*

The activity of inhibitor-1 was measured by its ability to inhibit the phosphorylase phosphatase activity of protein phosphatase-1 [12]. The reactions were carried out for only 5 min and initiated with protein phosphatase-1 rather than [^{32}P]phosphorylase *a*. These precautions were taken to avoid prolonged incubation of inhibitor-1 or its subfragments with protein phosphatase-1, which might have caused significant dephosphorylation and inactivation of these peptides. However, control incubations demonstrated that the dephosphorylation of each subfragment was negligible during the assays (< 5%). The specific radioactivity of inhibitor-1 and its subfragments was 20–50-fold lower than that of [^{32}P]phosphorylase *a*, and accounted for < 1% of the ^{32}P-radioactivity in the assays. The inhibitory activity of all peptides was measured ≥ 5 times over a 2000-fold concentration range (from nM–μM).

2.4. *Other analytical procedures*

Peptide mapping, gel filtration, amino acid analysis and automated sequencing were done as in [8].

3. RESULTS AND DISCUSSION

The activities of peptide subfragments derived from inhibitor-1 are given in table 2 and their structures are summarised in fig.2. The smallest peptide that was fully active was L1b comprising only 28% of the molecule (residues 9–54). However peptides L1bA and L1cA, comprising residues 23–54 and 23–60 respectively, were inactive, as were peptides comprising residues 32–60 or 32/33–69. Inhibitor-1 as normally isolated is substantially phosphorylated on Ser^{67} [8]. This phosphoserine does not appear to be involved in the activity since the fragments that were fully active do not contain this residue. Furthermore a peptide comprising residues 61–71 was inactive (table 2).

The results presented in table 2 and fig.2 demonstrate that the region comprising residues 9–22 is essential for the activity of inhibitor-1. The finding that a region not in the immediate vicinity of Thr^{35} is required for activity, is consistent with the kinetic studies that indicate inhibitor-1 is not a competitive inhibitor [11]. Interestingly, residues 9–22 is the most hydrophobic region of inhibitor-1, a protein with a very low content of hydrophobic amino acids [8]. Application of the predictions of Chou and Fasman [16] to the structure of inhibitor-1 suggest that inhibitor-1 contains a limited amount of secondary structure (mostly β-sheet) between residues 8–47, while the rest of the molecule contains little-ordered structure. These predictions are consistent with the results of circular dichroism studies [13]. Thus the region that contains secondary structure can be equated with the active part of the protein.

Peptides L1b (residues 9–54) and L1c (9–60) were both fully active indicating that the hydrophobic stretch from residues 55–60 (fig.2) is not required for activity. A chymotryptic peptide com-

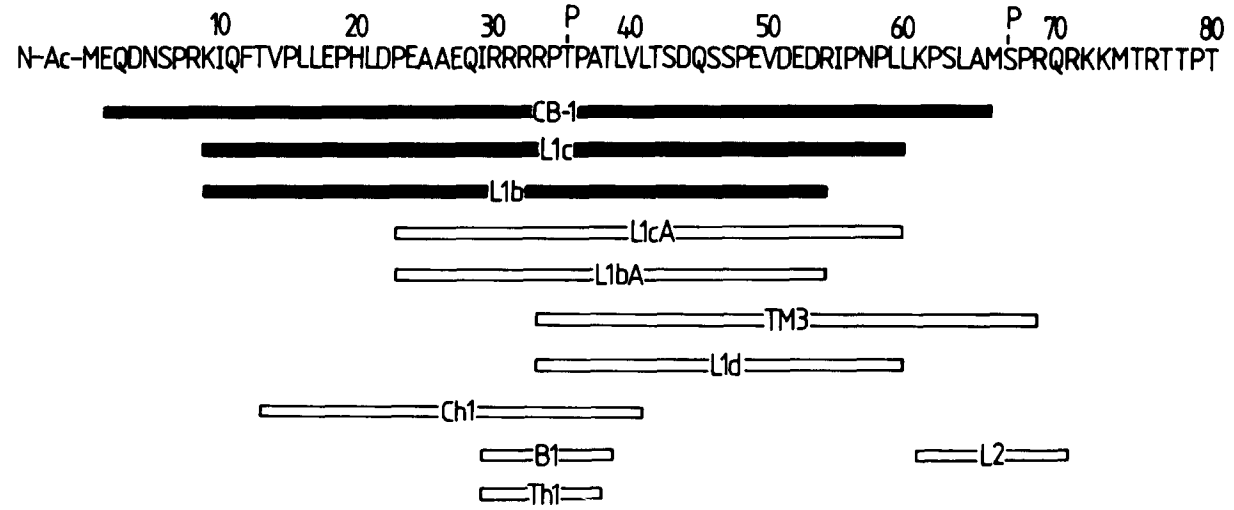

Fig.2. N-Terminal 80 residues of inhibitor-1 containing the active fragments CB-1, L1b and L1c: (■) active fragments; (▭) inactive fragments.

prising residues 13–39/41 was completely inactive. This might indicate that the region from residues 9–13 is crucial for activity, but it is perhaps more likely that some or all of the residues from 42–54 are essential. The resolution of this important question would require the isolation of peptides comprising residues 9–41 and 13–54, but it has not yet proved possible to obtain a specific cleavage between residues 41–54 without cleaving other peptide bonds between residues 9–32 (table 2).

Table 2

Activities of the subfragments of inhibitor-1

Digest	Subfragment	Activity (%)	Position in sequence	Size (residues)
None	inhibitor-1	100	1–165	165
Cyanogen bromide	CB-1	100[a]	2– 66	65
Lysine-specific proteinase	L1c	100	9– 60	52
	L1b	100	9– 54	46
pH 2.9 hydrolysis of L1c	L1cA	0.2	23– 60	38
of L1b	L1bA	0.1	23– 54	32
Trypsin	TM3	<0.1	32/33– 69	37–38
Lysine-specific proteinase	L1d	<0.1	32– 60	29
Chymotrypsin	Ch1	<0.1	13/17– 39/41	23–29
Subtilisin	B1	0.2	29– 39	11
Thermolysin	Th1	0.1[b]	29– 38	10
Lysine-specific proteinase	L2	<0.1	61– 71	11

[a]From [14]; [b]from [7]

Peptides L1b, L1bA, L1c, L1cA, Ch1 and B1 were purified as in section 2, peptides CB1, TM3, and L1d as in [8] and Th-1 as in [7]

Further progress in defining the smallest active fragment of inhibitor-1 would appear to depend on the chemical synthesis of appropriate phosphopeptides. This would also allow interesting substitutions to be made, such as serine for threonine at position 35. It would additionally be of interest to examine the rates of phosphorylation and dephosphorylation of such fragments by cyclic AMP-dependent protein kinase and protein phosphatases for which intact inhibitor-1 is a substrate [11,17,18].

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