Amino acid sequence at the major phosphorylation site on bovine kidney branched-chain 2-oxoacid dehydrogenase complex

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Received 28 September 1983

Inactivation of branched-chain 2-oxoacid dehydrogenase complex correlates with phosphorylation at one site on the α subunit of the E_1 component. The amino acid sequence surrounding this phosphorylated serine residue has now been determined. This sequence shows certain similarities with the sequence surrounding phosphorylated residue(s) on pyruvate dehydrogenase complex.

Branched-chain 2-oxoacid dehydrogenase complex (Bovine kidney cortex) *Phosphorylation site* *Amino acid sequence*

1. INTRODUCTION

The activity of the mitochondrial branched-chain 2-oxoacid dehydrogenase complex is regulated by covalent phosphorylation [1]. Phosphorylation of the α subunit of the E_1 component by a protein kinase intrinsic to the complex causes complete loss of activity [2–4].

We have reported previously that three distinct sites on the α subunit are phosphorylated by the kinase. These phosphorylation sites are recovered on 3 tryptic peptides designated T1, T2 and T3. Inactivation correlates closely with phosphorylation of one site [5]. We here report isolation of the tryptic phosphopeptide T1 containing this site and we have determined the amino acid sequence surrounding the phosphorylated serine residue. Homology with the reported sequence surrounding the phosphorylation sites on pyruvate dehydrogenase complex [6,7] is discussed.

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2. MATERIALS AND METHODS

2.1. *Materials*

Bovine kidney branched-chain 2-oxoacid dehydrogenase complex, with endogenous kinase activity, was purified, phosphorylated and desalted as in [4,5]. Trypsin (TPCK-treated) was from Worthington, subtilisin from Sigma and [γ-^{32}P]ATP was synthesised as in [8].

2.2. *Digestion with proteases*

Phosphorylated branched-chain 2-oxoacid dehydrogenase (25 mg) was digested with trypsin (0.5 mg) for 30 min at 20°C in 0.2 M NH_4HCO_3 (pH 8.4). Phosphopeptide T1 (24 nmol) was subdigested with subtilisin (100 μg) for 5 h at 37°C in 0.1 M NH_4HCO_3 (pH 7.8). This digestion was terminated by lyophilisation and the sub-peptides were purified by reverse phase HPLC.

2.3. *Purification of peptides*

High voltage paper electrophoresis at pH 1.9 was carried out as in [5]. Two-dimensional peptide mapping on cellulose sheets was as in [9]. This involved electrophoresis in pyridine/acetic acid/water (pH 3.5), followed by ascending chromatography in pyridine/acetic acid/water/butanol (10 : 3 : 12 : 15).

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

Peptides were also purified by HPLC on a Waters μbondapak C_{18} reverse-phase HPLC column [10]. A linear gradient (0–40%) of water/acetonitrile, containing 0.1% trifluoroacetic acid, was used, with an increase in acetonitrile concentration of 1%/min. The flow rate was 1.0 ml/min and 0.5 ml fractions were collected. The effluent was monitored at 215 nm and by Cerenkov counting for ^{32}P-labelled peptides.

2.4. *Amino acid and automatic sequencer analysis*

Amino acid analyses were carried out on an LKB Biochrom 4400 amino acid analyser. Automatic sequencer analysis was done on a Beckman 890C sequencer using a 0.2 M quadrol programme. Polybrene (2 mg) and heparin (2 mg) were added to the sequencer cup to help prevent washout of peptides. Conversion to Pth derivatives was by incubation with 20% trifluoroacetic acid for 8 min at 80°C. Residues were identified by HPLC and by back-hydrolysis [9,10]. To identify the phosphorylated species in phosphopeptides, partial acid hydrolysis was carried out for 2 h at 110°C in 6 N HCl and the products separated by high voltage electrophoresis at pH 1.9.

3. RESULTS

3.1. *Purification of peptide T1 from branched-chain 2-oxoacid dehydrogenase*

Following tryptic digestion of phosphorylated branched-chain 2-oxoacid dehydrogenase, the digestion was terminated by addition of 0.1 vol. 100% (w/v) trichloroacetic acid. After standing for 10 min on ice, the mixture was centrifuged at 10 000 × *g* for 2 min. The supernatant, containing over 95% of the initial radioactivity but only 15% of the initial protein (as judged by absorbance at 280 nm), was extracted 5 times with diethyl ether and lyophilised. Alternatively digestion was terminated by lyophilisation and the phosphopeptides were separated by high voltage electrophoresis at pH 1.9. After autoradiography the peptides were eluted in 7% (v/v) formic acid and lyophilised.

Further purification was achieved by reverse phase HPLC. Peptide T1 emerged as one major peak of radioactivity at 24% acetonitrile. It was then subjected to two-dimensional peptide mapping. T1 was detected by autoradiography and eluted in 1.0 M acetic acid. Staining of the map with fluorescamine indicated that T1 was free of contaminating peptides.

Table 1

Amino acid composition of peptides

Amino acid	Tryptic peptide T1	Subtilisin peptides		
		$T1_a$	$T1_b$	$T1_c$
Aspartic acid	1.85(2)			
Threonine	1.05(1)		1.36(1)	
Serine	3.76(4)		1.87(2)	0.25(0)
Glycine	1.12(1)	0.98(1)		0.30(0)
Alanine	0.98(1)			1.30(1)
Isoleucine	1.04(1)	0.89(1)		
Tyrosine	1.18(1)			1.07(1)
Histidine	1.80(2)	2.13(2)		
Arginine	0.99(1)			0.69(1)
Residues	14	4	3	3
^{32}P-Radioactivity	+	−	+	−

Peptides were hydrolysed for 24 h at 110°C in 6 N HCl containing 2 mM phenol. Values for serine and threonine were corrected for 10 and 5% destruction, respectively

Table 2

Automatic sequencer analysis of peptides

Step	Peptide T1 (20 nmol)		Peptide $T1_c$ (18 nmol)
	HPLC	Amino acid analysis	HPLC
1	Ile	Ile (11.8)	Ala
2	Gly	Gly (13.3)	Tyr
3	His	His (2.8)	Arg
4	His	His (2.5)	
5	Ser	Ala (1.0)	
6	Thr	α-Aba (2.0)	
7	Ser	Ala (0.9)	
8	Asp	Asp (1.7)	
9	Asp	Asp (1.6)	
10	Ser	Ala (1.0)	
11	–	–	
12	Ala	–	
13	Tyr	–	
14	–	–	

Serine and threonine were identified as alanine and α-aminobutyric acid (α-aba) respectively, on amino acid analysis following back hydrolysis. Figures in parentheses indicate recovery in nmol

3.2. *Sequence analysis of peptide T1*

Amino acid analysis of the phosphopeptide T1 indicated that it consists of 14 residues (table 1). Automatic sequencer analysis allowed residues 1–10 to be identified by HPLC and amino acid analysis after hydrolysis (table 2). Residues 12 and 13 were identified by HPLC. The sequencer analysis was carried out twice. As T1 is a tryptic phosphopeptide containing only a single arginine this would be expected to be at the carboxyl terminus of the peptide, namely residue 14. The sequence of residues 12–14 was confirmed by analysis of a subtilisin sub-fragment $T1_c$ (tables 1,2). No residue was positively identified at position 11, although a trace of Pth-dehydroalanine was detected by HPLC. This breakdown product of Pth-serine has a characteristic absorbance at 313 nm. As amino acid analysis of T1 indicated the presence of 4 serine residues and as all other residues and 3 of the serine residues were positively assigned other positions in T1, it was concluded that residue 11 was serine. The complete sequence of T1 is shown in table 3.

3.3. *Identification of the phosphorylated residue of T1*

Partial acid hydrolysis of T1 (and of T2 and T3) followed by high voltage electrophoresis at pH 1.9 showed the presence of phosphoserine. No phosphothreonine or phosphotyrosine was detected (not shown). Automatic sequencer analysis of T1 showed that a peak of ^{32}P-radioactivity was released at the fifth cycle. No other peak was observed (fig.1). The serine residue at position 5 was

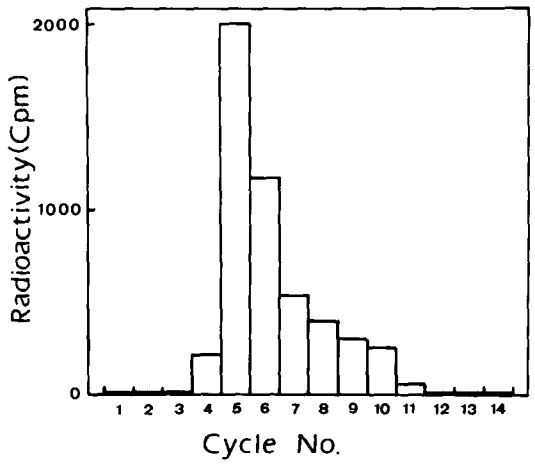

Fig.1. Automatic sequencer analysis of peptide T1. ^{32}P-radioactivity extracted from the sequencer cup into butyl chloride at each cycle of Edman degradation, was determined by Cerenkov counting.

Table 3

Amino acid sequences at the major (inactivating) phosphorylation site on branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes

Protein	Sequence
Branched-chain 2-oxoacid dehydrogenase (this work)	Ile-Gly-His-His-Ser(P)-Thr-Ser-Asp-Asp-Ser-Ser-Ala-Tyr-Arg
Pyruvate dehydrogenase [6,7]	Tyr-His-Gly-His-Ser(P)-Met-Ser-Asx-Pro-Gly-Val-Ser-Tyr-Arg

confirmed as the only phosphorylated residue in T1 by sub-digestion with subtilisin. Three subpeptides were recovered by HPLC (table 1), only one of which, $T1_b$, was radioactive. This peptide corresponds to residues 5–7 of T1. After one cycle of Edman degradation, all the radioactivity was removed from $T1_b$ and was detected as inorganic [^{32}P]phosphate following paper electrophoresis at pH 1.9 and autoradiography. It is therefore concluded that Ser-5 is the only phosphorylated residue in the phosphopeptide T1.

4. DISCUSSION

Inactivation of branched-chain 2-oxoacid dehydrogenase complex correlates closely with phosphorylation of one site on the α subunit of the E_1 component [5]. We report here the amino acid sequence surrounding the phosphorylatable serine residue. This sequence shows considerable homology with the sequence surrounding the serine residue on pyruvate dehydrogenase complex (table 3), phosphorylation of which causes inactivation of that complex [6,7]. Six of the 14 residues in the tryptic phosphopeptides are identical. Furthermore 4 of the 5 residues immediately surrounding the phosphorylated serine are identical in both complexes.

We have noted previously [5] several similarities between the regulation by phosphorylation of the 2 complexes, including the phenomenon of multisite phosphorylation. Phosphorylated preparations of each complex yield 3 tryptic phosphopeptides designated T1, T2 and T3 [5,6]. With pyruvate dehydrogenase complex, peptide T2 is a diphosphorylated form of peptide T1 whereas T3 is a distinct phosphopeptide. In preliminary work with branched-chain 2-oxoacid dehydrogenase complex we have found that a phosphopeptide corresponding in electrophoretic mobility to T2 has the same sequence as T1 but possesses an additional hydrophobic region at its carboxyl terminus (unpublished). The sequence of this hydrophobic region and identification of the phosphorylated residues in the peptide has yet to be determined. Preliminary analysis of T3 indicates that it is a distinct peptide from T1 and T2.

Knowledge of the primary structure surrounding the phosphorylation sites will facilitate further detailed investigation of the covalent regulation of the branched-chain 2-oxoacid dehydrogenase. Of particular interest will be studies using synthetic peptides to investigate the specificity requirements of the respective kinases and phosphatases which act on the branched-chain 2-oxoacid dehydrogenase and pyruvate dehydrogenase complexes.

ACKNOWLEDGEMENTS

This work was supported by grants from the Medical Research Council, UK to S.J.Y. and to Professor Philip Cohen, whom we thank for providing facilities for part of this work. R.L. is the recipient of a Research Studentship from the Science and Engineering Research Council, UK.

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