Amino acid sequence round the site of phosphorylation in isocitrate dehydrogenase from *Escherichia coli* ML308

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Isocitrate dehydrogenase from *Escherichia coli* is regulated by a reversible phosphorylation mechanism. We report here the amino acid sequence round the phosphorylation site; this is the first such sequence to be reported for a bacterial protein kinase. The sequence does not resemble sequences phosphorylated by cyclic AMP-dependent protein kinase.

Isocitrate dehydrogenase *Protein phosphorylation* *Amino acid sequence* *Phosphorylation site*

1. INTRODUCTION

The NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) of *Escherichia coli* can be regulated by reversible phosphorylation [1,2]: this is the first example of control of a key metabolic enzyme by phosphorylation in a prokaryote (review [3]). The phosphorylation and dephosphorylation of isocitrate dehydrogenase are mediated by a single bifunctional enzyme, isocitrate dehydrogenase kinase/phosphatase, and cause inactivation and activation, respectively [4–6]. The metabolic role of the phosphorylation system is to resolve the competition between isocitrate dehydrogenase and the glyoxylate bypass enzyme isocitrate lyase that occurs during growth on acetate (review [3]).

The only phosphoamino acid present in phosphorylated isocitrate dehydrogenase is a phosphoserine residue [2,5,7]. We have shown that, either in vivo or in vitro, the enzyme is phosphorylated at a single site contained within a unique 22-residue chymotryptic peptide [7]. To compare the specificity of isocitrate dehydrogenase kinase with those of other protein kinases we have subjected this peptide to automated sequence analysis. We now report the sequence of the first 14 residues of the chymotryptic peptide, including the phosphoserine residue.

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Abbreviations: Pth-, phenylthiohydantoin derivative of an amino acid; HPLC, high-performance liquid chromatography

2. EXPERIMENTAL

2.1. *Materials*

The active form of isocitrate dehydrogenase was purified from *E. coli* ML308 [8] and phosphorylated using isocitrate dehydrogenase kinase/phosphatase and [γ-^{32}P]ATP [5]. The material was carboxymethylated and digested with chymotrypsin and the ^{32}P-containing chymotryptic peptide was isolated as in [7]. Thermolysin was obtained from Boehringer (Lewes, England). The materials for automated sequence analysis were obtained as in [9,10]. The sources of other materials were as given in [5,7,8].

2.2. *Automated sequence analysis*

This was carried out as in [9,10] using a Beckman 890C sequencer. The peptides were applied to the sequencer cup containing Polybrene [9,11]. Conversion to Pth-amino acids was carried out as described in the text.

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2.3. *Identification of Pth-amino acids and amino acid analysis*

The Pth-amino acids were identified by reversed-phase HPLC as in [9,12]. Samples were hydrolysed in 5.5 N HI and subjected to amino acid analysis on an LKB Biochrom 4400 analyser.

2.4. *Sub-digestion with thermolysin*

The ^{32}P-containing chymotryptic peptide (10 nmol) was incubated with 40 μg thermolysin in 0.1% ammonium bicarbonate containing 1 mM $CaCl_2$ for 16 h at 37°C. The material was freeze-dried, dissolved in 0.1% trifluoroacetic acid and applied to a Waters C_{18} μbondapak reversed phase column (30 cm × 3.9 mm). Peptides were eluted with a linear gradient of 10–35% acetonitrile in 0.1% trifluoroacetic acid (0.5% acetonitrile/min, flow rate 0.5 ml/min) and detected by absorbance measurements at 215 nm.

3. RESULTS AND DISCUSSION

Amino acid analysis of the 22-residue chymotryptic phosphopeptide from isocitrate dehydrogenase revealed that it contained only one serine residue [7]. The peptide was subjected to sequence analysis on a Beckman 890C sequencer. In the first experiment the Pth-amino acids were generated by treatment with 0.1 M HCl for 10 min at 80°C [9], and were detected and quantitated after HPLC by absorbance measurements at 254 nm. The first 14 residues were assigned successfully with the exception of residue 10 (table 1). No Pth-amino acid was detected at this turn. However, a peak of ^{32}P radioactivity was released at this turn (table 1), suggesting that residue 10 is the phosphoserine residue. The trail of ^{32}P at subsequent turns is probably caused by poor extraction of P_i from the sequencer cup. The provisional assignment of phosphoserine at residue 10 is consistent with the failure to detect a Pth-amino acid at this turn because Pth-phosphoserine breaks down readily to give Pth-dehydroserine, which does not absorb significantly at 254 nm. The low yields of threonine at turns 1 and 2 in this experiment were caused by the relatively harsh conditions used for the generation of the Pth-amino acids [9].

In a second experiment, the Pth-amino acids were generated by treatment with 20% trifluoroacetic acid for 8 min at 80°C; when serine or threonine residues were expected treatment was for 10 min at 60°C [11]. The HPLC effluents were monitored at both 254 and 313 nm [11] to allow detection of the dehydro derivatives of serine and threonine. The first 8 turns confirmed the assignments made in the first sequencer run. However, the 3 glycine residues at positions 5–7 caused a considerable reduction in yield and no residues beyond position 8 could be identified.

To circumvent this problem, the chymotryptic peptide was digested further with thermolysin and the resulting peptides were separated using HPLC. Only two peptides, ChT I and ChT II, were detected; these were eluted from the column at 19 and 25% acetonitrile, respectively. All of the recovered ^{32}P radioactivity was associated with peptide ChT II (not shown). The amino acid compositions of these two peptides are shown in table 2. The data in tables 1 and 2 suggest that these peptides correspond to residues 1–7 and 8–12 of the chymotryptic phosphopeptide. Cleavages of

Table 1

Sequence analysis of chymotryptic phosphopeptide

Residue no.	Pth-amino acid	Recovery (nmol)	^{32}P released (cpm)	Amino acid from back-hydrolysis
1	Thr	7.7	980	Thr
2	Thr	5.5	0	Thr
3	Pro	31.7	26	Pro
4	Val	20.4	0	Val
5	Gly	21.5	4	Gly
6	Gly	20.1	0	Gly
7	Gly	22.4	0	Gly
8	Ile	–	0	Ile
9	Arg	23.2	79	Arg
10	–	–	493	–
11	Leu	13.1	332	Leu
12	Asn	10.3	276	Asn
13	Val	3.0	205	–
14	Ala	0.8	25	–

Sequencing of the peptide (50 nmol, 450000 cpm) and analysis and quantitation of the Pth-derivatives were as in [9]. The total ^{32}P released at each turn was calculated by liquid scintillation counting. The remainder of each Pth-derivative was subjected to back-hydrolysis and amino acid analysis: threonine was detected as α-aminobutyric acid

Table 2

Amino acid compositions of peptides ChT I and ChT II

Amino acid	Peptide ChT I	Peptide ChT II
Asp	0.3 (0)	1.0 (1)
Thr	1.9 (2)	0.25 (0)
Ser	–	1.02 (1)
Pro	1.0 (1)	
Gly	3.2 (3)	0.30 (0)
Val	0.7 (1)	–
Ile	–	0.85 (1)
Leu	0.3 (0)	0.97 (1)
Arg	–	0.99 (1)

The peptides were hydrolysed with 6 N HCl for 24 h at 110°C. Values for serine and threonine have been corrected for destruction by 10 and 5%, respectively. Values are expressed as molar ratios relative to Pro (for ChT I) and Asp (for ChT II). Impurities below 0.2 mol have been omitted

Gly–Ile and Asn–Val bonds are in accordance with the known specificity of thermolysin. It is not clear why we did not detect another peptide (or peptides) corresponding to the remainder of the chymotryptic peptide; fluctuations in the baseline absorbance during the HPLC run may have been responsible.

Another isolation of peptide ChT II was made and the peptide (10 nmol) was subjected to automated sequence analysis as for the second experiment described above. The first 4 amino acids were identified by HPLC of the Pth-derivatives as isoleucine, arginine, serine and leucine, respectively; the serine was detected as Pth-dehydroserine by absorbance measurements at 313 nm. The assignment of serine was confirmed by back hydrolysis and amino acid analysis, which gave alanine.

The evidence presented here, together with the amino acid composition given in [7], shows that the sequence of the chymotryptic phosphopeptide derived from phosphorylated isocitrate dehydrogenase is Thr-Thr-Pro-Val-Gly-Gly-Gly-Ile-Arg-Ser(P)-Leu-Asn-Val-Ala(Asx, Glx, Glx, Pro, Leu, His, Lys, Tyr).

We have recently learned that authors in [13] have sequenced a 7-residue tryptic phosphopeptide derived from the isocitrate dehydrogenase of a K12 strain of *E. coli* that had been phosphorylated in vivo. The first 5 residues of this peptide (Ser(P)-Leu-Asn-Val-Ala) are identical to residues 10–14 of the chymotryptic peptide that we have sequenced.

This is the first reported sequence round a phosphorylation site for a bacterial protein kinase. The sequence is not similar to those at sites phosphorylated by cyclic AMP-dependent protein kinase, which usually contain two basic residues on the amino-terminal side of the phosphorylated residue and separated from it by one or two intervening residues (e.g. [14]). The finding that isocitrate dehydrogenase cannot be phosphorylated by cyclic AMP-dependent protein kinase (unpublished) is consistent with this. One unusual feature of the sequence of the phosphopeptide from isocitrate dehydrogenase is the presence of one proline residue and a run of 3 glycine residues in a 5-residue stretch on the amino-terminal side of the phosphorylation site. This region of the polypeptide is likely to form a flexible random coil and it may play a role in the enzyme/substrate recognition process.

We have suggested that isocitrate dehydrogenase may be phosphorylated close to or at its NADP-binding site [15]. In this context it is interesting to note that the sequence Arg-Ser-Leu-Asn is also found in another NADP-binding enzyme, dihydrofolate reductase from chicken liver [16]. This sequence, which is in the highly conserved amino-terminal portion of the molecule, is very close to the NADP-binding site [16]. We are currently trying to identify residues at the NADP-binding site of isocitrate dehydrogenase so that we can test directly the suggestion above.

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