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A NOVEL GLYCOGEN SYNTHASE PHOSPHATASE FROM CANINE HEART*

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SUMMARY: Two molecular forms (M_r=49,300 and 26,000) of a divalent cation-dependent (Mg^{2+}=Mn^{2+}>Co^{2+}) protein phosphatase, which represent the major glycogen synthase phosphatase activity in canine heart extracts, have been partially purified and characterized. Although a general protein phosphatase of M_r=35,000 is also active toward synthase D, it represents the major phosphorylase phosphatase activity in heart muscle. The present findings indicate that the dephosphorylation of synthase D and phosphorylase a may be regulated by two distinct phosphatases rather than by a single nonspecific enzyme.

INTRODUCTION

In recent years, several papers have appeared supporting the concept that a single protein phosphatase is responsible for the activation of glycogen synthase D and inactivation of phosphorylase a (1-8). Evidence indicates that a general protein phosphatase of M_r=31-35,000, which has been purified to homogeneity from several tissues (6,7,9-11) as phosphorylase phosphatase, can also function as a synthase phosphatase (6,7). Previously (9) we reported that protein phosphatases of canine heart may be divided into two classes. Class I isozymes include several molecular forms of divalent cation-independent, nonspecific phosphatases which appear to consist of a common catalytic subunit of M_r=35,000. Class II isozymes include two divalent cation phosphatases with narrow substrate specificity. One of the class II isozymes (M_r=49,300) is specific for phosphocasein (9). We now report that this casein phosphatase is in fact the major synthase phosphatase activity in the cardiac muscle.

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MATERIALS AND METHODS

Preparation of substrates. Rabbit skeletal muscle glycogen synthase was prepared by the method of Nimmo et al (12). ^{32}P-Synthase D was prepared by incubating 0.3-0.6 mg/ml of synthase I with 20 mM potassium phosphate; pH 6.8, 10 mM magnesium acetate, 0.2 mM ethyleneglycol tetraacetic acid, 10 mM NaF, 0.2 mM [γ-^{32}P] ATP (1-1.9 cpm/nmol) and the catalytic subunit of bovine heart adenosine 3',5'-monophosphate (cyclic AMP)-dependent protein kinase (purified according to Erlichman et al (13)) at 30° for 5 hrs. The resulting ^{32}P-synthase D was separated from the unreacted [γ-^{32}P] ATP by gel filtration followed by dialysis. The final preparation contained about 0.7 mol ^{32}P/90,000 g of synthase. Rabbit skeletal muscle phosphorylase b (Sigma) and vitamin free casein (Nutritional Biochemicals) were phosphorylated with [γ-^{32}P] ATP and cyclic AMP-dependent protein kinase as described previously (14).
Enzyme assays. Synthase phosphatase activity was determined either by measuring the ^{32}P released from ^{32}P-synthase D or by measuring the conversion of the D to I form of synthase. The reaction was carried out at 30° in an incubation volume of 25 µl containing 50 mM Tris, pH 7.4, 1 mg/ml rabbit liver glycogen, 1 mg/ml bovine serum albumin, 1.3 µM ^{32}P-synthase D (based on ^{32}P concentration) or 2 units/ml non-radioactive synthase D in the absence or presence of 10 mM $MgCl_2$. When the release of ^{32}P was measured, the reaction was terminated and ^{32}P was separated from the remaining ^{32}P-synthase by a chromatographic procedure described previously (14). When the D to I conversion of synthase was followed, the reaction was terminated as described by Kato and Bishop (3) and the synthase activity was measured according to Smith et al (15) except that ^{14}C-glycogen formed was separated from UDP-[^{14}C]-glucose as described by Huang et al (16). One unit of synthase phosphatase activity was defined as that amount catalyzing the conversion of 1 unit of synthase D to I per min. The activity of phosphorylase and casein phosphatase was determined by measuring the ^{32}P released from the corresponding ^{32}P-protein as described previously (9).
Isolation of glycogen synthase phosphatase. Canine heart extracts were fractionated with 55% $(NH_4)_2SO_4$, DEAE-Sephadex A-50 and treated with 80% ethanol at 25° as described previously (9). Subsequent ion exchange and gel filtration chromatography procedures are described in the legends to the figures. The following buffers were used during the chromatography procedures: 20 mM Tris, pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 50 mM KCl (Buffer A), and 20 mM Tris, pH 7.4, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol and 0.1 M KCl (Buffer B). Protein concentration was determined according to Lowry et al (17), using bovine serum albumin as a standard.

RESULTS

Purification of synthase phosphatase. As shown in Fig. 1, chromatography of the ethanol-treated $(NH_4)_2SO_4$ fraction on DEAE-cellulose resulted in two distinct peaks of synthase phosphatase activity. Peak I could be detected only in the presence of $MgCl_2$ and co-eluted with a casein phosphatase (data not shown). Peak II was active either in the absence or presence

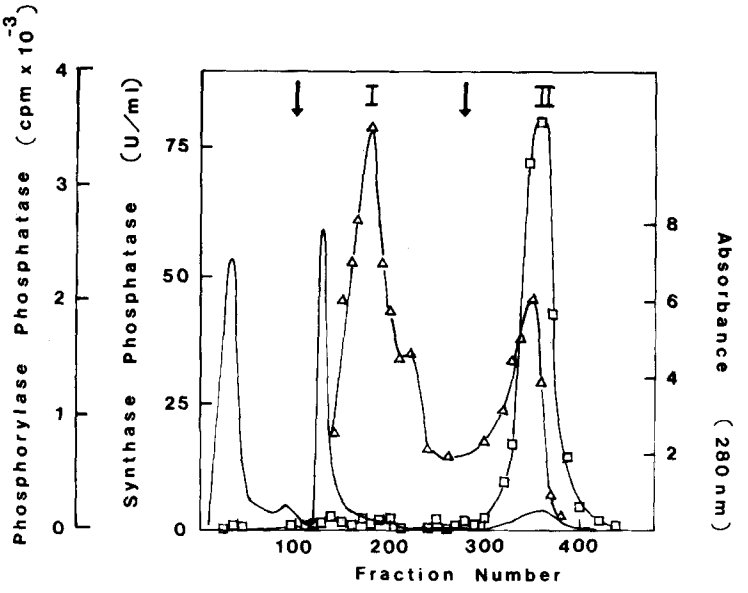

Fig. 1. Column chromatography of the ethanol precipitation fraction on DEAE-cellulose.

The ethanol precipitation fraction (193 ml, 30 mg/ ml) was applied on a DEAE-cellulose column (2.5 X 50 cm) equilibrated with buffer A. After sample application, the column was first eluted by buffer A followed by 2 ℓ of buffer A plus 0.05 M KCl and 1.5 ℓ of an increasing KCl gradient from 0.1 to 0.4 M in buffer A (9) as indicated. Fractions of 11 ml were collected. The enzyme activity toward synthase D (D to I conversion, Δ-Δ) and phosphorylase a (□-□) was determined with aliquotes from the indicated fractions as described in the text. (⟷) indicates absorbance at 280 nm.

of $MgCl_2$ and co-eluted with phosphorylase phosphatase (Fig. 1) and casein phosphatase activity (data not shown). Fig. 1 clearly shows that peaks I and II represent the major synthase and phosphorylase phosphatase activities, respectively. Further purification of the Peak II enzyme to apparent homogeneity by a procedure described previously (9) resulted in a general phosphatase of M_r=35,000 which was not dependent on metal ions for its activity.

As seen in Fig. 2, DEAE-Sephadex A-50 chromatography resolved the peak I synthase phosphatase into a minor (peak I-A) and a major (peak I-B) active fraction. Gel filtration of peak I-A on Sephacryl S-200 resulted in a major and minor synthase phosphatase peak

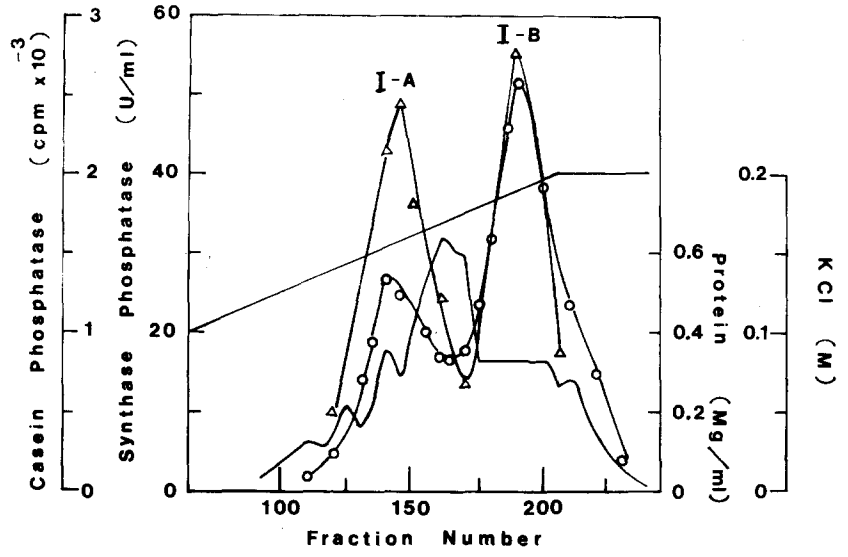

Fig. 2. Column chromatography of synthase phosphatase (peak I) on DEAE-Sephadex A-50.

The concentrated peak I (45.5 ml, 8.2 mg/ml) obtained from DEAE-cellulose chromatography (Fig. 1.) was applied on a DEAE-Sephadex A-50 column (1.6 X 20 cm) equilibrated with buffer A plus 0.05 M KCl. After rinsing the column with this buffer, the enzyme was eluted with a linear gradient of KCl from 0.1 to 0.2 M in Buffer A. Fractions of 6.4 ml were collected. The enzyme activity toward phosphocasein, (o-o), and synthase D (D to I conversion , Δ-Δ), was determined with aliquots from the indicated fractions as described in the text. (⟷), indicates protein concentration.

corresponding to M_r=49,300 and 26,000, respectively (Fig. 3A). Gel filtration of peak I-B on the same column resulted in a single active species of M_r=49,300 (Fig. 3B). As indicated in Figs. 2 and 3, the enzyme activity toward phosphocasein coincided with that toward synthase D. At this stage of purification, the M_r=49,300 form obtained from peak I-B, which represents the major casein and synthase phosphatase activity recovered from the DEAE-cellulose chromatography, had been purified about 1,000-fold from the canine heart extracts in terms of casein phosphatase activity as reported previously (9). Although the M_r=49,300 preparation was not homogeneous as judged by polyacrylamide gel electrophoresis, it showed a single activity band on the gel with a mobility slower than that of the

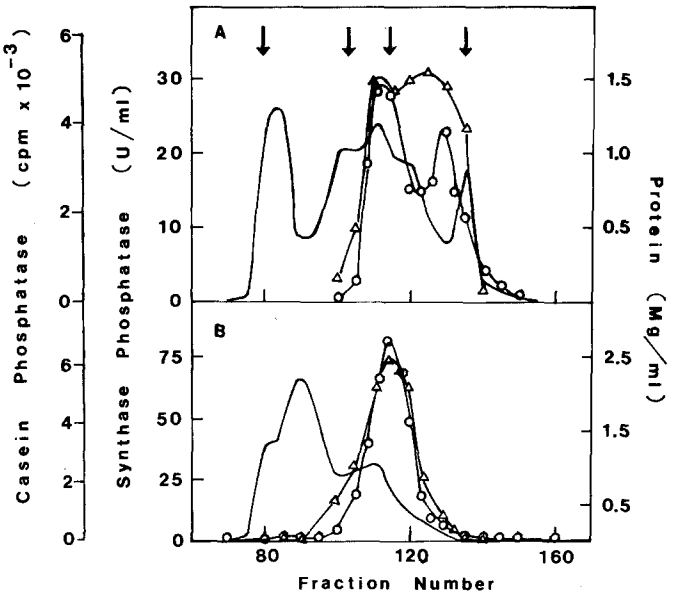

Fig. 3. Gel filtration of synthase phosphatase on Sephacryl S-200. The concentrated peaks I-A (9.7 ml, 12.8 mg/ml) and I-B (7.2 ml, 22 mg/ml) obtained from DEAE-Sephadex chromatography (Fig. 2.) were, separately, applied on a Sephacryl S-200 column (2.6 X 90 cm) equilibrated with buffer B for elution with the same buffer. Fractions of 2.6 ml were collected. Enzyme activity toward synthase D (D to I conversion, Δ-Δ) and phosphocasein (o-o) was determined with aliquotes from the indicated fractions as described in the text. The arrows mark the elution volume of the following: blue dextrin, bovine serum albumin (68,000), ovalbumin (44,000), myoglobin (17,000). (▬) indicates protein concentration. Figs. 3A and 3B indicate the elution profile of peaks I-A and I-B, respectively.

phosphatase of M_r=35,000 (data not shown). The M_r=49,300 preparation was used in the following studies.

Catalytic properties of synthase phosphatase. The enzyme is inactive in the absence of Mg^{2+} (Fig. 4). The Mg^{2+}-activation reaction followed Michaelis-Menten kinetics with an apparent Km of about 0.8-1.0 mM. In the presence of saturating concentration of $MgCl_2$ (10 mM), the saturation curve for ^{32}P-synthase was rectangular-hyperbolic with an apparent Km of about 0.6 μM (based on ^{32}P concentration). The enzyme was also activated by Mn^{2+} and Co^{2+} (Mg^{2+}=Mn^{2+}>Co^{2+}). The divalent cation

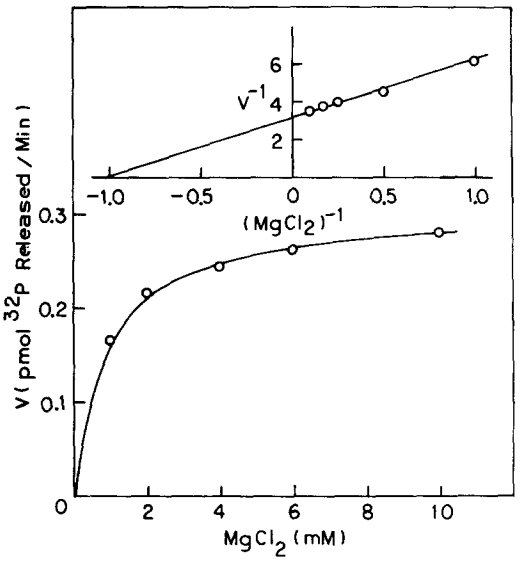

Fig. 4. Effects of $MgCl_2$ concentrations on synthase phosphatase activity. Synthase phosphatase activity was measured at 30° in an incubation volume of 25 µl, containing 1.3 µM ^{32}P-synthase D and other components as described in the text in the absence and presence of various concentrations of $MgCl_2$ as indicated. The insert indicates double reciprocal plots of the same data.

activation effects were found to be the same when the enzyme activity was measured either by the rate of ^{32}P release from ^{32}P-synthase or by the rate of conversion of synthase D to the I form. The enzyme had an optimum pH=7.5 (data not shown). When phosphocasein was used as a substrate, the response of the enzyme activity to divalent cations was the same as described above. Both the M_r=49,300 and 26,000 preparations showed little activity toward phosphorylase a or phosphohistone, either in the presence or absence of Mg^{2+}.

By contrast, the M_r=35,000 species purified from peak II was active toward various phosphoproteins in the absence of added divalent cation. Comparative studies on the substrate specificity of the M_r=49,300 and the M_r=35,000 species in the presence of 10 mM $MgCl_2$ showed that the ratio of activity toward synthase D versus activity toward phosphorylase a

to be 20 times higher in the former than in the later preparation. The M_r=49,300 species was much more susceptible to heat inactivation than the M_r=35,000 enzyme. After incubating the M_r=49,300 species at 40° for 5 min with 20 mM Tris, HCl, pH 7.4, 0.2% bovine serum albumin, more than 65% of the activity toward synthase D and phosphocasein was lost. In contrast, incubation of the M_r=35,000 enzyme at 45° for 5 min in the same incubation mixture resulted in less than a 20% loss of its activity.

DISCUSSION

Current thinking on glycogen metabolism seems to favor the hypothesis that the M_r=35,000 species or its holoenzyme form(s) is (are) responsible for the dephosphorylation of both glycogen synthase D and phosphorylase a (6,7,18,19). On the other hand, a few laboratories (20-22) have recently noted that synthase and phosphorylase phosphatase activities in rat and mouse liver may be separated by ion-exchange and/or gel filtration chromatography. Since the data concerning molecular weight, metal ion dependency and other catalytic properties of these liver synthase phosphatases have not been reported, it is not known whether they are similar to the canine heart enzyme described here.

Our results indicate that the heart synthase phosphatase, unlike the class I isozymes (9), can not be converted to the M_r=35,000 form either by treating with 80% ethanol at 25° or by treating with 4 M urea (unpublished observations). Therefore, we concluded that the M_r=49,300 species is not a holoenzyme form of the M_r=35,000 species. In addition, the possibility that the synthase phosphatase may be a de-metallized form of the M_r=35,000 species has also been excluded. We have found that the de-metallized form of the M_r=35,000 enzyme can be activated by Mn^{2+} or Co^{2+} but not by Mg^{2+} (23). Thus, the M_r=49,300 and 26,000 enzymes represent a novel synthase phosphatase and divalent cations may play an important role in regulating their activity. Further studies

are required to clarify whether the M_r=26,000 species is a monomeric form of the M_r=49,300 enzyme.

Recent studies indicate that the skeletal muscle synthase may be regulated by two different kinases, namely cyclic AMP-dependent and -independent protein kinase, which phosphorylate different sites on the enzyme (24-29). The present findings show that both the metal ion-dependent synthase phosphatase and the general phosphatase of M_r=35,000 can dephosphorylate and activate synthase D. Whether these two classes of phosphatases have different specificities toward different sites on synthase D, which have been phosphorylated by different kinases, is currently under investigation.

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