Activation of a D-form of rabbit muscle glycogen synthase by Ca^{2+}-activated protease

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Glycogen synthase isolated from rabbit skeletal muscle as a D-form (synthase D_2) is activated by a rat liver cytosolic protein differing from any of the known protein phosphatases (D_2 activase). Although reversible by phosphorylation by cyclic AMP-dependent protein kinase, the activation is a result of limited proteolysis by D_2 activase, which has been identified as the Ca^{2+}-activated protease.

(Liver, Skeletal muscle) *Glycogen synthase* *Protein phosphatase* *Ca^{2+}-activated protease*

1. INTRODUCTION

Previously we characterized three types of protein phosphatase, IA, IB and II, from rat liver cytosol [1–5]. During the course of investigating their substrate specificity, we found that glycogen synthase isolated from rabbit skeletal muscle as a D-form (synthase D_2) was only a poor substrate for these phosphatases but was activated by a different protein present in rat liver cytosol (D_2 activase). In the present study, we identified D_2 activase as the Ca^{2+}-activated protease. Although the action of the protease on glycogen synthase may not be important physiologically, it will constitute a valuable tool for studying the structure-function relationship of synthase.

2. MATERIALS AND METHODS

All the preparative procedures described below were performed at 4°C. 1 U synthase (D or I) was defined as in [5]. Cyclic AMP-dependent protein kinase (the catalytic subunit) was purified from rabbit skeletal muscle as in [9] but up to the CM-Sepharose step. 1 U of the kinase was defined as the amount which catalyzed the incorporation of 1 nmol P_i into a histone mixture at 30°C in 1 min [10]. Protein was estimated as in [11].

2.1. *Preparation of labeled synthase D_1*

Purified rabbit skeletal muscle synthase I (4.8 U/ml) was incubated at 30°C with cyclic AMP-dependent protein kinase (10 U/ml), 0.05 mM [γ-^{32}P]ATP (9.6×10^7 cpm/μmol), 3 mM Mg acetate, 19 mM glycerol 2-P, 11 mM mercaptoethanol, 1 mM EDTA (pH 7.0), 0.1 mM EDTA and 0.19% glycogen. After 6 min, 0.25 vol. of 250 mM Tris-HCl (pH 7.5), 50 mM EDTA and 0.06 vol. of 0.8 M NaF were added, and synthase was precipitated with 10% polyethylene glycol. Labeled synthase D_1 thus obtained was dissolved in 50 mM Tris-HCl (pH 7.5), 15 mM mercaptoethanol, desalted by Biogel P-6, and stored. It had an activity ratio ($\pm$10 mM glucose 6-P) of 0.15.

The method described in [12] and modified by [13,14] was adopted to prepare synthase I used above but after the following modifications: extraction and acid precipitation were performed at pH 7.5 and 5.9, respectively; dephosphorylation was conducted with 5 mM $MnCl_2$ at pH 7.7; and the calmodulin-Sepharose step was omitted. After fractionation with polyethylene glycol, the enzyme

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was passed through Blue Sepharose equilibrated with buffer A (50 mM Tris-HCl, pH 7.5, 15 mM mercaptoethanol, 1 mM EDTA) containing 20% glycerol and precipitated with 10% polyethylene glycol in the presence of 0.2% glycogen. The final preparation had a specific activity of 14 U/mg and an activity ratio of 0.8.

2.2. *Preparation of synthase D_2*

The method employed was the same as that described for synthase I except that buffers for steps 1–2 contained 25 mM NaF and the dephosphorylation step was omitted. Synthase D_2 thus obtained was dissolved in buffer A, mixed with 2/3 its volume of 50 mM glycerol 2-P, 30 mM mercaptoethanol, 2 mM EDTA (pH 7.0), precipitated with 10% polyethylene glycol, dissolved in buffer A minus EDTA, desalted by Biogel P-6, and stored. The enzyme had a specific activity of 19 U/mg and an activity ratio of 0.1.

2.3. *Purification of D_2 activase*

Crude cytosolic protein phosphatase fraction prepared from 317 g rat liver by the method described in [5] was applied to a 4.2×10 cm DEAE-cellulose column equilibrated with buffer A containing 0.15 M NaCl and developed with a linear 0.15–0.35 M NaCl gradient in buffer A (500 ml) (step 1). After rechromatography on DEAE-cellulose (step 2), D_2 activase was applied to a 1.5×3.5 cm aminohexyl-Sepharose-4B column equilibrated with buffer A containing 40 mM NaCl. The column was washed with buffer A containing 0.15 M NaCl and developed with a linear 0.15–1.0 M NaCl gradient in buffer A (500 ml) (step 3). After rechromatography on aminohexyl-Sepharose-4B (step 4), the active fractions were pooled, dialyzed against buffer A and applied to a 2.5×3 cm Blue Sepharose-4B column equilibrated with buffer A containing 40 mM NaCl. The column was washed with buffer A containing 0.15 M NaCl and developed with buffer A containing 1.0 M NaCl (100 ml) (step 5). The active fractions were pooled, concentrated to 3 ml over a small DEAE-cellulose column and applied to a 2.5×80 cm Sephacryl S-300 column equilibrated with buffer A containing 40 mM NaCl; elution was made with the same buffer (step 6). The active fractions were pooled, concentrated and chromatographed on a second Sephacryl S-300 column (2.5×40 cm) (step 7).

2.4. *Assay of D_2 activase*

With synthase D_2 as substrate, D_2 activase was assayed as if it were a synthase phosphatase, measuring the formation of synthase I from D_2. The standard assay mixture contained 50 mM Tris-HCl (pH 7.3), 5 mM $MnCl_2$, 0.1% bovine serum albumin, 1 mM dithiothreitol, 0.2 mM glucose 6-P, 10% glycerol, 0.6 U/ml of synthase D_2 and D_2 activase. After 10 min at 30°C, 0.05 ml was used to determine synthase I [5]. When labeled synthase D_1 was the substrate, the formation of synthase I was assayed as above, or ^{32}P release was determined after incubation was terminated by addition of 0.5 vol. of 30% trichloroacetic acid. The acidic mixture was centrifuged, and the supernatant was either spotted on filter paper and counted, or its 100 μl was mixed with 25 μl 5% ammonium molybdate. In the latter case, the mixture was agitated with 200 μl isobutanol/benzene (1:1) and the organic phase was spotted on filter paper and counted. The proteolytic activity of D_2 activase was assayed as [6] using casein as substrate.

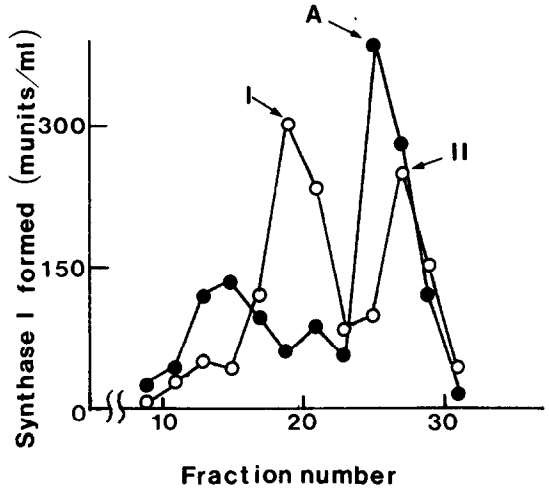

Fig.1. Activation of synthase D_2 by D_2 activase. Crude protein phosphatase fraction prepared from 180 ml of liver cytosol [5] was applied to a 2.5×15 cm DEAE-cellulose column equilibrated with 10 mM Tris-HCl (pH 7.5), 5 mM mercaptoethanol, 10% glycerol, 5 mM $MgCl_2$, 40 mM NaCl, and developed with a linear 40–500 mM NaCl gradient in the same buffer (400 ml). 10 ml fractions were collected and synthase activation was assayed with synthase D_1 (○) and D_2 (●) as substrate. I, II and A indicate protein phosphatase I and II and D_2 activase, respectively.

3. RESULTS

When rat liver cytosolic fraction was chromatographed on DEAE-cellulose, synthase D_1, muscle synthase D prepared by in vitro phosphorylation of synthase I, was activated by phosphatases I (IA plus IB) and II while a peak of protein eluting somewhat earlier than II was chiefly responsible for the activation of synthase D_2, synthase isolated from muscle as a D-form (fig.1). This protein was therefore designated 'D_2 activase'. As shown in fig.2, the activation of synthase D_2 by D_2 activase was readily and completely reversed by incubation with ATP, Mg^{2+} and cyclic AMP-dependent protein kinase. The three components were all essential for the reversal.

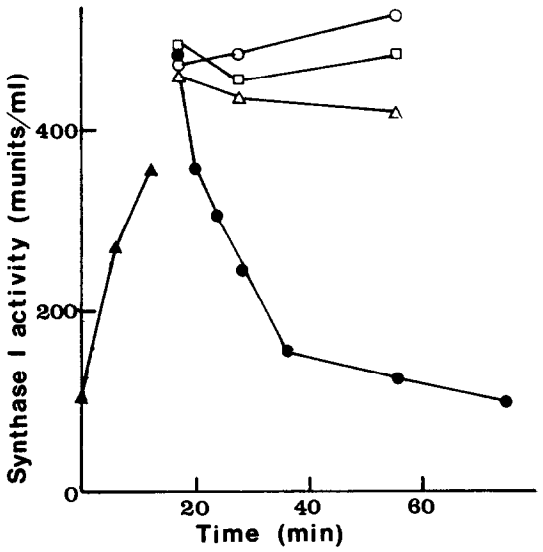

Fig.2. Reversal of D_2 activase-catalyzed synthase D_2 activation by phosphorylation. D_2 activase purified by DEAE-cellulose and Sephacryl S-300 was incubated with synthase D_2 (1.2 U/ml) at 30°C using the standard assay mixture but 0.2 mM $CaCl_2$ plus 0.5 mM $MnCl_2$ substituting 5 mM $MnCl_2$; at times indicated, aliquots were assayed for synthase I (▲). After 16 min, an equal volume of 17 mM glycerol 2-P, 0.66 mM EDTA, 0.1 mM EGTA, 10 mM mercaptoethanol, 0.2 mM ATP, 6 mM Mg acetate and 10 U/ml of cyclic AMP-dependent protein kinase catalytic subunit was added, and the incubation continued. At times indicated, aliquots were assayed for synthase I: ●, △, ○ and □ represent the complete system and the systems lacking ATP, Mg^{2+} and the kinase, respectively.

While these findings suggested D_2 activase to be a novel protein phosphatase, its failure to be inhibited by NaF (not shown) was against this view. To answer this question, we purified D_2 activase from rat liver cytosol. At each step of the purification, D_2 activase eluted as a single peak, where more than 50% of the applied activity could be recovered. Fig.3 shows the elution profile of step-7 enzyme. The enzyme was then run on nondenatured gel electrophoresis at pH 9.5, yielding a major protein band with R_f 0.47 (fig.3, inset a, arrow). On SDS gel electrophoresis, the same enzyme gave two major bands, M_r 67 000 and 26 000 (fig.3, inset b, arrows). D_2 activase must be a heterodimer since the gel filtration study (fig.3) gave M_r 90 000.

In attempts to characterize D_2 activase further, step-7 enzyme was incubated with labeled synthase D_1, leading to almost no activation of the synthase (not shown). When incubation was terminated by trichloroacetic acid, on the other hand, the acid supernatant was found to contain a considerable

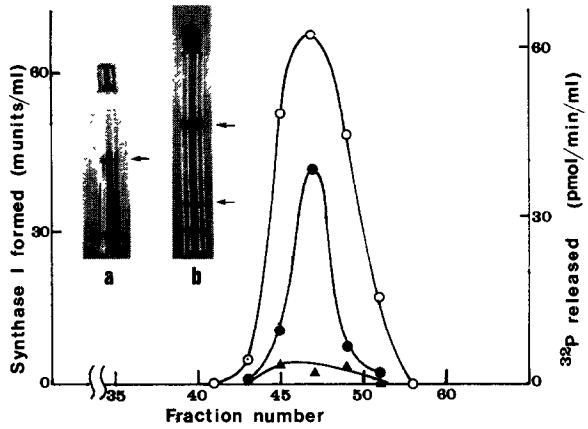

Fig.3. Purification of D_2 activase by the second Sephacryl S-300 chromatography. Step-6 enzyme was chromatographed as described in text (step 7). Fractions (2.7 ml) were collected and assayed for activation of synthase D_2 (○) or ^{32}P release from labeled synthase D_1. ^{32}P was measured in trichloroacetic acid supernatant (●) or after complex formation with ammonium molybdate (▲). The pooled active fractions (13.5 ml) were concentrated to 1 ml using an Amicon PM-10 membrane, and 0.14 ml aliquot was electrophoresed on 7% acrylamide gel at pH 9.5 using the buffer system of Davis [15] (inset a) and 0.1 ml aliquot according to Neville [16] (inset b). The gels were stained with 0.125% Coomassie blue.

amount of ^{32}P, more than 90% of which, however, failed to form a complex with ammonium molybdate and thereby was (a) phosphopeptide(s) rather than P_i (fig.3). From these, it followed that D_2 activase may be a protease.

As shown in table 1, D_2 activation by D_2 activase requires Mn^{2+} or Ca^{2+}, the most effective concentration being 5 and 0.2 mM, respectively. Mg^{2+} failed to substitute Mn^{2+}. The mechanism for the Mn^{2+} effect, however, may not be identical to that for the Ca^{2+} effect, since 5 mM Mn^{2+}, in comparison with 0.2 mM Ca^{2+}, caused more D_2 activation but less phosphopeptide release (table 1). The most probable explanation is that although Ca^{2+} stimulates D_2 activase more intensely, only Mn^{2+} is able to interact with the activated synthase molecule to increase its stability.

The results presented so far suggest that D_2 activase is a class of Ca^{2+}-activated protease widely distributed in tissues [6–8] including liver [17]. In fact, when D_2 activase was chromatographed on aminohexyl-Sepharose-4B, caseinolytic activity co-eluted with D_2-activating activity (fig.4). The caseinolysis here was Ca^{2+}- or Mn^{2+}-dependent, Ca^{2+} being more effective than Mn^{2+} (fig.4b) as in the case of ^{32}P release (table 1). Moreover, increasing concentrations of antipain (Protein Research Foundation, Osaka, Japan), a potent inhibitor of the Ca^{2+}-activated protease [6], inhibited both synthase D_2 activation and ^{32}P release from synthase D_1 in a progressive manner. In either case, half-maximal inhibition was attained at 3 μg/ml of the inhibitor.

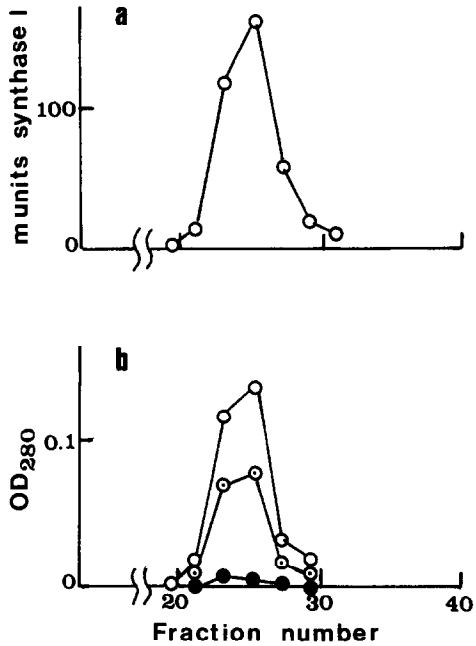

Fig.4. Co-purification of D_2 activase with Ca^{2+}-activated protease. D_2 activase purified by DEAE-cellulose was chromatographed on aminohexyl-Sepharose-4B as described in text. 10 ml fractions were collected and assayed for D_2 activation (a) and caseinolysis (b). The latter was performed with no metal (●), 1 mM $CaCl_2$ (○) or 5 mM $MnCl_2$ (⊙) present.

Table 1

Effects of Mn^{2+} and Ca^{2+} on activity of synthase D_2 activase[a] towards synthase D_2 and ^{32}P-labeled synthase D_1

Divalent cations added	Synthase I[b] from synthase D_2	^{32}P-peptide[c] from ^{32}P-labeled synthase D_1
None	0.010	0.007
150 μM Mn^{2+}	0.038	0.008
200 μM Ca^{2+}	0.074	0.460
150 μM Mn^{2+}, 200 M Ca^{2+}	0.250	0.510
5 mM Mn^{2+}	0.340	0.260

[a] Purified by DEAE-cellulose followed by aminohexyl-Sepharose-4B
[b] mU/min
[c] Equivalents to pmol phosphate/min

4. DISCUSSION

In the present paper, a rat liver cytosolic enzyme capable of converting synthase D_2 to an apparent I-form was described as D_2 activase. Although we at first thought D_2 activase to be a novel protein phosphatase (see fig.2), subsequent studies have made it clear that the enzyme is the protease requiring a millimolar level of Ca^{2+} for activity: its synthase D_2-activating activity requires Ca^{2+} (table 1), is co-purified with Ca^{2+}-activated caseinolytic activity (fig.4) and is effectively blocked by antipain. Although D_2 activase is also activated by Mn^{2+}, Mn^{2+} has been shown to substitute Ca^{2+} in the activation of the Ca^{2+}-activated protease [18,19]. In addition, D_2 activase is a heterodimer

like the Ca^{2+}-activated protease [6,17]. Values reported for the M_r of the Ca^{2+}-activated protease (100 000–120 000) [6], however, are somewhat greater than M_r 90 000 assigned for D_2 activase. The Ca^{2+}-activated protease from rat liver was shown to consist of two subunits, of M_r 80 000 and 28 000, respectively [17]. The reason for the lower M_r of D_2 activase remains unclear.

It therefore appears that limited proteolysis converts synthase D_2 to an apparent I-form, which is still capable of being phosphorylated by cyclic AMP-dependent protein kinase thus returning to a D-form. If this is just the case, however, the present paper contradicts a number of previous papers showing that proteolysis causes the conversion of synthase I to a D-form [20–23]. One exception is [24] which reports the activation of yeast synthase D upon trypsinization. Since synthase is a multisite phosphorylatable protein [25–27], various forms of synthase D are supposed to exist so that the activation by D_2 activase may be a phenomenon highly specific for synthase D_2. In support of this, we were unable to activate synthase D_1 by D_2 activase, although a considerable amount of phosphopeptides was liberated. Also noteworthy is our finding that trypsin, chymotrypsin and subtilisin all failed to activate synthase D_2 (not shown). These results suggest that the Ca^{2+}-activated protease and its action towards glycogen synthase will constitute a valuable tool for elucidating the structure-function relationship of glycogen synthase.

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