AMINO ACID SEQUENCE OF A PHOSPHORYLATION SITE IN SKELETAL MUSCLE GLYCOGEN SYNTHETASE

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SUMMARY

Rabbit skeletal muscle glycogen synthetase was phosphorylated by incubation with [γ-^{32}P]ATP, Mg^{++} and cyclic AMP-dependent protein kinase catalytic subunit from the same source. One of the major phosphorylation site peptides was isolated following brief tryptic-hydrolysis, and shown to have the sequence Ser-Asx-Ser(p)-Val-Asp-Thr-Ser-Ser-Leu-$\overset{10}{\text{Ser}}$-Pro-Pro-Thr-Glu-Ser-Leu-Ser-Ser-Ala-$\overset{20}{\text{Pro}}$-Leu-Gly-Glu-Gln-Asp-Arg.

INTRODUCTION

Glycogen synthetase is capable of existing in two forms: 1) the non-phosphorylated or physiologically active I form and 2) the phosphorylated, or less active D form, which requires the presence of glucose-6-P as a cofactor for full activity. For the muscle enzyme it has been reported that somewhere between two and six mol of phosphate are incorporated per mol of synthetase subunit in the conversion of the I-form to the D-form (1-3). A phosphorylated hexapeptide with the sequence, $\overset{\text{(Arg)}}{\text{Lys}}$-Glu-Ile-Ser(P)-Val-Arg, was isolated from the latter form of the synthetase (4).

In the present communication it is shown that in a reaction catalyzed by homogeneous cyclic AMP-dependent protein kinase up to two mol of phosphate are introduced per mol of glycogen synthetase subunit. One of two major ^{32}P-containing tryptic peptides has been isolated and its amino acid sequence determined. This phosphorylated site is different from that reported by Larner and Sanger (4).

MATERIALS AND METHODS

Glycogen synthetase I was isolated from rabbit skeletal muscle according to a previously described method (5). After the Sepharose-4B step the enzyme

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was further purified by absorbing it on DEAE-Sephadex-A-50 previously equilibrated with 150 mM Tris-HCl, 1 mM EDTA, 45 mM β-mercaptoethanol and 5% sucrose at pH 7.5. The absorbed enzyme was then eluted with a gradient established from 0 to 0.8 M KCl. Catalytic subunit of the cyclic AMP-dependent protein kinase purified from rabbit skeletal muscle was a gift from Drs. P. J. Bechtel and J. A. Beavo of this laboratory. [γ-^{32}P]ATP was prepared according to Glynn and Chappel (6) as described previously (7). For the phosphorylation of glycogen synthetase I, the enzyme at a concentration of 1-2 mg/ml was incubated with the catalytic subunit of cyclic AMP-dependent protein kinase at concentrations ranging from 3-50 μg/ml at 30°C in the presence of 50 mM Tris-HCl (pH 7.5), 10 mM $Mg(Ac)_2$, 45 mM β-mercaptoethanol, 5% sucrose, 100 mM NaF and 0.3 mM [γ-^{32}P] ATP (76 cpm/pmole). Phosphorylation was also carried out in an identical fashion except in the absence of catalytic subunit of cyclic AMP-dependent protein kinase. The reaction was stopped by adding an excess of EDTA to the reaction mixture. ^{32}P-labelled glycogen synthetase was precipitated by adding solid $(NH_4)_2SO_4$ (0.25 g/ml) to the solution. The pellet was collected by centrifugation, dissolved in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 45 mM β-mercaptoethanol and 5% sucrose, (Buffer A) and then dialyzed against 2-3 changes of the same buffer overnight to remove excess [γ-^{32}P]ATP. For measurement of the conversion of synthetase I to Synthetase D, conditions identical to those used for the enzymatic phosphorylation were employed, except that nonlabeled ATP was used instead of [γ-^{32}P]ATP. The reaction was stopped by diluting an aliquot of enzyme 50 to 100 fold in cold buffer containing 50 mM Tris-HCl, 1 mM EDTA, 0.25 mg/ml bovine serum albumin, 45 mM β-mercaptoethanol, pH 7.5. Synthetase activity was assayed by the method of Thomas et al. (8), in the presence and absence of glucose-6-P. Specific activity is defined as the incorporation of 1 μmol of glucose from UDP-glucose into glycogen per min per mg of protein under the assay condition at 30°. Partial tryptic hydrolysis of ^{32}P-labelled synthetase was carried out at 30° in solutions of the latter protein at a concentration of 10 mg/ml in Buffer A at a substrate to trypsin ratio of 25 to 1. After 5 min the reaction was terminated by adding concentrated trichloroacetic acid to a final concentration of 10%. The mixture was cooled on ice for 30 min and then centrifuged to separate the soluble ^{32}P-peptide from the core enzyme precipitate. The pellet was washed once with cold 5% trichloroacetic acid and the wash was combined with the TCA supernatant. This solution was extracted several times with ether to remove trichloracetic acid and lyophilized. Further isolation and purification of tryptic ^{32}P-peptides involved sephadex gel filtration, high voltage paper electrophoresis. Further proteolytic digestion using thermolysin and chymotrypsin were as described previously (9). Peptides eluted from Sephadex columns were detected using fluorescamine (Fluram, Roche Diagnostics Corp.) (10). Amino acid sequence studies were performed by the manual Edman method (11) and digestion with Carboxypeptidase A.

RESULTS

Phosphorylation and conversion - Phosphorylation of glycogen synthetase by cyclic AMP-dependent protein kinase catalytic subunit resulted in the incorporation of up to 2 mol of phosphate per mol of synthetase subunit (MW 90,000)(Fig. 1). In a typical experiment, the synthetase (1.4 mg/ml) was incubated at pH 7.0 with 5 μg/ml protein kinase catalytic unit, 0.3 mM [γ-^{32}P]ATP and 10 mM Mg^{++} at 30°. Within less than 2 min one mol of ^{32}P was incorporated and the enzyme, which originally had approximately 70% "I activity" was partially converted to the D-

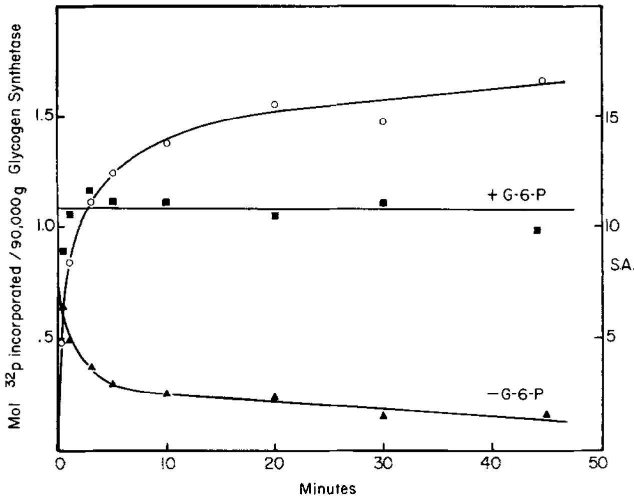

Figure 1. Phosphorylation and glycogen synthetase I to D conversion by cyclic AMP-dependent protein kinase catalytic subunit in the presence of ATP and Mg^{++}. Reaction conditions were as described under "Materials and Methods" with 1.4 mg/ml of synthetase I, containing 5 μg/ml protein kinase catalytic subunit and 0.3 mM ATP (■ , ▲), or 0.3 mM [γ-^{32}P]ATP (76 cpm/pmol) (O). Synthetase activity in the presence of glucose-6-P (■) and absence of glucose-6-P (▲) is expressed as specific activity (S.A.).

form with a remainder of 15% I-form after 45 minutes. By 45 min 1.6 mol of ^{32}P had been introduced and the ratio of activity (-glucose-6-P) to activity (+glucose-6-P) had decreased to 0.15. It is noteworthy that the rate of ^{32}P incorporation and the change in enzyme activity slowed appreciably after the first few minutes. In a control reaction carried out in the absence of the protein kinase catalytic subunit there was no significant phosphorylation of the synthetase, i.e., only 0.12 mol of phosphate per synthetase subunit in a 60 min. incubation.

Tryptic hydrolysis of ^{32}P-labelled synthetase - For this study ^{32}P-labelled glycogen synthetase containing variable amounts of ^{32}P was utilized, but essentially identical peptide patterns were obtained whether the enzyme contained only 1 mol of ^{32}P/mol of synthetase or whether the amount of ^{32}P approached 2 mol/mol of subunit. In one particular preparation, 120 mg of ^{32}P-labelled synthetase containing one mol of ^{32}P per mol of enzyme subunit was digested with 4.8 mg of

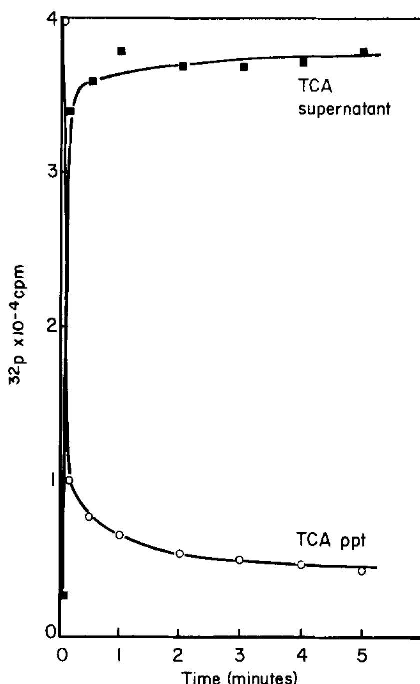

Figure 2. Tryptic hydrolysis of ^{32}P-labelled glycogen synthetase. Conditions were described in "Materials and Methods." Synthetase (10 mg/ml) was hydrolyzed with TPCK-treated trypsin (0.4 mg/ml) at 30°C for 5 min. Radioactivity released into the trichloracetic acid supernatant (■) and radioactivity remaining in the pellet (O).

TPCK-treated trypsin for 5 min. Approximately 90% of the radioactivity was found in the trichloroacetic acid supernatant (Fig. 2). The soluble ^{32}P-peptides were then fractionated on a Sephadex G-50 column (1.9 x 150 cm) as described in Methods. One of two major ^{32}P-peptides, Peak I from the Sephadex G-50 column (Fig. 3) was isolated and further purified by high voltage electrophoresis as previously described (9). Purification was greatly facilitated by using high concentrations of trypsin and short digestion times. This permitted the solubilization of 90% of the radioactivity while leaving most of the protein mass in the pellet (Fig. 2).

Sequence determination of the tryptic ^{32}P-peptide (Peak I) - To determine which amino acid residues were phosphorylated, the ^{32}P-labelled tryptic-peptide was further digested with thermolysin and chymotrypsin. By combination of

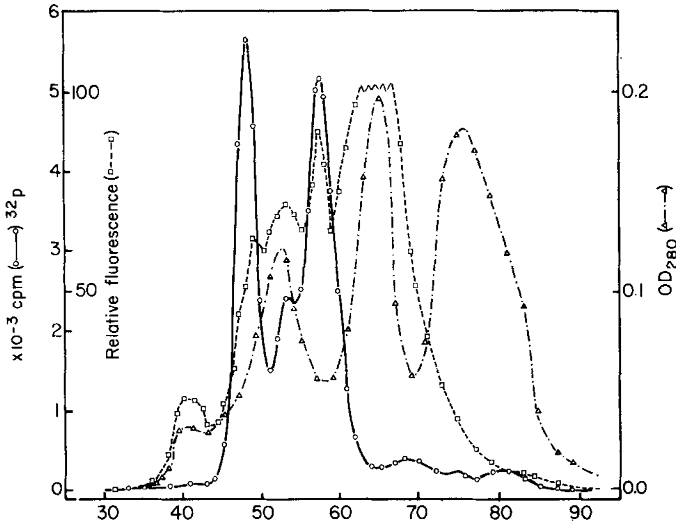

Figure 3. Fractionation of tryptic peptides by gel filtration. A tryptic hydrolysate of ^{32}P-labelled glycogen synthetase was applied to a Sephadex-G-50 column (1.9 x 150 cm), previously equilibrated with 30% acetic acid and eluted with the same buffer. 5.6 ml/fractions were collected, and 5 μl from each fraction was used to measure radioactivity (0) and relative fluorescence (□). Absorbance was measured at 280 μm (Δ).

manual Edman degradation and carboxypeptidase A hydrolysis, the phosphorylated site sequence was determined as shown in Figure 4. To confirm that Ser(p) is the third residue from NH_2-terminal of the peptide, the ^{32}P-tryptic-peptide and ^{32}P-Th-1 peptide left after three steps of Edman degradation were examined by electrophoresis at pH 1.9. Peptides were stained with ninhydrin, and the ninhydrin positive spot was cut out and counted for ^{32}Pi. Neither fragment contained significant amounts of radioactivity.

DISCUSSION

In the present study it was shown that the catalytic subunit of the cyclic AMP-dependent protein kinase catalyzes a reaction in which up to 2 mol of ^{32}P can be transferred from [γ-^{32}P]ATP to the glycogen synthetase subunit. This reaction occurs at catalytic subunit concentration in the range of 3-50 μg/ml of reaction mixture and is accompanied by a change in the -glucose-6-P to

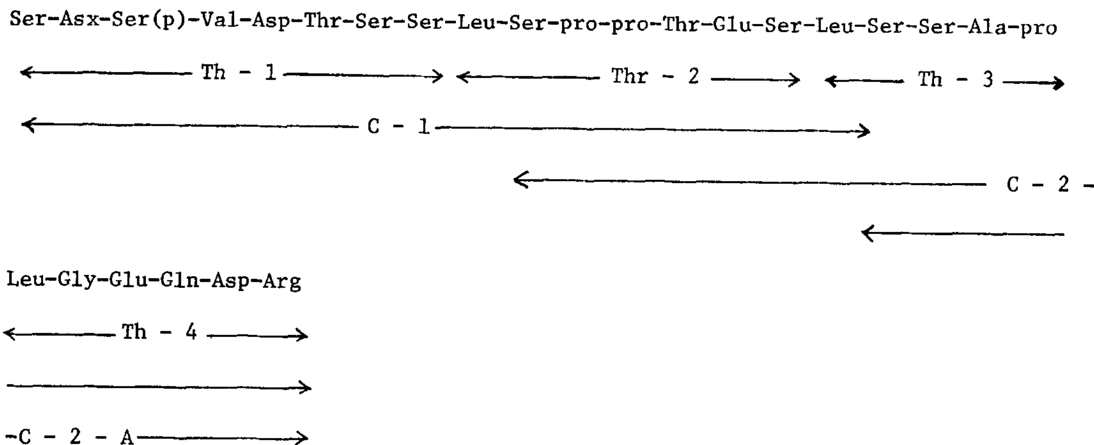

Figure 4. Structure of one of the major phosphorylation sites in glycogen synthetase phosphorylated by the cyclic AMP-dependent rpotein kinase catalytic subunit. Th = peptides derived from thermolysis hydrolysis; c = peptides derived from chymotrypsin hydrolysis.

+glucose-6-P activity ratio from 0.70 to 0.15. The conditions for the reaction and the results obtained are essentially in agreement with those reported by Soderling (2) who found that 2 mol of phosphate were incorporated per mol of synthetase subunit when pure protein kinase catalytic subunit was employed at concentrations ranging from 10-30 μg/ml of reaction mixture. The results are different from the report by Nimmo et al. (12), who using partially purified cAMP-dependent protein kinase, were able to incorporate only one mol of phosphate per mol of synthetase subunit. In Soderling's study, however, the amount of residual synthetase I activity was only 5 per cent when 2 mol of phosphate had been incorporated.

In the glycogen synthetase phosphorylation reaction the first mol of ^{32}P is incorporated rapidly but then the reaction rate slows appreciably as though a second site was being phosphorylated at a much slower rate than the first site.

Nonetheless, when tryptic peptides are examined at a time when only 1 mol of ^{32}P has been introduced, two major peptides are present as shown in Fig. 3. The peptide pattern is essentially identical when the amount of ^{32}P introduced per mol of synthetase subunit approaches 2 (not illustrated in the paper but confirmed by a number of experiments). No explanation is available for this phenomenon but it is possible that serines at two different sites in the synthetase molecule are equally susceptible to phosphorylation, but a reaction involving one of them affects the other. Experiments to ascertain whether one or both of the phosphorylated sites are required for the conversion of synthetase I to D-form, as well as the determination of the amino acid sequence of the other ^{32}P-peptide (Peak II in Fig. 3) are currently in progress.

Inspection of the sequence of the phosphorylated peptide reported in this communication reveals an unusually high proportion of side chain hydroxyl groups. Of a total of 26 amino acid residues, there are ten with hydroxyl groups, including eight serine and two threonine residues, yet only one of the eight serine residues was phosphorylated by the cyclic AMP-dependent protein kinase catalytic subunit. In view of the important role of arginine residues in determining the substrate specificity of the cyclic AMP-dependent protein kinase, and in keeping with the fact this is a tryptic peptide, it would be anticipated that Arg-Arg, Lys-Arg, or Arg-Lys would be the next residues present on the Amino terminal end of the sequence (13, 14).

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REFERENCES

1. Smith, C. H., Brown, N. E., and Larner, J. (1971) Biochim. Biophys. Acta, 242, 81-88.
2. Soderling, T. R. (1975) J. Biol. Chem. 250, 5407-5412.
3. Roach, P. J., Takeda, Y., and Larner, J. (1976) J. Biol. Chem. 251, 1913-1919.
4. Larner, J., and Sanger, F. (1965) J. Mol. Biol. 11, 491-500.

5. Soderling, T. R., Hickenbottom, J. P., Reimann, E. M., Hunkeler, F. L., Walsh, D. A., and Krebs, E. G. (1970) J. Biol. Chem. 245, 6317-6328.
6. Glynn, J. M., and Chappel, J. B. (1964) Biochem. J. 90, 147-149.
7. Walsh, D. A., Perkins, J. P., Bronstrom, C. O., Ho, E. S., and Krebs, E. G., (1971) J. Biol. Chem. 246, 1968-1976.
8. Thomas, J. A., Schlender, K. K., and Larner, J. (1968) Anal. Biochem. 25, 486-499.
9. Huang, T-S., Bylund, D. B., Stull, J. T., and Krebs, E. G. (1974) FEBS Lett. 42, 249-252.
10. Nakai, N., Lai, C. Y., and Horecker, B. L. (1974) Anal. Biochem. 58, 563-570.
11. Edman, P. (1950) and (1953) Acta Chem. Scand. 4, 283-293; 7, 700-701.
12. Nimmo, H. G., Proud, C. G., and Cohen, P. (1976) Eur. J. Biochem. 68, 31-44.
13. Kemp, B. E., Graves, D. J., and Benjamini, E. (1976) Fed. Proc. 35, Abs. 139.
14. Zetterqvist, Ö., Ragnarsson, V., Humble, E., Berglund, L., and Engström, L. (1976) Biochem. Biophys. Res. Comm. 70, 696-703.