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Studies on Uridine Diphosphate Glucose:α-1,4-Glucan α-4-Glucosyltransferase. VIII. Catalysis of the Phosphorylation of Muscle Phosphorylase and Transferase by Separate Enzymes*

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ABSTRACT: Paper III of this series demonstrated the interconversion of the two forms of muscle uridine diphosphate glucose:α-1,4-glucan α-4-glucosyltransferase by a phosphorylation–dephosphorylation reaction sequence. These reactions were analogous to those leading to the interconversion of the two forms of glycogen phosphorylase. Both phosphorylase and transferase appear to be under the control of epinephrine and adenosine 3′,5′-phosphate. In both cases activation of the kinase reaction has been implicated as an intermediate reaction in the hormone effect. It was, therefore, of interest to determine whether a single kinase or separate kinases catalyzed the phosphorylation of the two glycogen metabolizing enzymes. Phosphorylase *b* kinase was purified from rabbit muscle, and each fraction was examined for both kinase activities. There was a progressive loss of transferase I kinase activity during purification. The ratio of activities phosphorylase *b* kinase/transferase I kinase increased 100-fold during purification.

Purified phosphorylase *b* kinase had little or no activity with transferase I as substrate. Activated phosphorylase *b* kinase, which was produced by incubation with adenosine triphosphate and adenosine 3′,5′-phosphate was also without significant affect upon transferase I.

Muscle uridine diphosphate glucose-α-glucan glucosyltransferase I kinase (transferase I kinase) catalyzes the conversion of transferase I (glucose-6-P independent form) to transferase D (dependent form) in a reaction, the stoichiometry of which is unknown (Friedman and Larner, 1963) (reaction I).[1,2]

$$\text{transferase I} + n\text{ATP} \rightarrow \text{transferase D } (PO_4^{2-})n + n\text{ADP} \quad \text{(I)}$$

Muscle glycogen phosphorylase *b* kinase catalyzes the conversion of phosphorylase *b* to *a* according to reaction II (Krebs *et al.*, 1958).

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[1] Abbreviations used are UDPG, uridine diphosphate glucose; glucose-6-P, glucose 6-phosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; transferase, UDPG-α-glucan glucosyltransferase, UDPG:α-1,4-glucan α-4-glucosyltransferase.

[2] I activity is measured in the absence of glucose-6-P. Total activity is determined in the presence of excess added glucose-6-P. D activity is estimated by subtracting I activity from total.

2 phosphorylase + 4ATP → 2 phosphorylase *b* + 4ADP (I)

phosphorylase $(PO_4^{2-})_4$ → 4 phosphorylase *a* (II)

The phosphorylated forms of both phosphorylase and transferase can be dephosphorylated by phosphatases.

The conversion of phosphorylase *b* to *a* is accelerated by epinephrine *via* its intermediary adenosine 3′,5′-phosphate (Sutherland and Rall, 1960). In muscle a mechanism for the effect of the hormone has been localized to the activation of phosphorylase *b* kinase (Krebs and Fischer, 1960; Krebs *et al.*, 1959). It is now clear that transferase is also under the influence of epinephrine. Studies have demonstrated that extracts prepared from rat diaphragms that had been incubated with epinephrine contain decreased transferase I (Craig and Larner, 1964) and total (Craig and Larner, 1964; Belocopitow, 1961) activities. The I form is more sensitive to low hormone concentrations than the D form (Craig and Larner, 1964). In partially purified transferase preparations of dog and rat, adenosine 3′,5′-phosphate increases the rate of the ATP-Mg^{2+} dependent conversion of the I form to the D (Rosell-Perez and Larner, 1964b; Appleman *et al.*, 1964).

It would appear, therefore, that reactions I and II are both subject to hormonal control by epinephrine. The over-all effect of epinephrine would be to stimulate the breakdown of glycogen *via* phosphorylase while simultaneously decreasing its synthesis *via* transferase. This dual control can be accounted for by three possible mechanisms: (a) a single enzyme may exist which catalyzes both kinase reactions; (b) there may be two discrete but similar kinases both subject to control by a common mechanism; or (c) there may be two kinases, only one of which is under hormonal control and has activity with both substrates. The finding of Larner and Sanger (1965) that the sequence of 5, and probably 6, amino acids surrounding the phosphorylation site of transferase is identical with that of phosphorylase is consistent with the possibility of a single kinase. On the other hand, these authors reported the phosphorylation of transferase with ^{32}P-labeled ATP under conditions in which there was no label incorporated into phosphorylase, suggesting separate kinases. However the presence of phosphorylase phosphatase could have produced the same result and was not ruled out.

The present study indicates that when phosphorylase *b* kinase is purified there is loss of transferase I kinase activity and that purified phosphorylase *b* kinase exerts little or no effect upon transferase.

Materials and Methods

Enzymes. Purified phosphorylase *b* kinase was kindly supplied by Dr. E. G. Krebs or was prepared by the method of Krebs *et al.* (1964). Phosphorylase *b* was crystallized by the method of Fischer and Krebs (1962). For use in the kinase assay, the crystalline suspension was centrifuged at 0° and the precipitate was dissolved in neutral 0.015 M cysteine to give a concentration of 20,000 units/ml.[3]

Transferase I activity from rabbit skeletal muscle was prepared with Dr. C. Villar-Palasi according to the procedure of Rosell-Perez and Larner (1964a) with several modifications. Frozen rabbit muscle (900 g), obtained from Pel-Freez Biologicals, Inc., was used instead of fresh rabbit muscle. Fresh rat muscle, 15% by weight, was mixed with the rabbit muscle as a source of transferase D phosphatase in order to ensure conversion of the enzyme to the I form. For the conversion of D to I, the crude 16,000 × *g* supernatant fluid (pH 6.9) was incubated for 1 hr at 30° in the presence of 0.05 M mercaptoethanol and 0.005 M EDTA. The enzyme was purified further by ammonium sulfate precipitation, differential centrifugation, and DEAE column chromatography as described by Rosell-Perez and Larner (1964a). The pooled 0.25 M NaCl fractions from the column were precipitated with ammonium sulfate at 70% of saturation, and the precipitate was dissolved in 5 ml of 0.05 M Tris-HCl, 0.005 M EDTA, pH 7.8. The solution was dialyzed for 4 hr against the same buffer, frozen in liquid nitrogen, and stored at −70°. The purified solution had a protein content of 25.8 mg/ml and 5.3 units[4]/ml of total transferase activity, of which 77% was in the I form as determined by measurement without glucose-6-P. The preparation contained 200 units of transferase I kinase activity/ml, an amount that caused a conversion of 14% of the I in 3 min under the conditions of the kinase assay. This preparation was used as substrate for assay of transferase I kinase activity in all but the earliest experiments with a sample of the kinase obtained in the frozen state from Dr. E. G. Krebs. In these experiments a partially purified preparation of transferase, carried up to the DEAE column step (Rosell-Perez and Larner, 1964a), was used, and the final concentration of transferase I in the assay was 0.06 unit/ml.

Analytical. Phosphorylase *b* kinase was assayed by the method of Krebs *et al.* (1964) except that the kinase reaction mixture contained 4000 units of phosphorylase *b* rather than 8000 units. Unless otherwise stated, the assay was run at pH 8.2. A unit of phosphorylase *b* kinase was as defined by Krebs *et al.* (1964), *i.e.*, that amount of kinase that gave rise to 100 units of phosphorylase *a*/ml of kinase reaction mixture in 5 min under the assay conditions. Assay of phosphorylase was by the method of Cori *et al.* (1943), modified as described by Krebs *et al.* (1964). Phosphate was estimated by the procedure of Lowry and Lopez (1946).

The method for assaying transferase I kinase activity was based upon measurements of the decrease in I activity in the presence of ATP and Mg^{2+}. All kinase assays were run in duplicate and the reaction mixtures contained 0.10 ml of 0.10 M Tris-HCl, containing 0.10 M mercaptoethanol (pH 8.2, unless otherwise indicated),

[3] One unit is calculated as described by Cori *et al.* (1939).

[4] One unit is defined as that amount of enzyme catalyzing the addition of 1 μmole of glucose from UDPG to glycogen in 1 min under the specified assay conditions.

TABLE I: Transferase I Kinase and Phosphorylase *b* Kinase Activities at Each Stage in the Purification of Phosphorylase Kinase.[a]

Fraction	Volume (ml)	Protein (mg/ml)	Phosphorylase *b* Kinase: Total Activity (units × 10^{-6})	Phosphorylase *b* Kinase: Specific Activity (units/mg)	Transferase I Kinase: Total Activity (units × 10^{-4})	Transferase I Kinase: Specific Activity (units/mg)	Phosphorylase *b* Kinase/Transferase I Kinase
Extract	900	20.7	6.2	333	13.0	7.0	47.6
Acid precipitate	150	7.6	8.5	7,500	2.4	21.0	357
Acid supernatant	850	17.6	0.4	25	7.5	5.3	4.7
30,000 × *g* supernatant	130	1.8	4.7	20,300	0.7	30.3	676
30,000 × *g* precipitate	35	23.8	2.6	3,150	0	0	
40,000 × *g* supernatant	125	1.2	2.1	13,750	0.06	4.0	3,437
40,000 × *g* precipitate	10	3.8	3.0	79,300	0.06	17.0	4,665

[a] Phosphorylase *b* kinase was purified by the method of Krebs *et al.* (1964). The assay procedures are described in Methods.

0.04 ml of purified transferase I (4.1 units/ml), and 0.10 ml of kinase, diluted appropriately with 0.015 M cysteine, pH 7.0. Controls without kinase were always included and contained 0.10 ml of 0.015 M cysteine, pH 7.0. The mixtures were incubated for 5 min at 30° before the kinase reaction was started by the addition of 0.06 ml of 0.025 M ATP, 0.05 M $MgCl_2$, or 0.06 ml H_2O. After 3-min incubation at 30°, 0.05-ml aliquots were removed and added to 0.10 ml of a reaction mixture for the assay of transferase I activity. The components of this reaction mixture and the subsequent steps in the transferase assay have been described previously (Friedman and Larner, 1963). Reaction mixtures did not contain glucose-6-P. The kinase activity was taken as the difference between the I activity of the tubes containing ATP–Mg^{2+} and that of the water controls. Corrections were made for kinase present in the transferase I preparation (27 kinase units/ml of reaction mixture). The amount of kinase used was such that not more than 35% of the transferase I activity was converted in the assay. There was a linear increase in the reaction rate (Figure 1) with increasing kinase concentrations up to about 60% conversion. Although an incubation period of 3 min was used for the assay, the kinase reaction rate was constant for 6 min. One unit of transferase I kinase activity was defined as the amount of enzyme catalyzing a decrease in $^1/_{1000}$ unit of transferase I/ml of kinase reaction mixture in 1 min under the stated conditions. Protein was estimated by the biuret method (Layne, 1957).

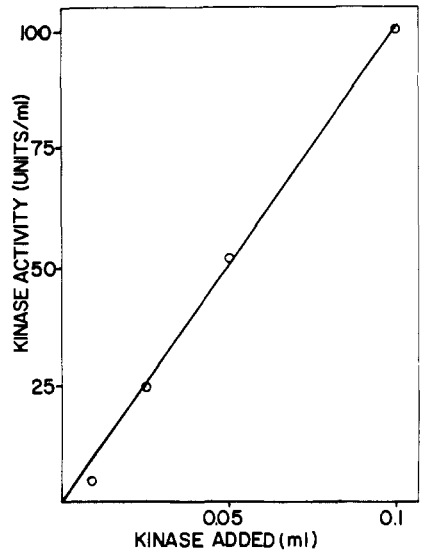

FIGURE 1: Transferase I kinase activity as a function of enzyme (kinase) added. The conditions of the assay are described in the text. The source of kinase for this experiment was a rabbit muscle preparation carried through the transferase purification procedure up to, but not including, DEAE-cellulose column chromatography. One pellet was suspended in 3 ml of 0.05 M mercaptoethanol, pH 7.8, and used as source of kinase.

Results

Purified phosphorylase *b* kinase containing 10^6 units/ml (19 mg of protein/ml) was received in the frozen state from Dr. E. G. Krebs. It was thawed immediately and tested on the partially purified preparation of transferase. It was found that 100 kinase units/ml caused no observable conversion of transferase I to D. One thousand units produced a small but definite conversion of 0.023 unit of transferase I to D, whereas 10,000 units produced complete conversion (0.06 unit) in 3 min. Control experiments ruled out the presence of

ATPase activity in the transferase preparation and the presence of transferase phosphatase in either the transferase or the kinase preparation. This experiment suggested that phosphorylase *b* kinase had low but definite activity in converting transferase I to D.

This question was studied further. Phosphorylase *b* kinase was purified and each fraction was analyzed for kinase activity with both phosphorylase *b* and transferase I as substrate (Table I). It was found that the two activities were partially separated by acid (pH 6.1–6.2) precipitation of the crude extract. Whereas over 100% of phosphorylase *b* kinase activity appeared to be recovered in the precipitate, only 22% of the transferase I kinase activity was present. The subsequent steps in the purification led to an increase in the ratio of phosphorylase *b* kinase to transferase I kinase activity. The ratio was 47.6 in the crude extract and 4665 in the purified preparation. The acid supernatant fraction was enriched in transferase I kinase activity and had a ratio of 4.7.

When varying dilutions of the most highly purified preparation (40,000 × *g* precipitate) were tested for transferase I kinase activity it was found that the small activity did not vary with change in concentration. The same low level of decrease in I activity (0.037 unit/ml) occurred with the undiluted kinase (100,000 phosphorylase kinase units/ml) or with dilutions of two-, four-, and eightfold. This result suggested that there was actually no transferase I kinase present in the purified preparation, but rather some other factor which acted in a nonstoichiometric fashion. One possibility is that this factor may alter the activity of the small amount of transferase I kinase present in the transferase I preparation itself.

In order to determine whether the active form of phosphorylase *b* kinase has any activity toward transferase I, the purified fraction (40,000 × *g* precipitate) was activated by incubation with ATP, Mg^{2+}, and adenosine 3′,5′-phosphate according to the procedure of Krebs *et al.* (1964). There was an increase in phosphorylase *b* kinase activity, as measured at pH 6.8, from 4500 units/ml to 55,000 units/ml. In contrast there was no significant increase in apparent transferase I kinase activity measured at pH 7.0.

Discussion

The results presented in this paper demonstrate that during purification of phosphorylase *b* kinase there is a progressive loss of transferase I kinase activity. This is best explained by the separation of two enzymes.

In the early experiments, the purified phosphorylase *b* kinase preparation catalyzed a definite low level of conversion of transferase I to D. It is possible to calculate the relative activity of the kinase toward the two substrates in terms of the rate of phosphorylation, using the established value of phosphate content of phosphorylase (Krebs *et al.*, 1958) and the preliminary values for transferase (Friedman and Larner, 1963). The ratio of phosphorylation in the early experiment was 10,000:1 in favor of phosphorylase. In the purified fraction of Table I the ratio was about 400,000:1. This low level of activity toward transferase was highly questionable because it did not vary with change in concentration of kinase. It appears unlikely, therefore, that phosphorylase *b* kinase has any significant activity toward transferase. The difference between the early and the later experiments may be explained by the slightly better purification obtained in the latter case. The specific activities of the two preparations were 52,000 and 79,300 units/mg, respectively.

The experiments of Trayser *et al.* (1962) indicate that phosphorylase *b* kinase is activated by phosphorylation with ATP and that under certain conditions adenosine 3′,5′-phosphate stimulates this reaction. It would now be of interest with regard to hormone mechanisms to determine whether the same mechanism acts on transferase I kinase.

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