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MOLECULAR CHARACTERISTICS OF THE TOTALLY DEPENDENT AND INDEPENDENT FORMS OF GLYCOGEN SYNTHASE OF RABBIT SKELETAL MUSCLE

II. SOME CHEMICAL CHARACTERISTICS OF THE ENZYME PROTEIN AND OF ITS CHANGE ON INTERCONVERSION

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SUMMARY

Preparations of both D and I forms of glycogen synthase (UDPG:glycogen α-4-glucosyltransferase, EC 2.4.1.11) have been demonstrated to be virtually homogeneous by gel electrophoresis in sodium lauryl sulfate. The subunit found on electrophoresis has a molecular weight of approx. 90 000 determined by its mobility.

The D form contains 7 moles of alkali-labile phosphate per 100 000 g and the I form virtually none. Both forms contain 7 moles of sulfhydryl groups per 100 000 g and neither contains pyridoxal phosphate. The analytical data suggest the presence of a smaller subunit as a component of the one which has been detected.

INTRODUCTION

With the studies reported in the preceding paper, glycogen synthase (UDPG: glycogen α-4-glucosyltransferase, EC 2.4.1.11) can now be prepared in either the completely glucose 6-phosphate dependent (D) or independent (I) form[1-3].

Accordingly, it is now possible to investigate the relationship between the chemistry of the enzyme protein and its catalytic activity. Understanding of this relationship can be expected to clarify the mechanism of enzymatic catalysis and its regulation by effector molecules. Equally important, it will permit description in molecular terms of the action of the cyclic AMP stimulated kinase and the phosphatase which catalyze interconversion of the two protein forms.

Study of this relationship must begin with investigation of the essential aspects of the chemistry of the enzyme protein. Phosphorylation occurs at a serine hydroxyl. The sequence of a hexapeptide at this site is already known and is identical or

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essentially identical with that of phosphorylase[4]. The previous paper[3] described the preparation and determination of molecular weight of the D form of the enzyme. As the next step in this investigation, this paper describes other essential features of the chemistry of the enzyme protein: the establishment of homogeneity of both D and I preparations, the molecular weight of a major subunit, the sulfhydryl content, and the change in phosphate content on interconversion of the I and D forms.

MATERIALS AND METHODS

Amylase treatment and gel filtration

The I form was prepared as previously described[2] and the D form as described in the preceding paper[3]. After the 15% ethanol precipitations, both forms were redissolved and treated with α-amylase at room temperature as described in the preceding paper. The subsequent gel filtration procedure at room temperature, however, was modified as follows: after neutralization to pH 7.8, the mixture was applied to a Sepharose 4-B column, 75–90 cm in length. The diameter of the column (1.5–5 cm) was selected so that the volume of the gel bed was 20–25 times the volume applied, an increase of 2–3-fold over that previously employed[3]. Approx. 70 fractions were collected whose total volume was equal to that of the gel bed. The fractions were assayed for synthase activity and the principal active fractions were pooled and concentrated at 4°, using Ficoll as already described. The increased bed volume of the Sepharose gel column resulted in virtually homogeneous preparations of the D and I forms and made unnecessary the subsequent Sephadex filtration.

Electrophoresis

The general procedures of WEBER AND OSBORN[5] were employed with slight modification. Samples were incubated routinely in 10 mM phosphate (pH 7.1) containing 0.1% sodium lauryl sulfate and 50 mM mercaptoethanol. Gels were prepared with 10% acrylamide and 0.27% methylene-bis-acrylamide. After electrophoresis, proteins were fixed overnight in 20% sulfosalicylic acid and stained 2–4 h in 0.25% aqueous Coomassie blue[6]. For molecular weight determination, the crystalline protein standards employed were rabbit muscle phosphorylase A_7, aldolase[8], pyruvate kinase (Boehringer), bovine serum albumin, bovine pancreatic carboxypeptidase A (Worthington), and horse heart cytochrome *c* (Boehringer). Mobility was measured relative to cytochrome *c*.

Phosphate determinations

Synthase preparations, after amylase treatment, gel filtration and dialysis, were analyzed using the procedures of AMES[9] for total and inorganic phosphate. To increase sensitivity, volumes were reduced 5-fold maintaining concentrations as described. For determination of total protein phosphate, in most instances, protein was precipitated with trichloroacetic acid, redissolved in 0.1 M NaOH, reprecipitated and washed twice with trichloroacetic acid and finally redissolved in 0.1 M NaOH (ref. 10). In other instances, total phosphate and inorganic phosphate were determined directly on the dialyzed enzyme without precipitation and the organic phosphate calculated by subtraction of inorganic from total phosphate. Since the results, using the two procedures were similar, both values were included in Table II as total organic phos-

phate. For analysis of alkali-labile phosphate, protein was always precipitated, redissolved and washed as described. NaOH was added to a final concentration of 0.25 M and the washed redissolved precipitate incubated for 20 h at 37°. A blank containing the same concentration of alkali without enzyme was incubated simultaneously to correct for phosphate leached from the glass tube. Concentrated trichloroacetic acid was added to sample and blank tubes to a final concentration of 300 mM free acid, and the precipitated protein removed by centrifugation. Alkali-labile phosphate was determined by analyzing aliquots of each supernatant for inorganic phosphate.

Sulfhydryl determinations

Sulfhydryl content was determined with ELMAN's reagent[11]. The purified enzymes obtained after amylase treatment and gel filtration were incubated 30 min at 30° in a solution of 50 mM Tris–5 mM EDTA–50 mM mercaptoethanol (pH 7.8) to reduce any disulfide groups present. They were then dialyzed 24–48 h at 4° against the same buffer with mercaptoethanol omitted. Removal of non-protein sulfhydryl material was followed by testing the dialysate with ELMAN's reagent[11] and the dialysis was continued for at least 8 h beyond the time at which reaction was last observed. Protein sulfhydryl content was measured in a mixture containing 50 mM Tris–5 mM EDTA–0.2% sodium lauryl sulfate, in a final volume of 0.2 ml. Elman's reagent (5 μl of a 10 mM solution) was added and the total increase in absorbance at 412 nm measured. Reaction was complete within 20–30 min. The increase on addition of reagent to a corresponding solution from which synthase had been omitted was subtracted as a blank and the protein sulfhydryl content calculated from the known molar extinction coefficient of the reduced reagent[11].

Analytical procedures

Protein was determined spectrophotometrically[12]. Pyridoxal phosphate in protein was determined fluorometrically[13]. Glycogen synthase activity was assayed with [^{14}C]UDPG using the filter paper technique of THOMAS *et al.*[14], with 10 mM Na_2SO_4 added to the reaction mixture[15,16].

RESULTS

Gel electrophoresis

Past attempts to perform conventional electrophoresis of glycogen synthase preparations in this laboratory have been unsuccessful. When bound to glycogen, the enzyme would not enter the gel, and after treatment with amylase, it aggregated, causing streaking. The addition of sodium lauryl sulfate facilitated successful electrophoresis (Fig. 1). The preparation of glycogen-bound enzyme resulting from the 15% ethanol precipitation[3] was impure and consistently showed 2 heavy bands near the origin and a number of lighter ones along the length of the gel (Fig. 1a). After amylase treatment and gel filtration, one heavy band was present. When large amounts of material were applied (Figs. 1b and c) only a faint suggestion of other material was seen. The relatively heavy intensity of the principal band indicated that the preparations were virtually homogeneous. D and I preparations gave only a single band with the same mobility for both forms.

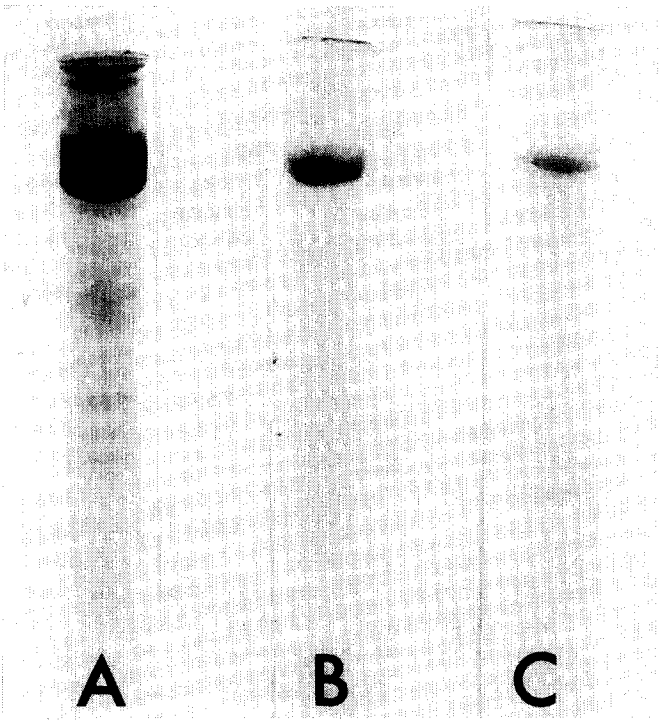

Fig. 1. Photographs of electrophoretic gels stained with Coomassie blue (10–30 μg protein). a. D preparation after second 15% ethanol precipitation. b. D preparation after amylase digestion and gel filtration. c. I preparation after amylase digestion and gel filtration.

Molecular weight

The glycogen synthase subunit had a mobility very close to that of the phosphorylase monomer. Molecular weight, determined from a logarithmic plot, was 90 000 (Fig. 2). Attempts to cause further disaggregation by incubation overnight in 8 M urea with sodium dodecyl sulfate concentrations up to 1%, and by the addition of urea to the gel, failed to change the mobility of the synthase protein band.

Pyridoxal phosphate analyses

Because of the presence of pyridoxal phosphate as a component of phosphorylase[17], synthase preparations were analyzed for pyridoxal phosphate. During purification of the enzyme the pyridoxal phosphate concentration decreased markedly after the DEAE-column step when phosphorylase is removed (Table I). Only trace amounts were found after ethanol precipitation and these varied from preparation to preparation (D 1, D 2, and I, Table I). Furthermore, one of the preparations (D 1) with high pyridoxal phosphate content was found to contain contaminating phosphorylase sufficient to account for its pyridoxal phosphate content. These analyses, therefore, demonstrated that pyridoxal phosphate is not a component of glycogen synthase.

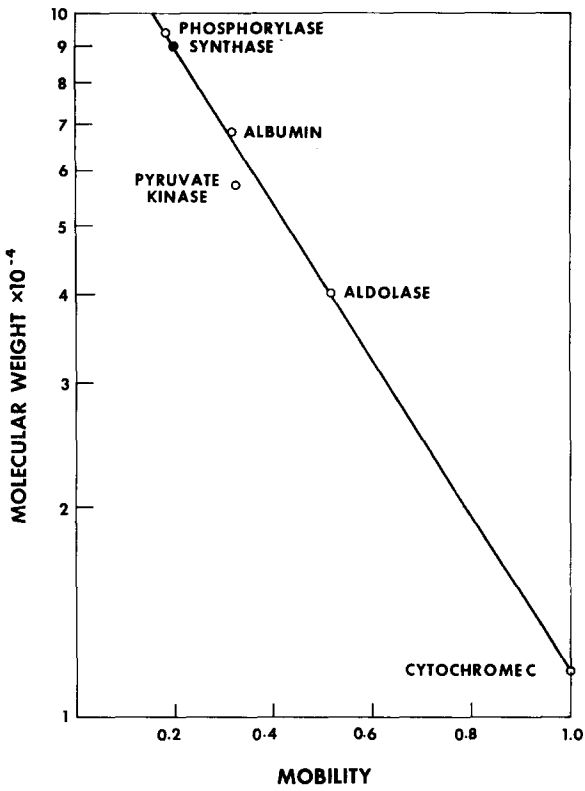

Fig. 2. Determination of molecular weight of synthase subunit by electrophoresis in sodium lauryl sulfate. Mobility was measured relative to cytochrome *c*.

TABLE I

PYRIDOXAL PHOSPHATE CONTENT

	nmoles/ unit activity	*mmoles/ 10^5 mg protein (determined)*[a]	*mmoles/ 10^5 mg protein (calculated)*[b]
Preparations			
D 1	0.21	0.21	0.43
D 2	0.047	0.086	0.095
I	0.18	0.018	0.36
Purification			
Extracted glycogen pellet	16.9	0.614	33.8
DEAE eluate	0.022	—	0.045
Final 15% ethanol precipitate	0.023	0.110	0.046

[a] Based on measured protein content.

[b] Based on enzyme activity and assumed maximum specific activity of 20 units/mg protein.

Phosphate and sulfhydryl content

The phosphate content of the virtually homogeneous D and I preparations resulting from amylase treatment and gel filtration are given in Table II. By both procedures employed, the D form consistently contained 6–8 mmoles of phosphate per 100 000 mg. The content determined by total organic phosphate analysis was similar to or perhaps slightly greater than found as alkali-labile phosphate. The phosphate content of I preparations was markedly less than that of the D preparations. When the content of alkali-labile phosphate of the I form was analyzed, it was extremely low and approached the limit of detection with the amount of protein available.

TABLE II

PHOSPHATE AND SULFHYDRYL CONTENT OF GLYCOGEN SYNTHASE

Values expressed as mmoles/100 000 mg protein

	Total organic phosphate		*Alkali-labile phosphate*		*Sulfhydryl content*	
	D	*I*	*D*	*I*	*D*	*I*
	9.8	1.3	6.7	0.9	7.3	8.0
	9.0	3.7	6.9	0.5	6.9	6.9
	10.0	3.2	9.3	0.2	6.6	
	6.3					
	8.0					
	8.7					
	7.2					
Mean ± S.E.	8.42 ± 0.51	2.73 ± 0.71	7.61 ± 0.82	0.52 ± 0.20	7.13 ± 0.24	
D − I	5.9 ± 0.91		7.09 ± 0.85			

The alkali-labile phosphate analysis measures protein serine phosphate more specifically[18] and is less susceptible to potential error by contaminating material which might still persist through trichloroacetic acid precipitation. Therefore, the difference in alkali-labile phosphate content of the D and I forms is taken as the best measure of the phosphate incorporated in the total conversion reaction. Over the entire range from total I to total D, this amount is approx. 7 mmoles/100 000 mg or 1 mole/14 000 g.

The sulfhydryl content of the D and I forms was 7.13 mmoles/100 000 mg.

DISCUSSION

The I and D forms of glycogen synthase have now been isolated as preparations whose protein components are virtually homogeneous by gel electrophoresis and ultracentrifugation. Both show a marked tendency to aggregate after removal of glycogen. This makes preparation more difficult and has until recently[19] prevented electrophoresis of the undissociated protein.

Electrophoresis in sodium lauryl sulfate yielded a single component with molecular weight 90 000; and, after this investigation was completed, SODERLING *et al.*[19] reported a similar value. For the intact active enzyme, a molecular weight of 250 000 is reported in the accompanying paper[3] and by PIRAS AND STANELONI[20], and

a value of 400 000 by SODERLING *et al.*[19]. At the present time it is impossible to be certain whether the larger molecule is a trimer or tetramer.

The large difference in alkali-labile phosphate content of D and I forms, 1 mole/14 300 molecular weight, confirms now in homogeneous preparations, what was suggested by earlier studies using less pure preparations[2,21,22]. This value is approx. 6-fold greater than the difference between the a and b forms of muscle phosphorylase. Despite the essential identity of the amino acid sequence at their immediate sites of phosphorylation[4], synthase appears to differ from phosphorylase in its greater capacity for phosphorylation and in the absence of pyridoxal phosphate present in phosphorylase[17]. The differing pyridoxal phosphate content of the two enzymes, now demonstrated analytically, is in keeping with an earlier nutritional report[23].

Phosphorylase kinase, however, rather than phosphorylase, is the enzyme that might be expected to be most analogous to synthase, since its phosphorylation is catalyzed by the same cyclic AMP dependent kinase as synthase[2,19] and its dephosphorylation by a phosphatase which, like synthase phosphatase is glycogen-inhibited[24,25]. Interestingly, the maximum phosphate content of phosphorylase kinase, as so far prepared, does approach that of synthase[10].

Since the D and I synthase preparations in the current study were subjected to prolonged action of either phosphatase or kinase, the difference measured is probably the limit of phosphorylation of the enzyme. Conversion of glucose 6-phosphate dependence did (when followed) occur throughout most or all of the period of phosphatase or kinase action. In earlier studies with less purified enzyme, glucose 6-phosphate dependence varied linearly with phosphorylation over a wide range[22].

SODERLING *et al.*[19] have described conversion of the enzyme accompanied by a lesser degree of phosphorylation than that reported here. In their study[19] phosphorylation was measured by radioactive incorporation rather than chemical analysis. Conversion did not begin with 100% I form, and with the techniques used, it did not proceed beyond 75% glucose 6-phosphate dependency. In contrast, in the present study the phosphate content of 100% I form was compared with that of 100% D form. These were prepared by slow kinase or phosphatase action carried out in the cold (3–5°) and in the presence of glycogen to stabilize the enzyme under carefully controlled conditions[2,3]. The range of conversion is therefore clearly greater in the present study, although an exact comparison is impossible because in the published study activity was measured without anion activation of the I form[15,16]. More complete conversion would be expected to increase the reported value of 1.1 mmole/100 000 mg protein and cast some doubt on the concept of 1 mole/90 000 g subunit. Whether additional factors may underlie the apparent discrepancy between the two reports remains to be established by further experimentation.

The analytical data reported here would require that each subunit of 90 000 molecular weight contain 6 sulfhydryl groups and 6 moles of phosphate when fully phosphorylated. The most straight forward interpretation is that these phosphate groups occupy identical sites and that there is a small subunit of molecular weight approx. 15 000 which is a component of the larger 90 000 subunit.

Attempts to detect such a small subunit are in progress. Other possible interpretations that may be considered are (1) the existence of different phosphorylatable alkali-labile (serine) sites within the molecule, or (2) the presence of similar sites

joined in one peptide chain rather than the existence of separate subunit components. However, only one peptide sequence was found in earlier studies of synthase. With the possible exception of phosphorylase kinase[10,25] we know of no enzyme which conforms to either of these models*. The identity of sites in synthase is now being reinvestigated with preparations over a wide range of phosphorylation.

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* Non-enzymatic proteins such as phosphovitin are known to have numerous phosphoserine residues within the same peptide chain.