PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 177, 33–41 (1984)

Properties of a Phosphoprotein Phosphatase from Skeletal Muscle and Its Regulation in Diabetes[1] (41908)

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Although the major phosphorylase phosphatase activity in skeletal muscle (1) and in liver (2) chromatographs with an apparent molecular weight of 250,000–260,000, only low molecular weight forms (30,000 to 35,000) have been purified to homogeneity (see review (3)). Since the low molecular weight phosphatases have not been demonstrated in tissues, it is important to isolate native high molecular weight phosphatases to investigate their subunit structure, substrate specificity, and mode of regulation.

In skeletal muscle partially purified phosphatase preparations of molecular weight ranging from 30,000 to 140,000 have been reported (3–7) whereas in other tissues investigators have obtained highly purified high molecular weight multisubunit phosphoprotein phosphatases. Tsuiki and co-workers (8, 9) have isolated two high molecular weight phosphatases from rat liver (termed phosphatase IB and II). Phosphatase IB has an apparent molecular weight of 260,000 and consists of three polypeptide chains of 69,000, 58,000, and 35,000 (9). Phosphatase II is of lower molecular weight (160,000) and comprises two subunits of 69,000 and 35,000 (8). A Ca^{2+}–calmodulin-dependent protein phosphatase (termed phosphatase 2B) is composed of two polypeptide chains of 61,000 and 15,000 Da and is identical to a major calcium binding protein of neural tissue termed calcineurin (10). Two distinct protein phosphatases from turkey gizzard smooth muscle have been purified to apparent homogeneity (termed smooth muscle phosphatase I and II). Smooth muscle phosphatase I is composed of three polypeptide chains of 60,000, 55,000, and 38,000 Da (11) whereas the smooth muscle phosphatase II is composed of a single polypeptide chain of 43,000 Da (12). Furthermore, a protein phosphatase capable of catalyzing the dephosphorylation of the α subunit of eIf-2 has been shown to be composed of two polypeptide chains of 60,000 and 39,000 Da (13).

In skeletal muscle two heat-stable protein inhibitors of phosphoprotein phosphatase activity have been identified (4, 14). Inhibitor I is active only when phosphorylated by the cAMP-dependent protein kinase (14, 15). It has been suggested that inhibitor 2 is a subunit of an inactive phosphorylase phosphatase (6) and is a part of Fc (16) and can be phosphorylated by an activating factor termed Fa or glycogen synthase kinase 3 (6, 17). Phosphorylation of inhibitor 2 in the inactive phosphatase designated Fc leads to the activation of phosphatase activity (6, 17). Alternatively, the inactive phosphatase can be activated by limited trypsinization in the presence of $MnCl_2$ (6). Nevertheless, both inhibitor 1 and inhibitor 2 have been suggested to be involved in the regulation of type I phosphoprotein phosphatase (18, 19).

Fischer and co-workers (20) have postulated that the glycogen–protein complex isolated from the rabbit skeletal muscle extract by acid precipitation followed by differential centrifugation represents a functional unit of the cell. Since the glycogen–protein complex contains a major portion (50–80%) of the total phosphorylase phosphatase activity of the skeletal muscle, we have focused our studies on the high molecular weight enzyme present in the glycogen–protein complex. Most of the characterization studies were carried out on partially purified preparations whereas recently we have been able to obtain highly purified preparations of this enzyme.

Our earlier *in vitro* studies utilizing ^{32}P-synthase showed that synthase containing 2.5 P_i/subunit was a poor substrate compared to synthase containing 1.7 P_i/subunit for this high molecular weight phosphatase (21). This in conjunction with our recent studies which showed that the total phosphate content of

[1] This work was supported by National Institutes of Health, Public Service Grants AM 26334 and BRSG-RR05424.

0037-9727/84 $1.50

synthase is significantly increased in diabetes (22) suggested that synthase phosphatase may be regulated by substrate modification. Therefore, we decided to investigate the role of substrate modification on the glycogen synthase phosphatase activity in muscle extracts. The results of this investigation are discussed in the final section.

Experimental Procedures. *Phosphoprotein phosphatase assays.* The phosphoprotein phosphatase activity toward protein and nonprotein substrates was measured as described earlier (21, 23). The synthase phosphatase activity in muscle extracts was measured in a reaction mixture containing 50 m*M* triethanolamine buffer, pH 7.5; 0.5 mg/ml shellfish glycogen; 1 m*M* β-mercaptoethanol; 0.5 mg/ml BSA; 5 m*M* magnesium acetate; 5 m*M* manganese chloride, 10 μg of purified synthase D; and 60 μl of muscle extract in a final volume of 100 μl. The control assays contained either no extract (basal synthase I activity) or no added synthase D (endogenous synthase I activity). At 0, 5, 10, 15, and 20 min, 20-μl aliquots were diluted with 80 μl of synthase dilution buffer (50 m*M* Tris, pH 7.8, 50 m*M* sodium fluoride, 5 m*M* EDTA; 1 mg/ml BSA, and 30 m*M* β-mercaptoethanol) and assayed for synthase activity in the absence of glucose 6-phosphate as described previously (24). The rate of conversion of the exogenously added synthase D into I was calculated from the increase in total synthase I activity after subtracting the basal and endogenous synthase I activities. The linear portion of the curve was used to calculate the values. One unit of synthase activity is described as 1 μmole of glucose incorporated into glycogen per minute of incubation.

Preparation of high molecular weight phosphatase and glycogen synthase D. Both glycogen synthase D and high molecular weight phosphatase were prepared from the glycogen–protein complex as described previously with some modifications (21, 22). The glycogen synthase D was prepared free of phosphatase by running a salt gradient on DEAE-cellulose column and phosphocellulose chromatography (21, 22). The phosphoprotein phosphatase was further purified by chromatography on ACA-34 followed by Sepharose–histone (type IIA) and Blue–Sepharose chromatographies.

Determination of Stokes radius, sedimentation coefficient, frictional ratio, and molecular weight. Stokes radius was determined by chromatography on a Sephacryl S-300 column (0.9 × 60 cm) equilibrated in buffer containing 50 m*M* Tris, pH 7.5, 10% glycerol, 10% ethylene glycol, 100 m*M* KCl, 1 m*M* EDTA, and 30 m*M* β-mercaptoethanol. The standard proteins used were catalase (5.2 nm), aldolase (4.6 nm), BSA (3.5 nm), and ovalbumin (2.8 nm). The Stokes radius of the high molecular weight phosphatase was also determined by using HPLC on a Bio-Rad TSK-250 column as described by Beebe and Corbin (25). The sedimentation coefficient was determined by sucrose-density gradient centrifugation by the method of Martin and Ames (26) by using BSA and catalase as standards. The molecular weight and frictional ratio were calculated by the method of Siegel and Monty (27) by assuming a partial specific volume of 0.725 cm^3/g.

Preparation of muscle extracts. The rabbits were sacrificed and muscle powders were prepared as described previously (22). One-half gram of powder was homogenized with 1 ml of buffer containing 50-m*M* Tris, pH 7.5, 2 m*M* EDTA, 1 m*M* EGTA, 30% glycerol, 0.1 m*M* phenylmethylsulfonyl fluoride, and 30 m*M* β-mercaptoethanol with polytron for 1–2 min at a medium speed. The homogenate was immediately centrifuged at 20,000 rpm for 5 min in a Beckman J2-21 centrifuge and applied to a G-25 column (0.9 × 30 cm) equilibrated in a buffer containing 50 m*M* Tris, pH 7.5, 1 m*M* EDTA, 1 m*M* EGTA, 10% glycerol, and 30 m*M* β-mercaptoethanol. Fractions containing maximum protein were pooled.

Other methods. Other methods, i.e., preparations of ^{32}P-substrates, preparation and assay of inhibitor 1, polyacrylamide gel electrophoresis, etc., were described earlier (21).

Results and Discussion. *Physical properties and subunit structure.* The Stokes radius of the purified enzyme was determined by gel filtration on a Sephacryl S-300 column and also by HPLC using a Bio-Rad TSK-250 column. Both procedures gave similar values, and the average value was calculated to be 4.4 nm. The sedimentation coefficient ($s_{20,w}$) was 4.4 S ± 0.23 as determined by sucrose-density gradient centrifugation. From these

values the molecular weight and frictional ratio (f/f_0) were calculated to be 83,000 and 1.53, respectively.

The SDS–polyacrylamide gel electrophoresis of most of the preparations showed two major protein bands migrating at 38,000 and 75,000 Da and some other minor bands (see Fig. 2B, last track labeled before centrifugation). In the absence of SDS most preparations showed a major Coomassie staining band. The activity of the enzyme migrated with the major Coomassie staining band (data not shown). Two-dimensional polyacrylamide gel electrophoresis (see Fig. 1) of a preparation showed that this major protein band which had the phosphorylase phosphatase activity contained two polypeptides migrating at molecular weights of 75,000 (75K) and 38,000 (38K).

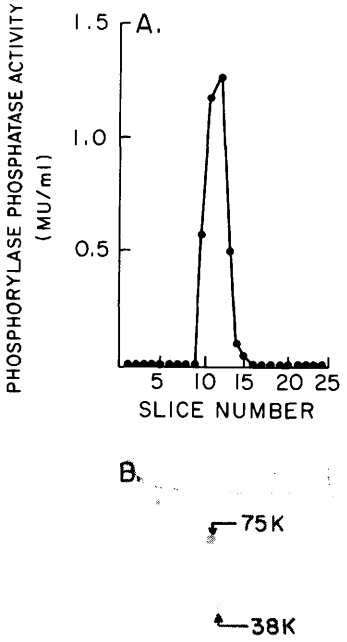

FIG. 1. Two-dimensional polyacrylamide gel electrophoresis of the phosphatase: Twenty-five micrograms of the phosphatase was electrophoresed on a 6% slab gel in the absence of SDS until the tracking dye migrated to the distal end. Following electrophoresis the track containing phosphatase was divided longitudinally into two. One-half was sliced and assayed for phosphorylase phosphatase activity (A) and the other incubated with stacking gel buffer containing 1.5 *M* β-mercaptoethanol and 5% SDS for 1 hr at 30°C. The gel was placed on the top of 10% polyacrylamide minislab gel and electrophoresed in the presence of SDS (B).

Further experiments showed that the 38K protein has the catalytic activity of the enzyme. Chromatography of the high molecular weight phosphatase on a reactive red-120 agarose column led to the dissociation of the enzyme. At this stage of purification the enzyme exhibited a Stokes radius of 2.4 nm and sedimentation coefficient of 4.1 S. Based on these values the molecular weight was calculated to be 42,000. SDS–polyacrylamide gel electrophoresis of this preparation showed a major protein band migrating at 38,000 Da, and the two-dimensional electrophoresis clearly showed that the activity migrates with the 38K protein. Since the 38K protein was also present in the high molecular weight phosphatase, it was concluded that this is the catalytic subunit of the high molecular weight phosphatase.

Since the molecular weight of the enzyme was determined to be 83,000, the results presented in the preceding section raise the question whether both the 75K and 38K are the subunits of the high molecular weight phosphatase. It was, therefore, decided to investigate the relationship between the enrichment of the two proteins (38K and 75K) and the enzyme activity following sucrose-density gradient centrifugation and HPLC. Figure 2 shows the SDS–polyacrylamide gel electrophoresis of fractions obtained after sucrose-density gradient centrifugation of the phosphatase. The results show that the 38K protein was enriched with the activity (see Fig. 2A) of the enzyme whereas the 75K protein migrates deeper into the gradient (Fig. 2B), suggesting that the 75K protein may be an impurity. When the peak tubes (tubes 5–7, Fig. 2A) were pooled to measure the Stokes radius, again a value of 4.4 nm was obtained, suggesting no shift in the molecular weight of the enzyme. Limited trypsinization of the fractions did not show the presence of a latent or inactive phosphatase activity. Polyacrylamide gel electrophoresis of the fractions obtained from the HPLC was also carried out. The results showed that the 38K protein enriched with the activity of the

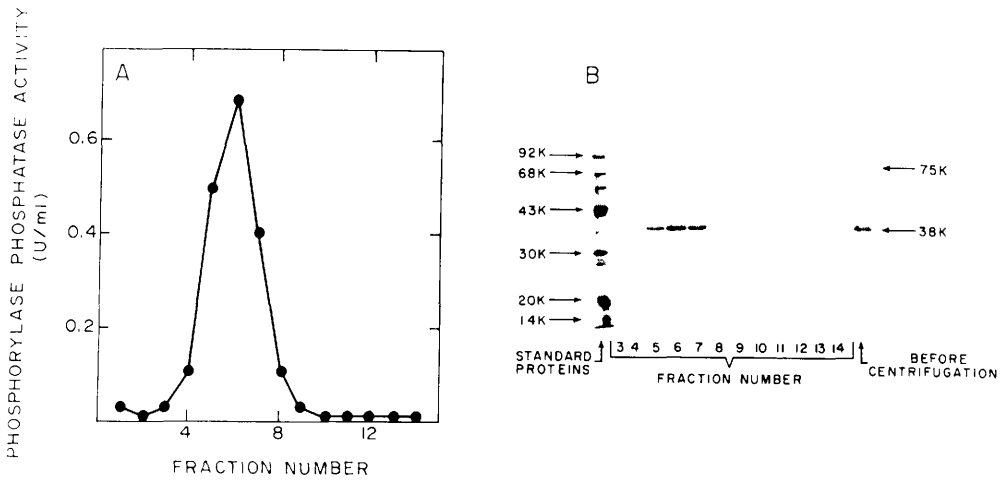

FIG. 2. SDS–polyacrylamide gel electrophoresis of the phosphatase following sucrose-density gradient centrifugation: Six hundred micrograms of phosphatase was applied on the top of a sucrose-density gradient (5–20%) and centrifuged at 1.9×10^5 g at 4°C for 18 hr. The gradient was fractionated into 30 fractions of 0.4 ml and assayed for phosphorylase phosphatase activity. The phosphatase activity peaked in fraction No. 6 from the top (A). Twenty microliters of the top fractions (3–14) was boiled with sample buffer and electrophoresed on a minislab polyacrylamide gel in the presence of SDS (B). Standard proteins were phosphorylase (92,000), bovine serum albumin (68,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), and lysozyme (14,000).

enzyme whereas the 75K protein was slightly separated (data not shown). From these experiments it was concluded that the 75K protein does not have the catalytic activity of the enzyme. Since this protein could be separated from the 38K protein without shifting the Stokes radius, it is suggested that the high molecular weight enzyme may consist of only 38K subunits.

The results presented in this section do not rule out the possibility of a loose association between the two proteins. Examples showing that subunits can be dissociated without significantly affecting the Stokes radius of the parent enzyme are available in the literature. Thus, the Stokes radius of the regulatory subunit of the type II cAMP-dependent protein kinase is not strikingly different from that of the holoenzyme (25, 28, 29). Therefore, more experiments are needed to reach a definitive conclusion as to whether both the 38K and the 75K proteins are the subunits of the high molecular weight phosphatase. Finally, a frictional ratio (f/f_0) of 1.53 for the high molecular weight phosphatase suggests that the enzyme is an asymmetric molecule. The earlier determinations of the molecular weight of the enzyme in extracts and also of purified preparations were based on the gel permeation data and could thus have been overestimated (1, 2, 5).

An inactive phosphatase from skeletal muscle has also been shown to contain a 38,000-Da catalytic subunit (6). Like Fc this inactive phosphatase preparation contains inhibitor 2 (31,000 M_r). Although our preparation also contains a 38,000-Da catalytic subunit and has substrate specificity very similar to Fc and inactive phosphatase (21, 30), it differs in many respects. First, this preparation binds to Blue–Sepharose whereas Fc and inactive phosphatase do not. The glycogen-bound enzyme is spontaneously active whereas these preparations were markedly activated by phosphorylation by Fa (or GSK-3) or by limited trypsinization in the presence of $MnCl_2$ (6). Our preparation is not markedly inhibited by inhibitor 1 whereas Fc is inhibited (29). Finally this preparation does not contain significant amounts of inhibitors where inhibitor 2 has been suggested to be a subunit of the inactive phosphatase (6). Since this preparation is prepared from the glycogen–protein complex and these other prepa-

rations are cytosolic, it is possible that our enzyme is a different phosphatase. However, the presence of a 38,000 protein in both preparations suggests that they may somehow be interrelated.

Activators and inhibitors. Phosphoprotein phosphatases are generally activated by divalent metals (31). The high molecular weight phosphatase from pellet is activated 1.5- to 3-fold by Mn^{2+} (23). Other metals except Co^{2+} are not very effective in activating the enzyme. Dialysis of the enzyme against potassium phosphate buffer or preincubation with free ATP or potassium fluoride leads to a reversible inhibition of the enzyme. The inhibition by all these ligands is reversed by a second preincubation with Mn^{2+} (apparent $K_a \sim 3\ \mu M$) prior to assay. Other divalent metals ($Co^{2+} > Zn^{2+} > Mg^{2+}$) are only partially effective in reversing the inhibition (23).

Substrate specificity. The relative activities (%) of the high molecular weight enzyme toward phosphorylase, phosphohistones, glycogen synthase, phosphorylase kinase, regulatory subunit of cAMP-dependent protein kinase, and phosphatase inhibitor 1 were 100, 40, 20, 13, 7, and 10, respectively (21). Furthermore, preliminary results show that the high molecular weight enzyme also dephosphorylates pyruvate kinase, 6-phosphofructo 1-kinase, fructose 1,6-bisphosphatase, and the bifunctional enzyme 6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase (Khatra and Pilkis, unpublished observations).

Although glycogen synthase phosphatase has been separated from phosphorylase phosphatase activity in liver (see review (32)), no such separations have been reported in skeletal muscle. In our preparations we were also unable to separate the synthase phosphatase activity from the phosphorylase phosphatase activity. The following results suggest that a single enzyme may be responsible for the dephosphorylation of the two substrates. Both activities comigrated on chromatography columns as well as polyacrylamide electrophoresis gels (21). The two activities showed similar heat denaturation patterns and were inhibited by ATP and then reactivated by Mn^{2+} in a similar fashion (21). Also, the apparent K_m values for these two substrates were very similar (5–6 μM), although the apparent V_{max} values differed by a factor of 2 to 3 (21). Seven phosphorylation sites have been identified in glycogen synthase (33). The high molecular weight enzyme dephosphorylates sites 1a, 2, and 3 (Khatra and Soderling, unpublished observations). Furthermore, this preparation showed high specificity toward the β subunit of phosphorylase kinase (21).

In addition the glycogen-bound enzyme shows some activity toward *p*-nitrophenyl phosphate. Results of many experiments, i.e., pH optima, polyacrylamide gel electrophoresis, heat denaturation patterns, competitive inhibition of phosphorylase phosphatase by *p*-nitrophenyl phosphate, etc., suggest that this activity toward *p*-nitrophenyl phosphatase may be an intrinsic property of this high molecular weight phosphatase (21).

Effect of Inhibitor 1. Hers and co-workers (34, 35) were the first to show that the phosphorylase phosphatase activity present in freshly prepared extracts of skeletal muscle and liver was insensitive to inhibition by heat-stable protein inhibitors. We have investigated the effect of inhibitor 1 on our purified preparations of high molecular weight phosphatase (Fig. 3) and at each stage of the purification of the glycogen–protein complex (Fig. 4). The results show that 60–80% of the phosphatase activity at each stage of the purification of glycogen–protein complex was insensitive to inhibition by this inhibitor. A limited trypsinization of each fraction increased the total activity 1.5- to 2-fold and converted the enzyme into a form which was potently inhibited by inhibitor 1 (Figs. 3 and 4). Similar results were obtained whether the glycogen–protein complex was prepared by pH 6.1 precipitation or direct centrifugation suggesting that this lack of inhibition is not an artifact produced by pH 6.1 precipitation or during the purification (Fig. 4). The possibility that the added inhibitor was rapidly inactivated was also excluded (21). When the purified preparations were analyzed for the presence of heat-stable inhibitors, an insignificant amount of inhibitor activity was observed. The mechanism of increase in the total phosphorylase phosphatase activity and the generation of inhibitor 1 sensitive phosphatase activity by limited trypsinization of

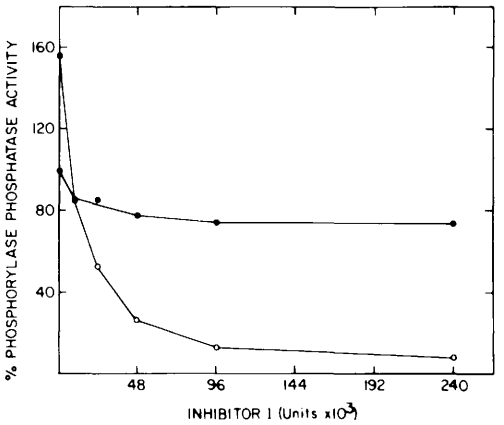

FIG. 3. Effect of inhibitor 1 on the high molecular weight phosphatase before and after trypsinization. Ten-microliter aliquots of the high molecular weight phosphatase were incubated for 20 min at room temperature (25°C) either without (●) or with (○) 50-μg/ml trypsin and diluted 100-fold in ice cold buffer (50-m*M* Tris, pH 7.5, 1-m*M* EDTA, 1-mg/ml BSA, 10% sucrose, and 30-m*M* β-mercaptoethanol) containing 25 μg/ml of soybean trypsin inhibitor. Each fraction was then assayed in the presence of the given concentration of Inhibitor 1. The given values are percentage activities of control which was 2 mU of phosphorylase phosphatase activity.

the extracts and purified high molecular weight phosphatase are not clearly understood. Various possibilities include (a) breakdown of our inhibitor insensitive high molecular weight phosphatase, (b) activation of an inactive or latent phosphatase (34, 35), or (c) proteolysis and destruction of a deinhibitor protein (36). Since the enzyme was activated only 1.5- to 2-fold by limited trypsinization, activation of a latent enzyme may not be occurring. The possibility of the presence of a deinhibitor protein cannot be excluded. A deinhibitor protein has been shown to bind to the glycogen–protein complex (36). We have observed that on further purification or storage in the absence of $MnCl_2$ some preparations show a variable degree of sensitivity toward this inhibitor. This could be explained by the loss of the deinhibitor protein or a change in the conformation of the enzyme. Work is in progress to study this effect in more detail.

Regulation of synthase phosphatase activity in diabetic rabbit skeletal muscle. It is known that the administration of insulin to both normal and diabetic animals leads to an increase in the rate of activation of glycogen synthase in various tissues (37–39). The mechanism of this action of insulin is not known. Based on kinetic data, it was suggested that adipose tissue from diabetic rats may contain highly phosphorylated forms of glycogen synthase which are poor substrates for the phosphatase (40). As mentioned earlier we have observed that total phosphate content of synthase prepared from diabetic rabbits is significantly increased when compared to synthase prepared from normal rabbits (22). Also, *in vitro* studies utilizing high molecular weight phosphatase showed that ^{32}P-synthase containing 2.5 P_i/subunit showed a lower apparent V_{max} compared to synthase containing 1.7 P_i/subunit (21). Based on these studies we decided to investigate the role of substrate modification on the regulation of phosphatase activity. Glycogen synthase phosphatase activity was measured in muscle extracts of normal and diabetic rabbits by measuring the rate of activation of exogenously added pure synthase D preparations which were isolated from normal and diabetic rabbits. The results of our study are shown in Table I. When glycogen synthase D isolated from normal rabbits was used as a source of substrate and muscle extracts prepared from normal and diabetic rabbits were used as sources of synthase phosphatase activity, the extracts from diabetic rabbits showed a significantly lower phosphatase activity. However, no significant differences were observed between phosphatse activities of the muscle extracts prepared from normal and diabetic rabbits toward synthase D prepared from diabetic rabbits. Furthermore, in each case synthase D prepared from diabetic rabbits was only 50% as good a substrate as synthase D purified from normal rabbits. The results presented in Table 1 thus indicate that (a) the glycogen synthase phosphatase activity is inhibited in diabetes and (b) the synthase D isolated from diabetic rabbits is a poor substrate. The inhibition of synthase phosphatase activity may lead to the observed increase in the phosphorylation state of glycogen synthase D in diabetes (22). Recent studies by Sheorain *et al.* (41) show that synthase D isolated from diabetic rabbits shows significant increase in the phosphorylation level of sites 2 and 3. In conjunction with these studies it may be

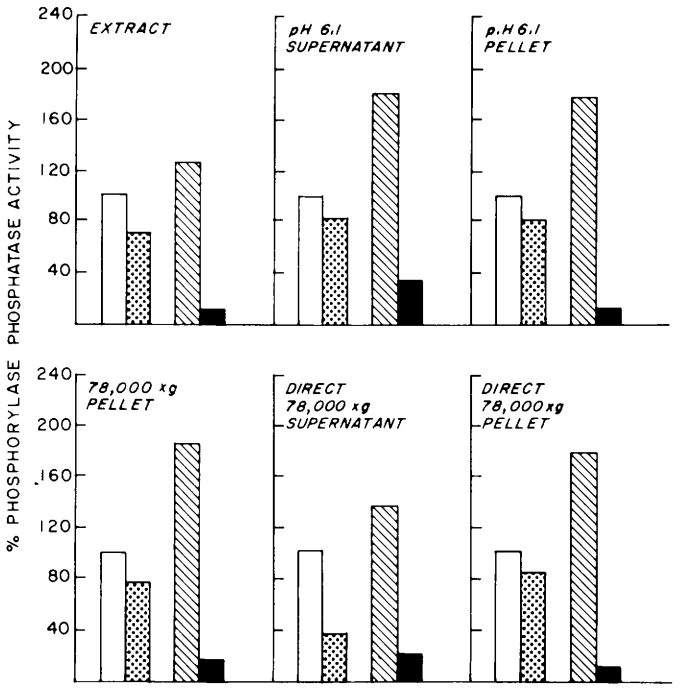

FIG. 4. Effect of inhibitor 1 on the phosphatase activity at different stages of the purification of glycogen–protein complex. The given fractions were incubated at room temperature (25°C) either without (open bars and dotted bars) or with (crossed bars and closed bars) 50 μg/ml trypsin and diluted 20-fold in buffer (see legend to Fig. 3) containing 25 μg/ml of soybean trypsin inhibitor. Each fraction was then assayed either in the absence (open and crossed bars) or in the presence (dotted and closed bars) of 96 mU of Inhibitor 1. The given values are the percentage activities of the control values which were in the range of 0.5 to 2 mU of phosphatase activity.

possible that insulin is required to maintain a normal level of phosphatase activity. In diabetes loss of phosphatase specific for these sites would shift the synthase interconversion equilibrium toward increased phosphorylation of these sites. As mentioned earlier our high molecular weight phosphatase showed high specificity toward the dephosphorylation of these sites and may thus be a potential target for the regulation by insulin. Finally a poor activation of glycogen synthase D isolated from diabetic rabbits suggests that synthase phosphatase activity may be regulated through substrate modification. Investigations are in progress to study the mechanism of this type of regulation. Other enzymes, i.e., pyruvate dehydrogenase phosphatase activity in heart, have also been suggested to be regulated through substrate modification (42).

TABLE I. GLYCOGEN SYNTHASE PHOSPHATASE ACTIVITY[a] IN NORMAL AND DIABETIC RABBIT MUSCLE EXTRACTS

	Source of extract	
Source of substrate	Normal rabbits	Diabetic rabbits
Normal rabbits	13.99 ± 1.67	9.51 ± 0.81*
Diabetic rabbits	6.50 ± 2.55	4.02 ± 1.09

Note. Preparation of muscle extracts and synthase phosphatase assays is described in detail under Experimental Procedures. Each value is a mean ± SE of six determinations. The asterisk denotes statistically significant difference ($P < 0.025$) when compared with extracts from normal rabbits utilizing synthase D isolated from normal rabbit muscle.

[a] mU of synthase D converted/5 min/mg.

The author is indebted to Mrs. Harriet M. Harrison for excellent technical assistance. I am grateful to Dr. J. D. Corbin, Dr. T. R. Soderling, and Dr. S. Beebe for

helpful discussion. Thanks are also due to Mr. Cobb and Dr. J. D. Corbin for performing HPLC.

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