SUBSTRATE SPECIFICITY OF GLYCOGEN SYNTHASE PHOSPHATASE FROM BOVINE HEART: ACTION ON PHOSPHORYLASE a AND HISTONE*

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SUMMARY: Glycogen synthase phosphatase has been purified from bovine heart. This preparation catalyzes conversion of synthase D into I and phosphorylase a into b and is able to dephosphorylate synthase D, phosphorylase a, active phosphorylase kinase, and phosphorylated histone and casein. The activity of phosphatase was assayed with synthase D, phosphorylase a, and histone as substrates after chromatography on Sephadex G-100, after sucrose gradient centrifugation, and after isoelectric focusing in a sucrose gradient. In all cases no separation of enzyme activity was observed with the above substrates. The phosphatase activity on all substrates was lost at the same rate by heat denaturation. These results indicate that this enzyme preparation contains a single phosphoprotein phosphatase which is responsible for the activity observed on the above substrates.

INTRODUCTION

Recent reports on glycogen synthase phosphatase purified from rabbit skeletal muscle indicate that the enzyme is not absolutely substrate specific but is also active on histone and active phosphorylase kinase (1,2). An earlier study by Hickenbottom (3) showed that dephosphorylation of synthase D to I could be catalyzed by phosphorylase kinase phsophatase from skeletal muscle and England et al. (4) recently demonstrated that skeletal muscle phosphorylase phosphatase catalyzed dephosphorylation of the inhibitor component of troponin. Since the foregoing data indicate some lack of substrate specificity for several protein phosphatases, we have investigated the substrate specificity of glycogen synthase phosphatase purified from bovine heart. The present paper reports some

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results of this study indicating that heart phosphatase acts on the same three substrates that are dephosphorylated by the skeletal muscle enzyme and also on casein and phosphorylase a.

MATERIALS AND METHODS

Bovine heart glycogen synthase phosphatase was prepared by procedures similar to those of Kato and Bishop (1), except that the Sephadex G-200 gel filtration step was omitted and Mn^{2+} was not included in the buffers used. We selected a fraction that eluted from DEAE-cellulose between 0.25 and 0.30 N NaCl for subsequent studies. Approximately 200-fold purification of the phosphatase was obtained.

Bovine heart synthase I (specific activity = 7 units/mg protein) was prepared by the method of Thomas and Larner (5), and was converted into ^{32}P-labeled synthase D (0.22 μCi/mg protein) as described by Thomas and Nakai (6). Phosphorylase b, phosphorylase kinase, and phosphorylase phosphatase purified from rabbit skeletal muscle (7,8,9) were supplied by Dr. D. J. Graves. Phosphorylase b was converted into ^{32}P-labeled phosphorylase a (0.11 μCi/mg protein) by the procedure of Krebs and Fischer (10). Calf thymus histone (Type IIA from Sigma) was phosphorylated with bovine heart protein kinase (11) by a method similar to that of Meisler and Langan (^{32}P-histone = 3 μCi/mg protein) (12). Casein was phosphorylated in a similar manner (^{32}P-casein = 50 nCi per mg protein).

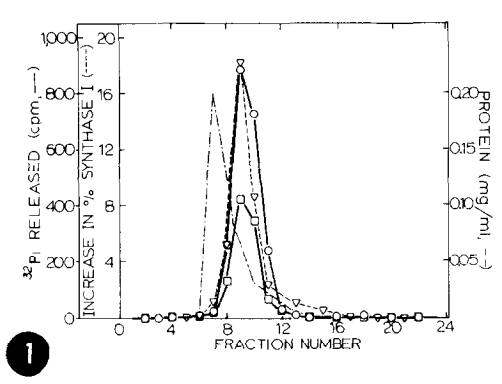
Phosphatase activity was assayed by release of ^{32}Pi from ^{32}P-labeled substrates at 30°C. Reaction mixtures contained 50 mM Tris (pH 7.5), 5 mM dithiothreitol (DTT), ^{32}P-labeled substrates (16 μg of synthase D, 27 μg of phosphorylase a, 36 μg of histone, or 400 μg of casein) and the phosphatase preparation in a volume of 50 μl. When histone was used as substrate, 100 mM KCl or $MgCl_2$ was added to the reaction mixture, and with synthase D or casein as substrate, 10 mM $MgCl_2$ was added. The reaction was started by the addition of phosphatase to the rest of the reaction mixture and stopped by the addition

of 20 µl of 100% (w/v) trichloroacetic acid (or 80 mM silicotungstic acid in 80 mN H_2SO_4 when histone was the substrate) at suitable time. All reaction mixtures were kept on ice for 20 min., then centrifuged at 2,600xg for 20 min. at 4°C. Fifty microliters of supernatant was spotted on a disc of filter paper (2 x 2 cms; Whatman ET31), dried and counted in a scintillation counter to determine ^{32}Pi released during the enzyme incubation. Control assays containing no phosphatase were run with each experiment in order to correct for non-covalently bound ^{32}P counts on the substrates. Phosphatase was also assayed by conversion of synthase D into I, and by phosphorylase a conversion into b as measured by changes in enzyme activity (6,8).

Protein was determined by the method of Lowry et al. (13) with bovine serum albumin as standard.

RESULTS

Our heart preparation gave one broad activity peak of phosphatase on DEAE-cellulose when assayed with either histone or phosphorylase a as substrate. We selected one fraction of the phosphatase peak which was eluted between 0.25 and 0.30 N NaCl for this present study, because it contained no glycogen synthase and very small amount of protein kinase. The purified preparation of glycogen synthase phosphatase was able to dephosphorylate glycogen synthase D, phosphorylase a, activated phosphorylase kinase, histone, and casein. In addition, synthase D was converted into I and phosphorylase a into b by the same purified synthase phosphatase. In a subsequent study of phosphorylase phosphatase, purified from rabbit skeletal muscle by the procedure of Hurd et al. (9) in Dr. D. J. Graves' laboratory, it was observed that bovine heart synthase D was readily converted into I (56% conversion in 20 min. at 30°C when 60 m units of synthase D was incubated with 10 µg of phosphorylase phosphatase). The above data indicated that phosphorylase phosphatase and synthase phosphatase might be the same enzyme. Therefore, we attempted to determine if our enzyme preparation contained one or several protein phosphatases.



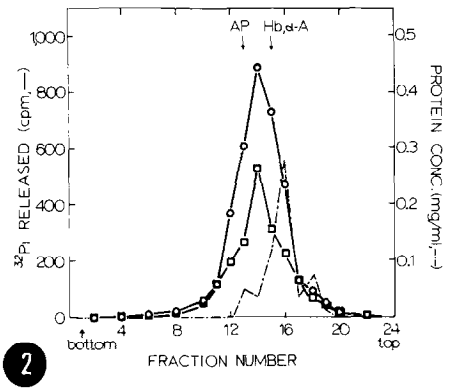
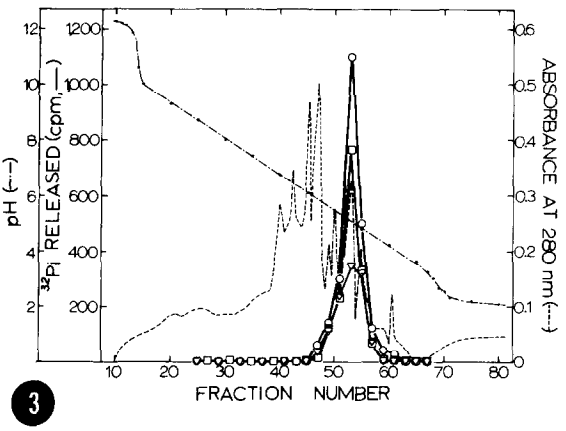


Figure 1. Sephadex G-100 column chromatography of phosphatase. Bovine heart synthase phosphatase (0.8 mg) was applied to Sephadex G-100 column (0.90 x 15 cms) equilibrated with 50 mM Tris-Cl, 5 mM DTT, pH 7.5. Fractions were assayed by dephosphorylation of histone (○—○) and phosphorylase a (□—□) as described in "MATERIALS AND METHODS". Synthase phosphatase (▽---▽) was assayed by conversion of synthase D into I (6). Protein (–·–·–) was determined by the Folin-Lowry method (13).

Figure 2. Sucrose gradient centrifugation of phosphatase. Bovine heart synthase phosphatase (0.16 mg) was loaded on a sucrose gradient (5-25%, 5 ml) in 50 mM Tris-Cl, 5 mM DTT, pH 7.5. Gradients were centrifuged in a Beckman SW39 rotor at 38,000 rpm for 18 hrs. at 4°C. Fractions were assayed for phosphatase activity with (○—○) histone (20 min.) and (□—□) phosphorylase a (60 min.) as in "MATERIALS AND METHODS". Protein (–·–·–) was determined by the Folin-Lowry method (13). The peak positions for human salivary α-amylase (α-A), bacterial alkaline phosphatase (AP), and bovine hemoglobin (Hb) are indicated by arrows.

The enzyme preparation was chromatographed on Sephadex G-100 as shown in Figure 1. The phosphatase was included indicating its molecular weight is lower than 100,000 and the phosphatase activities on three different substrates eluted with the same exclusion volume. Figure 2 shows the pattern of the phosphatase activity after sucrose gradient centrifugation. The phosphatase activity, measured with different substrates, migrated as one peak not coincident with the protein peak. From the activity peak of phosphatase and peak positions of human salivary α-amylase (MW 55,200), bovine hemoglobin (MW 65,000), and bacterial alkaline phosphatase (MW 77,500), the molecular weight of the bovine heart synthase phosphatase was estimated to be 65,000-70,000. The reported molecular weight of rabbit muscle phosphorylase phosphatase is approximately 50,000 (9), and since neither determination has been done accurately, they may in fact be the same. In contract, the molecular weight of a histone phosphatase from rat liver was estimated to be 190,000 (12).



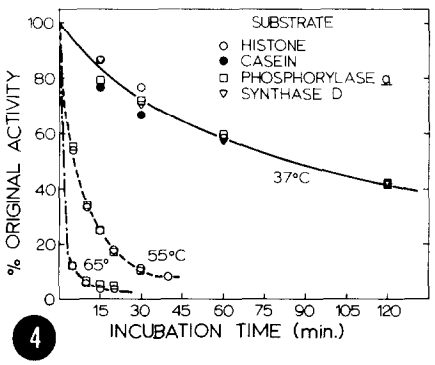


Figure 3. Isoelectric focusing of phosphatase. Isoelectric focusing was performed in an ISCO Model 212 preparative density gradient electrophoresis apparatus (3°C, 12 hrs., 400 V). The central column contained 20 mls of sucrose gradient (5-30%), 0.3 mg of bovine heart synthase phosphatase, 2.0% Ampholine (pH 3-10; LKB, Inc.), and 2 mM DTT. After pH measurements (—·—), fractions were neutralized with 2 M unneutralized Tris solution and assayed for phosphatase activity at 30°C for 60 min. with (○—○) histone, (▽—▽) synthase D, and (□—□) phosphorylase a. O. D. at 280 nm is also shown (- - - -).

Figure 4. Heat stability of phosphatase. Bovine heart synthase phosphatase was diluted in 50 mM Tris-Cl, pH 7.5 and incubated at 37°C, 55°C, or 65°C. At the times indicated in the figure, aliquots were removed and assayed for enzyme activity by dephosphorylation as in "MATERIALS AND METHODS" (30°C, 10 min. for the experiment at 37°C and 30°C, 4 min. when preincubated at 55°C or 65°C). The data points indicate activity remaining after incubation relative to the original activity when assayed with (○) histone, (●) casein, (▽) synthase D, and (□) phosphorylase a.

Isoelectric focusing (pH range 3-10) of the heart phosphatase preparation in a sucrose gradient (5-30%) is shown in Figure 3. The migration profile reveals only one phosphatase activity peak when assayed with three different phosphoproteins as substrates. The pI of the phosphatase was determined three times with several different substrates and an average value of 5.0 was obtained. Thus, the phosphatase activities on different substrates cannot be separated on the basis of isoelectric properties of the enzyme.

Figure 4 gives data on the stability of the phosphatase to heat denaturation. In the first experiment (37°C), the activity was assayed with four different phosphoprotein substrates and no differences in stability were apparent. In two additional experiments at 55°C and 65°C the enzyme preparation was assayed only with histone and phosphorylase a. Again there was no differ-

ence in the heat stability of the phosphatase activity on these two substrates. Thus, there was no heterogeneity evident in the heat stability of the phosphatase.

DISCUSSION

The bovine heart glycogen synthase phosphatase preparation used in these studies was capable of dephosphorylating glycogen synthase D, phosphorylase a, activated phosphorylase kinase, and phosphorylated histone and casein. Gel filtration, sucrose gradient centrifugation, and isoelectric focusing of this preparation failed to separate the different phosphoprotein phosphatase activities from each other and heat stability experiments could not distinguish these activities. Therefore, it is probable that this phosphatase preparation, purified as glycogen synthase phosphatase, contains one enzyme which is responsible for the observed activities on several different substrates.

The above conclusion is in good agreement with the results reported by Kato and Bishop (1) and Zieve and Glinsmann (2) for skeletal muscle synthase phosphatase, except that Kato and Bishop reported that phosphorylase a was not an alternative substrate for muscle synthase phosphatase. This discrepancy may be explained by differences in the source of synthase phosphatase or in assay conditions. Since our results are supported by the fact that bovine heart synthase D was converted into I by a phosphorylase phosphatase preparation from skeletal muscle (see "RESULTS"), there may be some differences in substrate specificity between heart and skeletal muscle phosphatase. This question remains to be answered by further experiments. In any case the heart enzyme might better be refered to as protein phosphatase until its specificity is defined more clearly.

It should be pointed out that the phosphatase described in this communication may not be the only phosphoprotein phosphatase present in heart tissue. As reported by Kato and Bishop (1) for skeletal muscle, the elution profile of heart phosphatase from DEAE-cellulose was rather broad. Since we selected a

narrow fraction of the phosphatase eluted from DEAE-cellulose for this present study, it is not clear whether other phosphoprotein phosphatases may yet be found in bovine heart. It should be noted that phosphatases reported to be specific for histone and protamine were found in rat liver (12) and brain (14).

The physiological significance of these results may be related to an observation by Stalman et al. (15) that indicated that phosphorylase a is an inhibitor of synthase phosphatase in liver. This may also be true in heart since, as shown in this report, one enzyme is capable of acting on both synthase D and phosphorylase a.

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REFERENCES

(1) Kato, K., and Bishop, J. S., J. Biol. Chem., 247, 7420 (1972).
(2) Zieve, F. J., and Glinsmann, W. H., Biochem. Biophys. Res. Commun., 50, 872 (1973).
(3) Hickenbottom, J., Ph.D. thesis, University of Washington (1968).
(4) England, P. J., Stull, J. T., and Krebs, E. G., J. Biol. Chem., 247, 5275 (1972).
(5) Thomas, J. A. and Larner, J., Biochim. Biophys. Acta, 293, 62 (1973).
(6) Thomas, J. A., and Nakai, C., J. Biol. Chem., 248, 2208 (1973).
(7) Fischer, E. H., and Krebs, E. G., Methods Enzymol., 5, 369 (1962).
(8) Brostrom, C. O., Hunkeler, F. L., and Krebs, E. G., J. Biol. Chem., 246, 1961 (1971).
(9) Hurd, S. S., Novoa, W. B., Hickenbottom, J. P., and Fischer, F. H., Methods Enzymol., 8, 546 (1966).
(10) Krebs, E. G., and Fischer, E. H., Biochim. Biophys. Acta, 20, 150 (1956).
(11) Reimann, E. M., Walsh, D. A., and Krebs, E. G., J. Biol. Chem., 246, 1986 (1971).
(12) Meisler, M. H., and Langan, T. A., J. Biol. Chem., 244, 4961 (1969).
(13) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).
(14) Maeno, H., and Greengard, P., J. Biol. Chem., 247, 3269 (1972).
(15) Stalmans, W., De Wulf, H., and Hers, H-G., Eur. J. Biochem., 18, 582 (1971).