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Primary structure of the site on bovine hormone-sensitive lipase phosphorylated by cyclic AMP-dependent protein kinase

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The primary structure of a region on hormone-sensitive lipase was determined to be: Lys-Thr-Glu-Pro-Met-Arg-Arg-Ser-Val-Ser-Glu-Ala-Ala-Leu-Thr-Gln-Pro-Glu-Gly-Pro-Leu-Gly-Thr-Asp-Ser-Leu-Lys. Ser-8 was the only residue in the intact protein phosphorylated by cyclic AMP-dependent protein kinase. However, Ser-10 also appeared to be present in a phosphorylated form, suggesting that it is a target for a distinct protein kinase in vivo.

Hormone-sensitive lipase; Protein phosphorylation; cyclic AMP; Amino acid sequence; Protein kinase

1. INTRODUCTION

Hormone-sensitive lipase (HSL) catalyses the rate-limiting step in adipose tissue lipolysis and its activity is under acute hormonal and neuronal control [1]. Noradrenalin, released from sympathetic nerve endings, and circulating adrenalin, corticotropin and glucagon, all stimulate lipolysis by raising the intracellular concentration of cyclic AMP. This leads to phosphorylation of HSL, causing activation of the enzyme and subsequent lipolysis [2]. The major anti-lipolytic hormone is insulin which reduces phosphorylation of HSL [2], and acts, at least in part, by lowering the level of cyclic AMP [3].

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Abbreviations: HSL, hormone-sensitive lipase; TFA, trifluoroacetic acid; Pth, phenylthiohydantoin

Enzymes: hormone-sensitive lipase or triacylglycerol lipase (EC 3.1.1.3); cyclic AMP-dependent protein kinase (EC 2.7.1.37); trypsin (EC 3.4.21.4); thermolysin (EC 3.4.24.4); *N*-Lys-specific proteinase (EC 3.4.99.32); *Staphylococcus aureus* V8 proteinase (EC 3.4.21.19)

Peptide mapping studies have suggested that HSL is phosphorylated at one site in vitro by cyclic AMP-dependent protein kinase [4]. This site is also that which is phosphorylated in isolated adipocytes in response to noradrenalin, consistent with the conclusion that this hormone acts via activation of cyclic AMP-dependent protein kinase [5]. A second site on HSL is also phosphorylated in the absence of lipolytic hormones. However, the extent of phosphorylation of this site does not change in the presence of lipolytic hormones [5] and its phosphorylation does not apparently have a direct effect on the activity of HSL [6].

Here, we report the primary structure of a region surrounding the serine residue phosphorylated in vitro by cyclic AMP-dependent protein kinase.

2. MATERIALS AND METHODS

2.1. *Materials*

HSL was purified from bovine perirenal adipose tissue, essentially as in [7], except that the final chromatography on heparin-Sepharose was omitted. The catalytic subunit of cyclic AMP-dependent protein kinase was purified to homogeneity from bovine heart or bovine adipose tissue [8]. [γ-^{32}P]ATP was from Amersham International, thermolysin and trypsin (treated with diphenyl carbamyl chloride) from Sigma, and

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Staphylococcus aureus V8 proteinase from Boehringer. *N*-Lys-specific proteinase from the fungus *Armillaria mellea* was provided by Dr S. Doonan (University of Cork, Eire). The non-ionic detergent $C_{13}E_{12}$ was from Berol Kemi (Sweden).

2.2. *Phosphorylation of HSL*

Prior to phosphorylation, HSL (approx. 15% pure) was dialysed against 5 mM imidazole-HCl, pH 7.0, containing 50 mM NaCl, 0.2% (w/v) $C_{13}E_{12}$, 30% (w/v) glycerol, 1 mM dithiothreitol, 0.1 mM benzamidine, 5 mg/l leupeptin and 1 mg/l pepstatin and then concentrated to approx. 5 mg/ml using an Amicon Centricon 30 centrifugal microconcentrator. Phosphorylation was carried out at 37°C for 15 min in the presence of 0.15 mM [γ-^{32}P]ATP (400–1000 dpm/pmol), 1 mM $MgCl_2$ and 3 μM catalytic subunit of cyclic AMP-dependent protein kinase. The reaction was terminated by addition of an equal volume of 0.3 M Tris-HCl (pH 6.8) containing 25% (v/v) glycerol, 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.0025% (w/v) bromophenol blue, followed by heating at 100°C for 5 min.

2.3. *Isolation and digestion of phosphorylated HSL*

Phosphorylated HSL was isolated by preparative SDS-polyacrylamide gel electrophoresis [9]. Following staining with Coomassie blue and destaining, the 84 kDa HSL polypeptide was excised from the gel and the protein eluted and precipitated with 20% (w/v) trichloroacetic acid, using 20 μg/ml fragmented DNA as carrier [10]. After centrifugation, the protein pellets were washed three times with ether/ethanol (3:1) and resuspended in 0.2 ml of 0.5% (w/v) NH_4HCO_3.

^{32}P-HSL (3 nmol) was digested with trypsin (20 μg/ml) for 1 h at 37°C in the presence of 2 mM $CaCl_2$, the reaction being terminated by the addition of trypsin inhibitor to 30 μg/ml. Following digestion, insoluble material was removed by centrifugation and the pellet extracted twice with 0.1 ml water to increase the recovery of soluble phosphopeptide.

HSL was also digested sequentially with V8 proteinase and *N*-Lys-specific proteinase. ^{32}P-HSL (2 nmol) was first digested for 5 h at 37°C with 1 mg/ml V8 proteinase, the reaction being terminated by heating for 2 min at 100°C. Insoluble material was removed by centrifugation and the pellet extracted with 0.2 ml of 0.5% (w/v) NH_4HCO_3. The resulting supernatants (0.4 ml) were then digested with 0.5 μg *N*-Lys-specific proteinase for 15 h at 37°C, the reaction being terminated as described above.

Digestion of HSL with thermolysin was carried out as described in [11].

2.4. *Purification and analysis of HSL phosphopeptides*

The phosphopeptides generated by proteolytic digestion were dried in a vacuum concentrator, resuspended in 1.0 ml of 1.0 M acetic acid and subjected to gel filtration on Sephadex G-25 Superfine (90 × 1.2 cm) equilibrated in the same solvent. After pooling and drying the radioactive fractions, further purification was achieved by reverse-phase HPLC on a Vydac 218TP54 C_{18} column (Phase Separations Group, Hesperia, CA) using a Gilson HPLC system equipped with on-line ultraviolet detector and radioactivity monitor. The peptides were applied to the column in 0.1% (v/v) TFA and eluted with a linear gradient from 0 to 30% acetonitrile in 0.1% TFA, with an increase in acetonitrile concentration of 0.33% per min. Further purification was achieved either by rechromatography on the Vydac column equilibrated in 10 mM ammonium acetate, pH 6.5, and eluted with a gradient of 0–30% acetonitrile, or by rechromatography in 0.1% TFA after incubating the peptide with ethanethiol in NaOH to convert phosphoserine residues to *S*-ethylcysteine [12]. *S*-Ethylcysteinyl peptides are eluted at higher acetonitrile concentrations than the phosphopeptides from which they were derived, and therefore resolved from impurities [13].

Amino acid analysis was carried out using a Waters Picotag system and sequencer analysis on an Applied Biosystems 470A sequencer equipped for on-line Pth-amino acid analysis. The method of Wang et al. [14] was used to identify residues to which [^{32}P]phosphate was attached, except that thin layer electrophoresis rather than HPLC was used to separate inorganic [^{32}P]phosphate from ^{32}P-peptides.

3. RESULTS

Tryptic digestion solubilised 70–80% of the ^{32}P radioactivity associated with HSL. Approx. 80% of this radioactivity was eluted as a single peak from Sephadex G-25 (K_{av} = 0.13, not shown) and reverse-phase HPLC (fig.1A). The tryptic peptide (T1) was pure, and its N-terminal sequence is given in table 1. All of the ^{32}P radioactivity associated with the peptide was released as inorganic

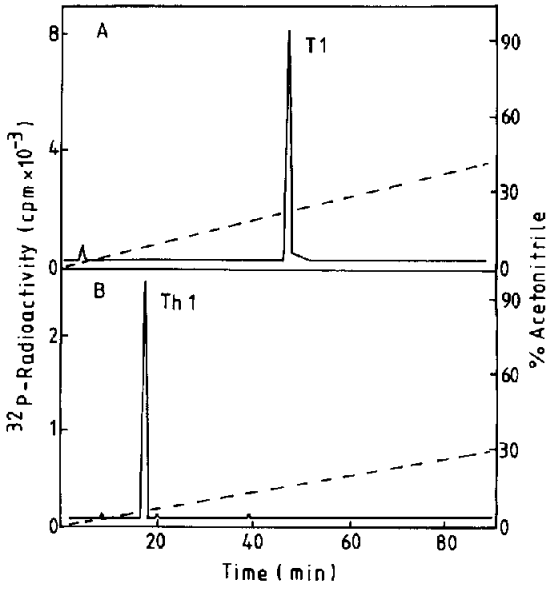

Fig.1. Separation of tryptic and thermolytic phosphopeptides on a Vydac C_{18} column. Phosphopeptides from Sephadex G-25 were chromatographed on a Vydac C_{18} column equilibrated in 0.1% (v/v) TFA as described in section 2. The elution profile of a tryptic digest is shown in panel A, and that of a thermolytic digest in panel B. ^{32}P radioactivity is denoted by the full line and the acetonitrile gradient by the broken line. No radioactivity was eluted after 90 min.

phosphate after one cycle of Edman degradation, demonstrating that the N-terminal serine was the residue phosphorylated in the intact protein by cyclic AMP-dependent protein kinase.

Although the tryptic phosphopeptide allowed determination of the primary structure on the carboxyl side of the phosphoserine, it is known that the important recognition feature for cyclic AMP-dependent protein kinase is the presence of basic residues on the amino-terminal side of the phosphorylated residue [15]. Therefore, different digestion conditions were employed to obtain this additional information. Digestion of HSL with thermolysin solubilized 80–90% of the ^{32}P radioactivity associated with HSL, and this radioactivity was eluted as a major peak from Sephadex G-25 (K_{av} = 0.27, not shown) or reverse-phase HPLC in the presence of TFA (fig.1B). This thermolytic peptide, termed Th1, after further purification by reverse-phase HPLC at pH 6.5, had the composition $Ser_{0.9}$, $Arg_{2.0}$, $Met_{0.7}$, and its N-terminal sequence was Met-Arg-Arg (table 1). This suggested that Th1 had the structure Met-Arg-Arg-Ser(P), and the complete absence of valine in the composition indicated that it was generated by cleavage of the phosphoseryl–valine bond in the tryptic peptide (table 1). These results were confirmed by digestion of a different HSL preparation with a combination of V8 proteinase and *N*-Lys-specific proteinase. The phosphopeptide generated in this way was eluted from Sephadex G-25 as a major peak (K_{av} = 0.26). However, following reverse-phase HPLC in the presence of TFA, two major radioactive peaks, VL1 and VL2, were obtained (fig.2B). When these peptides were rechromatographed on the Vydac C_{18} column, phosphopeptide VL1 yielded a single radioactive peak, while VL2 was resolved into two major peaks, termed VL2a and VL2b, the former comigrating with VL1. The sequences of VL1, VL2a and VL2b were identical (quantitation for VL1 only is shown on table 1). This information confirmed that the sequence of the thermolytic peptide was Met-Arg-Arg-Ser, and extended the sequence N-terminal to the phosphoserine by four residues.

A further preparation of HSL when digested with trypsin yielded two peaks of ^{32}P radioactivity on reverse-phase HPLC in the presence of TFA (fig.2A). One of these comigrated with peptide T1 (fig.1A), while the second, termed T2, was eluted

Table 1

Quantitation of gas-phase sequencer analyses

	T1		Th1		VL1		T2(S-Et-C)	
	Amino acid	pmol	Amino acid	pmol	Amino acid	pmol	Amino acid	pmol
1	S	63	M	70	K	28	S	90
2	V	31	R	30	T	13	V	85
3	S	16	R	26	E	51	S-Et-C	48
4	E	22			P	54	E	70
5	A	19			M	31	A	60
6	A	18			R	6	A	53
7	L	4			R	7	L	65
8	T	2			S	30	T	9
9	Q	2			V	17	Q	13
10	P	3			S	20	P	30
11	E	2			E	3	E	18
12							G	17
13							P	14
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15							G	10
16							T	1
17							D	3
18							S	4
19							L	5
20							K	1

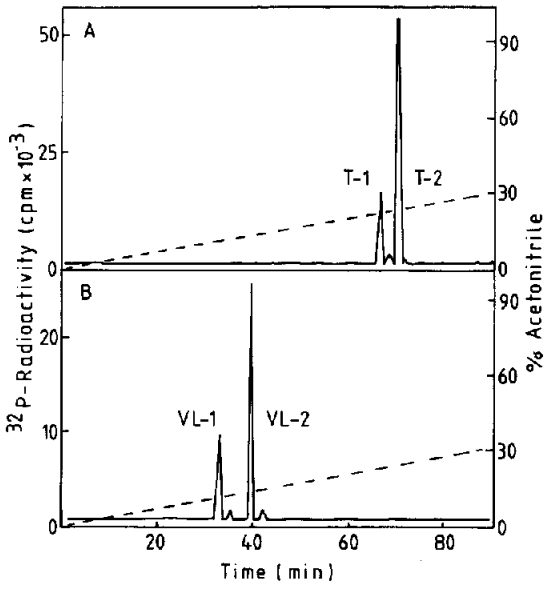

Fig.2. Separation of phosphopeptides by reverse-phase HPLC. Phosphopeptides generated by digestion of a different preparation of HSL with trypsin (A), or a combination of V8 proteinase and *N*-Lys-specific proteinase (B), were chromatographed on a Vydac C_{18} column as in fig.1.

at a slightly higher acetonitrile concentration. All of the ^{32}P radioactivity associated with T2 was released as inorganic phosphate after one cycle of Edman degradation, demonstrating that, as for T1, the N-terminal serine was the only residue phosphorylated by cyclic AMP-dependent protein kinase. In order to purify T2, this peptide was treated with ethanethiol in NaOH to convert phosphoserine to *S*-ethylcysteine, and rechromatographed on the Vydac C_{18} column under the same conditions. The pool of derivatised peptide was eluted 5–15 min later than before, and was therefore obtained in pure form. Sequencer analysis yielded the complete structure of T2 (table 1), and its N-terminal sequence was identical to that of T1 (table 1).

The analysis of T1, T2 and Th1 had established that the N-terminal serine of the tryptic peptide was the only residue in the intact protein phosphorylated by cyclic AMP-dependent protein kinase. It was therefore initially surprising to find that the serine at position 3 of T2, and not the N-terminal serine, was the residue converted to *S*-ethylcysteine (table 1). However, Meyer et al. [16] have reported that N-terminal (or C-terminal) phosphoserine residues do not convert to *S*-ethylcysteine following incubation with ethanethiol in NaOH. The presence of *S*-ethylcysteine at position 3 implies that this residue is esterified (presumably to phosphate) in the purified peptide. Since this phosphoserine is not ^{32}P-labelled, it would appear to represent an in vivo site of phosphorylation, presumably introduced by an enzyme distinct from cyclic AMP-dependent protein kinase.

Since peptides containing two phosphate groups are eluted from reverse-phase HPLC at lower acetonitrile concentrations than monophosphorylated derivatives [17], T1 and VL1 may represent peptides phosphorylated at both serines, while peptides T2 and VL2 may be monophosphorylated species containing phosphoserine at either position. The reason why peptide VL2 is resolved into two peaks by rechromatography at pH 6.5 is unclear, although partial oxidation of the methionine residue could underlie this observation.

4. DISCUSSION

From the sequences given in table 1 the primary structure of a 27-residue region of the HSL polypeptide containing the serine residue phosphorylated by cyclic AMP-dependent protein kinase, can be deduced. The sequence, KTEPMRRS(P)VSEAALTQPEGPLGTDSLK, is consistent with the known specificity of this protein kinase, in that two adjacent basic residues are present on the amino-terminal side of the phosphorylated serine [15]. The lack of an intervening residue between the phosphoserine and the two arginine residues is unusual, but occurs in cardiac troponin-I [18]. The very small acidic peptide that is generated by digestion with a combination of trypsin and V8 proteinase [4] is likely to comprise residues 8–11.

The sequence given here is the first reported data on the primary structure of HSL and may be valuable for the production of anti-peptide antibodies and for initiating cDNA cloning. It will also be of interest to investigate whether the second phosphorylation site that has been identified in this region corresponds to the serine residue that is phosphorylated in vivo, but which is unaffected by hormonal stimulation (see section 2).

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