# AMINO ACID SEQUENCE OF A REGION IN RABBIT SKELETAL MUSCLE GLYCOGEN SYNTHASE PHOSPHORYLATED BY CYCLIC AMP-DEPENDENT PROTEIN KINASE

Peter J. PARKER, Alastair AITKEN, Terence BILHAM, Noor EMBI and Philip COHEN
*Department of Biochemistry, Medical Sciences Institute, University of Dundee, Dundee, DD1 4HN, Scotland*

Received 15 December 1980

## 1. Introduction

Glycogen synthase can be phosphorylated in vitro by several protein kinases, producing forms of the enzyme that are more dependent on the allosteric activator glucose 6-phosphate (reviewed in [1,2]). One of these glycogen synthase kinases is the enzyme cyclic AMP-dependent protein kinase [3,4] which may underlie the inhibition of glycogen synthase that occurs in vivo in response to adrenaline [5,6].

Cyclic AMP-dependent protein kinase has been shown to phosphorylate glycogen synthase on three serine residues in vitro termed site-1a, site-1b and site-2 [7,8]. The initial rate of phosphorylation of site-1a is 7–10-fold faster than site-2 and 15–20-fold faster than site-1b; the activity of glycogen synthase is determined by the state of phosphorylation of site-2 as well as site-1a [8]. The phosphorylation of site-1b does not appear to have a direct effect on the activity [7,8]. Site-2, which is located seven residues from the N-terminus of glycogen synthase, is the major site phosphorylated by phosphorylase kinase [9,10].

Here, the amino acid sequence surrounding site-1a has been determined. This analysis has surprisingly demonstrated that sites-1a and 1b are separated by only 13 amino acids in the primary structure of glycogen synthase, which comprises ~770 residues [11].

## 2. Materials and methods

Glycogen synthase [11,12], the catalytic subunit of cyclic AMP-dependent protein kinase [13] and phosphorylase kinase [14] were purified from rabbit skeletal muscle by standard procedures. Trypsin (treated with tosylphenylchloromethylketone) was from Worthington, thermolysin from Boehringer, [$\gamma$-$^{32}$P]ATP, tritiated iodoacetic acid from the Radiochemical Centre and Sephadex G-50 (Superfine grade) from Pharmacia.

Glycogen synthase was phosphorylated at 1.0 mg/ml by cyclic AMP-dependent protein kinase or phosphorylase kinase as in [1]. Automated sequence analysis was carried out using a Beckman 890C sequencer as in [2] and amino acid analyses were performed on an LKB Biochrom 4400 amino acid analyser. Manual sequence analysis was carried out by the microdansyl Edman procedure in [15].

## 3. Results

### 3.1. *Isolation and structure of the tryptic phosphopeptide containing site-1a*

Glycogen synthase (60 mg) was phosphorylated to 2.4 mol/subunit with cyclic AMP-dependent protein kinase (10 U/ml) and contained approximately equal amounts of phosphate in sites-1a, 1b and 2 [8]. The reaction was stopped by the addition of 0.1 vol. 100 mM EDTA (pH 7.0) and 0.1 vol. 500 mM NaF. The native enzyme (0.8 mg/ml) was then incubated for 5 min with trypsin (0.02 mg/ml) at 30°C. This treatment releases sites-1a and 1b quantitatively, but does not release detectable amounts of site-2 [8]. The reaction was terminated by the addition of 0.05 vol. 100% (w/v) trichloroacetic acid, and after standing in ice for 10 min, the suspension was centrifuged at 15 000 × *g* for 5 min. The supernatant was extracted 5 times with ether to remove trichloroacetic acid and lyophilised. The material was then subjected to gel filtration on Sephadex G-50 (Superfine) equilibrated

 *Elsevier/North-Holland Biomedical Press*

in 1.0 M acetic acid. Site-1a was eluted at a $V_e/V_0$ of 1.9–2.0 and site-1b at $V_e/V_0$ of 1.4–1.5. Site-1a was pooled, lyophilised, performic acid oxidised as in [8] and further purified by peptide mapping on thin-layer cellulose [16] using electrophoresis at pH 3.5 in the first dimension and ascending chromatography (butan-1-ol/pyridine/acetic acid/water, 15:10:3:12) in the second dimension. Site-1a migrated extremely slowly during ascending chromatography and this step was repeated twice. The chromatography was repeated 4 times in butan-1-ol/pyridine/acetic acid/water, 10:15:3:12.

Autoradiography of the peptide map showed a major (T-1) and a minor (T-2) radioactive species, which were eluted with 1.0 M acetic acid. Peptide T-2 migrated slightly slower than T-1 during electrophoresis and slightly faster during chromatography. The amino acid compositions of these peptides were identical, except that T-1 contained two arginine residues whereas T-2 contained one (table 1).

Peptide T-1 was subjected to automated sequencer analysis and the results are summarised in table 2. The sequence was found to be:

Arg–Ala–Ser–(Cys)–Thr–Ser–Ser–Ser–Gly–

Gly–Ser–Lys–Arg

After the fourth cycle only traces of PTH cysteic acid were detected by HPLC or by amino acid analysis after back hydrolysis of the PTH-derivative. Dansyl cysteic acid was also detected after 3 cycles of manual Edman degradation. The evidence presented in section 3.2 confirmed that this residue was cysteine. All the $^{32}$P-radioactivity associated with peptide T-1 was lost after the third cycle of manual Edman degradation confirming this serine residue as the only site of phosphorylation [7].

Peptide T-2 was also subjected to automated sequencer analysis. The first 12 residues were identical to T-1. This indicated that T-2 lacks the C-terminal arginine present in T-1 (not shown).

It was then noticed that the last 7 residues of this peptide were identical to the first 7 residues of a phosphopeptide containing site-1b obtained with digestion by subtilisin [17]. The sequence of this peptide was reported to be:

Ser–Ser–Gly–Gly–Ser–Lys–Arg–Ser–Asn–

Ser(P)–Val–Asp–Thr–Ser

Table 1
Amino acid analyses of phosphopeptides derived from site-1a and site-1b of glycogen synthase

| Amino acid | T1 | T2 | Th-1 |
|---|---|---|---|
| Aspartate/asparagine | | | 2.1 (2) |
| Threonine | 0.95 (1) | 0.7 (1) | 1.7 (2) |
| Serine | 5.1 (5) | 4.2 (5) | 8.8 (9) |
| Glutamate/glutamine | | | 1.5 (1) |
| Proline | | | 1.9 (2) |
| Glycine | 2.2 (2) | 2.3 (2) | 1.95 (2) |
| Alanine | 1.0 (1) | 1.0 (1) | 1.9 (2) |
| Valine | | | 0.9 (1) |
| Isoleucine | | | 0.7 (1) |
| Lysine | 1.2 (1) | 1.2 (1) | 0.95 (1) |
| Arginine | 2.2 (2) | 1.1 (1) | 3.8 (4) |
| Cysteine | + (1) | + (1) | 0.95 (1) |
| Tryptophan | | | 0.7 (1) |
| Total | 13 | 12 | 29 |

Serine and threonine were corrected for 10% and 5% destruction, respectively, and cysteine was identified as cysteic acid after oxidation with performic acid. Hydrolyses were for 24 h or 72 h in 6 N HCl at 110°C, or for 72 h with 3 N mercaptoethane sulphonic acid at 110°C for the determination of tryptophan. The figures in parentheses represent residues detected by sequence analysis. Impurities below 0.1 residues are omitted

Table 2
Results of automated sequencer analysis on peptide T-1 (20 nmol)

| Step | HPLC | Amino acid analysis (nmol)[a] | Sequence residue |
|---|---|---|---|
| 1 | Arg | Arg (13.7) | Arg |
| 2 | Ala | Ala (8.7) | Ala |
| 3 | – | Ala[b] (1.8) | Ser |
| 4 | – | – | – |
| 5 | Thr | Thr[c] (3.8) | Thr |
| 6 | – | Ala[b] (1.6) | Ser |
| 7 | – | Ala[b] (1.1) | Ser |
| 8 | – | Ala[b] (1.2) | Ser |
| 9 | Gly | Gly (4.3) | Gly |
| 10 | Gly | Gly (6.2) | Gly |
| 11 | – | Ala[b] (0.8) | Ser |
| 12 | Lys | Lys (0.7) | Lys |
| 13 | Arg | Arg (0.7) | Arg |

[a] After back hydrolysis of the PTH-amino acids with HI, normalised to the recovery of PTH-norleucine internal standard
[b] PTH-serine is converted to alanine during back hydrolysis
[c] Identified as α-amino butyric acid

A hyphen denotes that no positive assignment could be made

Since the probability of obtaining 2 identical heptapeptides in a polypeptide chain of 770 amino acids is <1 in $10^6$, this suggested that site-1a and site-1b were only 13 residues apart in the primary structure.

### 3.2. *Isolation and structure of a thermolytic phosphopeptide containing site-1a and site-1b*

Glycogen synthase (50 mg) was phosphorylated to 2.0 mol/subunit with cyclic AMP-dependent protein kinase, precipitated with 45% ammonium sulphate, redissolved in 50 mM Tris–HCl (pH 7.0) containing 50 mM NaF and 0.1% (v/v) 2-mercaptoethanol, and dialysed against this buffer. The native enzyme (10 mg/ml) was then incubated with thermolysin (1.0 mg/ml) for 30 min at 30°C. Of the $^{32}$P-radioactivity 60–65% was released as peptides soluble in trichloroacetic acid. Since a control incubation using glycogen synthase phosphorylated by phosphorylase kinase showed that there was no release of site-2 under these conditions, this indicated that sites-1a and 1b had been released quantitatively.

The trichloroacetic acid supernatant was extracted 5 times with ether, lyophilised, redissolved in 5% formic acid and chromatographed on Sephadex G-50 (Superfine) equilibrated in the same solvent. The elution profile is shown in fig.1. A single major peak of $^{32}$P-radioactivity was eluted ($V_e/V_o$ = 1.9) separated from virtually all of the 280 nm absorbance. This peak (Th-1) was pooled and further purified by peptide mapping as in section 3.1. The amino acid composition of Th-1 is shown in table 1. The peptide was carboxymethylated by incubation with iodo[$^3$H]-acetate at pH 7.0 for 5 h at 35°C and subjected to automated sequencer analysis. The results are summarised in table 3. This extended the sequence of peptide T-1 by 8 amino acids in the N-terminal direction. A large burst of radioactivity after the 12th cycle and the identification of PTH-carboxymethylcysteine by HPLC confirmed that this residue was cysteine in the native glycogen synthase. Since the structure of peptide T-1 was established in section 3.1, the sequence of Th-1 was:

Ile–Arg–Ala–Pro–Gln–Trp–Pro–Arg–Arg–
Ala–Ser(P)–Cys–The–Ser–Ser–Ser–Gly–Gly–
Ser–Lys–Arg–($Asx_2$,$Thr$,$Ser_4$,Val)

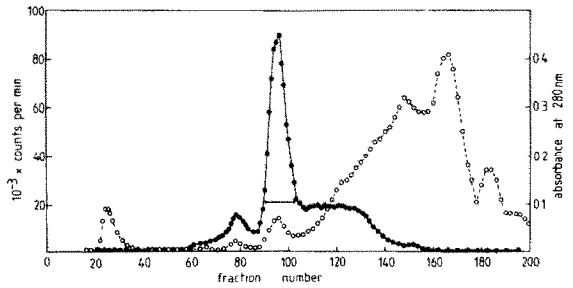


Fig.1. Gel filtration of a thermolytic digest of glycogen synthase on Sephadex G-50 (Superfine). The column (150 × 1.5 cm) was equilibrated in 5% (v/v) formic acid. The sample volume was 2.0 ml and the flow rate 15 ml/h. Fractions of 1.25 ml were collected, after 70 ml had passed through the column. The closed circles and full line show $^{32}$P-radioactivity, the open circles and broken line absorbance at 280 nm, and the horizontal bar the fractions pooled after elution.

Table 3
Results of automated sequencer analysis on *S*-carboxymethylated peptide Th-1 (20 nmol)

| Step | HPLC | Amino acid analysis (nmol)[a] | | Sequence residue |
|---|---|---|---|---|
| 1 | Ile | Ile | (15.6) | Ile |
| 2 | Arg | Arg | (6.6) | Arg |
| 3 | Ala | Ala | (12.2) | Ala |
| 4 | Pro | Pro | (13.0) | Pro |
| 5 | Gln | Glx | (12.6) | Gln |
| 6 | Trp | Gly + Ala[b] | (5.9) | Trp |
| 7 | Pro | Pro | (10.0) | Pro |
| 8 | Arg | Arg | (3.2) | Arg |
| 9 | Arg | Arg | (5.6) | Arg |
| 10 | Ala | Ala | (6.1) | Ala |
| 11 | – | Ala[c] | (4.1) | Ser |
| 12 | CM-Cys | – | | Cys |
| 13 | Thr (trace) | Thr[d] | (3.5) | Thr |
| 14 | – | – | | – |
| 15 | – | – | | – |
| 16 | – | – | | – |
| 17 | Gly | Gly | (2.6) | Gly |
| 18 | Gly | Gly | (6.5) | Gly |
| 19 | – | – | | – |
| 20 | Lys | – | | Lys |
| 21 | – | | | – |
| 22 | – | | | – |
| 23 | Asn (trace) | | | Asn |
| 24 | – | | | – |
| 25 | Val | | | Val |

[a] After back hydrolysis of the PTH amino acids with HI, normalised to the recovery of PTH-norleucine internal standard
[b] PTH-tryptophan is converted to both glycine and alanine during back hydrolysis
[c] PTH-serine is converted to alanine during back hydrolysis
[d] Identified as α-amino butyric acid

A hyphen denotes that no positive assignment could be made. Only cycles 1–20 were analysed after back hydrolysis

The composition of the final 8 residues, obtained by subtracting this sequence from the composition of Th-1, are identical to the first 8 residues of the tryptic phosphopeptide containing site-1b [7,18]. The identification of asparagine at position 23 and valine at position 25 of Th-1 (table 3) was also consistent with this conclusion.

In order to confirm that the final 8 residues represented site-1b, peptide Th-1 was subdigested with trypsin and subjected to peptide mapping on thin-layer cellulose using electrophoresis at pH 6.5 in the first dimension and ascending chromatography (butan-1-ol/pyridine/acetic acid/water, 15:10:3:12) in the second dimension. An acidic $^{32}$P-labelled peptide was eluted and its amino acid composition ($Asx_2$,$Thr_1$,$Ser_4$,Val) corresponded to the 8 C-terminal residues of Th-1. This proves that Th-1 contains site-1b as well as site-1a. The complete amino acid sequence in this region is shown in fig.2. Of the 46 residues in this sequence 16 are serine and threonine. Only the 2 serines C-terminal to each pair of adjacent basic amino acids are phosphorylated, in keeping with the known specificity requirements of cyclic AMP-dependent protein kinase [19].

Ile–Arg–Ala–Pro–Gln–Trp–Pro–Arg–Arg–Ala–Ser(P)–Cys–Thr–Ser–Ser–Ser–Gly–Gly–Ser–Lys–Arg–Ser–Asn–Ser(P)–Val–Asp–Thr–Ser–Ser–Leu–Ser–Pro–Pro–Thr–Glu–Ser–Leu–Ser–Ser–Ala–Pro–Leu–Gly–Glu–Gln–Asp–Arg

Fig.2. Amino acid sequence of the region containing site-1a and site-1b ([17,18], this report).

## 4. Discussion

The phosphorylation sites of physiological substrates for cyclic AMP-dependent protein kinase have mostly been found to fall into 1 of 2 classes. One class has the structure Arg–Arg–*x*–Ser, while the other has the structure Lys–Arg–*x*–*y*–Ser [17]. Examples of the first type include pyruvate kinase, the α-subunit of phosphorylase kinase and the regulatory subunit of type-2 cyclic AMP-dependent protein kinase. Examples of the second type include the β-subunit of phosphorylase kinase and histone H1 [17]. The present work has demonstrated that glycogen synthase contains both types of phosphorylation site (site-1a and site-1b). Of particular interest is the finding that site-1a and site-1b lie very close to one another in the primary structure and recent evidence suggests that the clustering of phosphorylation sites may be a common phenomenon. The 3 serine residues in glycogen synthase that are phosphorylated by glycogen synthase kinase-3 (sites-3a, 3b and 3c) are located within 9 residues of the primary structure [2], while 2 of the 3 serine residues on pyruvate dehydrogenase that are phosphorylated by pyruvate dehydrogenase kinase are separated by only 8 amino acid residues [20]. The presence of 2 phosphorylation sites in close proximity may help to amplify the effects of phosphorylation, or allow phosphorylation at one site to regulate the rate at which the other is phosphorylated or dephosphorylated. The finding that sites-1a and 1b are so close to one another in the primary structure will also facilitate studies of the phosphorylation of glycogen synthase in vivo which are in progress in this laboratory.

## Acknowledgements

This work was supported by grants from the Medical Research Council, London, the British Diabetic Association and the Cancer Research Campaign, London. P. P. is the recipient of a Postdoctoral Training Fellowship from the Medical Research Council, and Noor Embi acknowledges a postgraduate scholarship from the National University of Malaysia.

## References

[1] Embi, N., Rylatt, D. B. and Cohen, P. (1980) Eur. J. Biochem. 107, 519–527.
[2] Rylatt, D. B., Aitken, A., Bilham, T., Condon, G. D., Embi, N. and Cohen, P. (1980) Eur. J. Biochem. 107, 529–537.
[3] Schlender, K. K., Wei, S. H. and Villar-Palasi, C. (1969) Biochim. Biophys. Acta 191, 272–278.
[4] Soderling, T. R., Hickinbottom, J. P., Reimann, E. M., Hunkeler, F. L., Walsh, D. A. and Krebs, E. G. (1970) J. Biol. Chem. 245, 6617–6628.
[5] Craig, J. W. and Larner J. (1964) Nature 202, 971–973.
[6] Danforth, W. H. (1965) J. Biol. Chem. 240, 588–593.
[7] Proud, C. G., Nimmo, H. G., Yeaman, S. J. and Cohen, P. (1977) FEBS Lett. 80, 435–442.
[8] Embi, N., Parker, P. J. and Cohen, P. (1981) Eur. J. Biochem. in press.
[9] Rylatt, D. B. and Cohen, P. (1979) FEBS Lett. 98, 71–75.

[10] Embi, N., Rylatt, D. B. and Cohen, P. (1979) Eur. J. Biochem. 100, 339–347.
[11] Nimmo, H. G., Proud, C. G. and Cohen, P. (1976) Eur. J. Biochem. 68, 21–30.
[12] Caudwell, F. B., Antoniw, J. F. and Cohen, P. (1978) Eur. J. Biochem. 86, 511–518.
[13] Beavo, J. A., Bechtel, P. J. and Krebs, E. G. (1974) Methods Enzymol. 38, 299–308.
[14] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[15] Bruton, C. J. and Hartley, B. S. (1970) J. Mol. Biol. 52, 162–173.
[16] Aitken, A., Bilham, T., Cohen, P., Aswad, D. and Greengard, P. (1981) J. Biol. Chem. in press.
[17] Huang, T. S., Feramisco, J. R., Glass, D. B. and Krebs, E. G. (1979) Miami Winter Symp. 16, 449–461.
[18] Huang, T. S. and Krebs, E. G. (1979) Biochim. Biophys. Res. Commun. 75, 643–650.
[19] Cohen, P. (1978) Curr. Top. Cell. Reg. 14, 117–196.
[20] Yeaman, S. J., Hutcheson, E. T., Roche, T. E., Pettit, F. H., Brown, J. R., Reed, L. J., Watson, D. C. and Dixon, G. H. (1978) Biochemistry 17, 2364–2371.