The Conversion of Lobster Muscle Phosphorylase \(a\) to \(b\) and Phosphorylase \(b\) to \(a\)*

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Lobster muscle phosphorylase, in common with the phosphorylases of many other animal species, occurs in forms with different requirements for activity (1). Phosphorylase \(a\) is an active form; whereas phosphorylase \(b\) is active only in the presence of adenylic acid (AMP). It has been demonstrated with enzymes from mammalian species that phosphorylase may be converted from one form to another by reactions which are themselves enzyme catalyzed. One enzyme, from rabbit muscle (2) and dog heart muscle (3), catalyzes the conversion of phosphorylase \(a\) to phosphorylase \(b\) by removal of inorganic phosphate. This enzyme has been termed phosphorylase phosphatase (3). A second enzyme from dog (3) and rabbit (4) muscle catalyzes the conversion of phosphorylase \(b\) to phosphorylase \(a\). The latter reaction involves the transfer of phosphate from ATP to the phosphorylase molecule, and this enzyme has been named phosphorylase kinase (4) or phosphokinase (3).

The present paper will describe the extraction and partial purification of two analogous enzymes from lobster muscle which catalyze the conversion of lobster muscle phosphorylase \(a\) to \(b\); and the reverse reaction, that of lobster phosphorylase \(b\) to \(a\). The reactions catalyzed by these lobster enzymes have not been studied as thoroughly as those mentioned above for the mammalian enzymes; but, in view of the similarities that have been found for the lobster and mammalian enzymes, the names lobster phosphorylase kinase and lobster phosphorylase phosphatase will be used.

**EXPERIMENTAL**

**Methods**

ATP was obtained from Pabst Brewing Company as the chromatographically pure salt; other nucleotides were obtained from Nutritional Biochemicals Corporation. The sources and purity of other chemicals as well as the analytical procedures used were described in Paper I of this series (1). Lobster muscle phosphorylase \(a\) and phosphorylase \(b\) were prepared as the pure enzymes (1). Rabbit muscle phosphorylase \(a\) was prepared free of PR enzyme by the method of Cori et al. (5).

**Preparation of Lobster Phosphorylase Phosphatase**

The purification procedure for the rabbit muscle enzyme (PR enzyme) did not lead to good recoveries of the lobster muscle enzyme; therefore, the method described below was devised.

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**Step 1: Extraction of Enzyme**—The tail muscle of lobster (H. americanus) was removed and ground with 2 parts by weight of cold tap water* in a Waring Blender at high speed for 1 minute. All subsequent steps were conducted at 0-5° unless otherwise noted. The muscle slurry was stored for 30 minutes, and then centrifuged for 30 minutes at 7000 r.p.m. The supernatant extract was dialyzed overnight against tap water.

**Step 2: Ammonium Sulfate Fractionation**—For each 100 ml of dialyzed preparation, 20.6 g of (NH₄)₂SO₄ were added (to 0.35 saturated in ammonium sulfate). After 5 hours, the mixture was centrifuged for 30 minutes at 7000 r.p.m. and the precipitate was discarded. For each 100 ml of the supernatant fluid 5.1 g of (NH₄)₂SO₄ were added (to 0.45 saturated). After storage overnight, the mixture was centrifuged and the supernatant solution was discarded. The large precipitate was suspended in 0.005 M glycerol phosphate of pH 6.8 (\(\frac{1}{2}\) the volume of the original extract) and dialyzed overnight against the same buffer.

**Step 3: Selective Heat Denaturation**—The dialyzed solution from Step 2 was held for 10 minutes at 57° (water bath at 65°). The preparation was quickly cooled in an ice bath and centrifuged to remove a small amount of denatured material.

**Step 4: pH Precipitation**—The supernatant solution from Step 3 was adjusted to pH 5.5 (pH measured with a glass electrode pH meter) by addition of 1 M sodium acetate of pH 4.5. After 10 minutes at pH 5.5, a heavy precipitate was present, and the mixture was centrifuged for 5 minutes at 10,000 r.p.m. The supernatant fluid was discarded and the precipitate was quickly suspended in a small amount of 0.05 M imidazole of pH 7.0 (\(\frac{1}{2}\) the volume of the original extract).

**Step 5: Freeze-Thaw**—The solution from Step 4 was frozen at -15° and, upon subsequent thawing at 0°, a large, matlike precipitate formed. This precipitate was removed by centrifugation with little loss of enzyme (Table I). However, the enzyme at this stage was less stable to storage either frozen or at 0° than at Step 4.

**Preparation of Lobster Phosphorylase Kinase**

The tail muscle was removed from lobster† and ground for 1 minute with 2 parts of cold distilled water in a Waring Blender at high speed. All subsequent steps were conducted at 0-5°. The muscle slurry was centrifuged for 30 minutes at 7000 r.p.m.

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* Equally satisfactory sources of muscle were fresh lobsters; and the tails removed from lobsters, immediately frozen upon Dying, and stored frozen until used as much as several months later.

† Metal ions in tap water appeared to activate the enzyme, since dialysis against deionized (distilled) water led to preparations of lower specific activity at this step.
The supernatant extract was made 0.30 saturated in ammonium sulfate by the addition of saturated ammonium sulfate solution (0°) adjusted to pH 7 with ammonia. The mixture was stored overnight before centrifugation. Additional saturated ammonium sulfate solution was added to the supernatant solution to render it 0.35 saturated in ammonium sulfate. After storage overnight, the precipitate was packed by centrifugation and the supernatant fluid was discarded. A 60% loss of the kinase enzyme was suffered at this point in order to secure a preparation nearly free of phosphorylase, since the latter enzyme required a higher degree of saturation of ammonium sulfate for precipitation. The precipitate was suspended in 0.05 M glycerol phosphate of pH 6.8, and this was the preparation used for activation of phosphorylase b. One unit of enzyme was defined as that amount of enzyme capable of converting 1 unit of phosphorylase a to b per ml of reaction mixture per minute at 35°. The reaction mixture was composed of 0.1 ml of a solution containing 50 units of phosphorylase a, 0.1 ml of the phosphorylase phosphatase preparation, 0.1 ml of 0.05 M MnCl₂, and 0.7 ml of 0.05 M imidazole buffer of pH 6.5. Aliquots of the mixture, 0.1 ml, were routinely withdrawn for assay of phosphorylase a content (1) at 4, 5, and 15 minutes of incubation at 35°.

Assay of Phosphorylase Phosphatase

Activity was measured by the rate of conversion of purified lobster muscle phosphorylase a to phosphorylase b. One unit of phosphatase activity was defined as that amount of enzyme capable of converting 1 unit of phosphorylase a to b per ml of reaction mixture per minute at 35°. The reaction mixture was composed of 0.1 ml of a solution containing 50 units of phosphorylase a, 0.1 ml of the phosphorylase phosphatase preparation, 0.1 ml of 0.05 M MnCl₂, and 0.7 ml of 0.05 M imidazole buffer of pH 6.5. Aliquots of the mixture, 0.1 ml, were routinely withdrawn for assay of phosphorylase a content (1) at 4, 5, and 15 minutes of incubation at 35°.

Assay of Phosphorylase Kinase

Activity was measured by the rate of conversion of purified lobster muscle phosphorylase b to phosphorylase a; 1 unit of activity was defined as that amount of enzyme capable of converting 1 unit of phosphorylase b to a per ml of reaction mixture per minute at 35°. The reaction mixture was composed of 0.1 ml of a solution which contained 100 units of lobster phosphorylase b (1), 0.1 ml of the kinase preparation which had been pre-incubated with 0.015 M cysteine at pH 7.5 for 15 minutes, 0.1 ml of 0.05 M ATP, 0.1 ml of 0.05 M MnCl₂, 0.1 ml of 1.0 M NaF, and 0.5 ml of 0.05 M glycerol phosphate buffer of pH 7.5. The mixture was incubated at 35°, and 0.1-ml aliquots were withdrawn routinely at 4, 5, and 15 minutes for assay of phosphorylase a content (1). The dilution of the kinase and the high concentration of glucose 1-phosphate incident to the phosphorylase assay were sufficient to stop further action of the kinase at this point.

Results and Discussion

Lobster Phosphorylase Phosphatase—The purification of the phosphatase was marked by a 4-fold increase in total activity (Table I) and this increase in activity contributed to the apparent increase in specific activity of 200-fold during the purification. The enzyme preparation after Step 5 was free of phosphorylase, phosphorylase kinase, AMP deaminase, and any phosphatase capable of acting upon AMP or glucose 1-phosphate. Phosphorylase phosphatase catalyzed the complete conversion of phosphorylase a to phosphorylase b (Fig. 1), and optimal activity in 0.05 M imidazole buffer was at pH 0.5. The enzyme was inhibited by glycerol phosphate, ammonium sulfate, EDTA, sodium fluoride, and AMP (Table II and Fig. 2). The phosphatase was not inhibited by glycogen and only slightly by 5 × 10⁻⁵ M pyridoxal phosphate.

The phosphatase of lobster was not activated by cysteine; however, it was activated by Mn⁺⁺, Mg⁺⁺, Co⁺⁺, Pb⁺⁺, or Zn⁺⁺. For example, a purified preparation of phosphatase that had been dialyzed against 0.005 M EDTA was active, but the activity was increased by 0.005 M Mn⁺⁺, slightly inhibited by 0.01 M EDTA, and completely inhibited by 0.1 M sodium fluoride (Fig. 2).

In a number of ways the lobster phosphorylase phosphatase resembles the corresponding enzyme from rabbit muscle. Both enzymes convert an active form of phosphorylase to a form that requires AMP for activity (phosphorylase b); both have their activity increased by a variety of divalent cations; and both are inhibited by the same types of compounds (sodium fluoride, EDTA, phosphates, and sulfates). Furthermore, the lobster phosphatase was able to catalyze the conversion of rabbit muscle phosphorylase a to b (Fig. 3), and the rabbit phosphatase catalyzed the conversion of lobster muscle phosphorylase a to b (1).

Lobster Phosphorylase Kinase—A 7-fold increase in specific activity of the kinase was produced by the ammonium sulfate fraction and dialysis might account for the increased activity noted after the first two steps.
INCUBATION TIME (MINS.)

Fig. 1. Action of phosphorylase phosphatase on phosphorylase a. Purified lobster phosphorylase a, 50 units, and 9 units of phosphatase were incubated together at 35° in 2 ml of 0.05 M imidazole buffer at pH 6.5. Incubation mixture was assayed (1) for phosphorylase a (O--O) and phosphorylase b (+-+-+).

Fig. 2. Activators and inhibitors of phosphorylase phosphatase. (a) phosphatase incubated at 35° with lobster phosphorylase a in 0.02 M imidazole at pH 6.5; (b) as for (a) but in the presence of 0.01 M ethylenediaminetetraacetic acid; (c) as for (a) but in the presence of 0.1 M NaF; (d) as for (a) but in the presence of 0.005 M MnCl₂. Assays were for phosphorylase a.

Fig. 3. Action of lobster phosphorylase phosphatase on rabbit muscle phosphorylase a. Rabbit phosphorylase a (free of PR), 50 units, and 10 units of lobster phosphorylase phosphatase were incubated together at 35° in 1 ml of 0.05 M imidazole buffer at pH 6.5. Incubation mixture was assayed for phosphorylase a (O--O) and phosphorylase b (+-+-+) by the procedure of Cori et al. (6).

Fig. 4. Dependence of kinase activity upon ATP and Mn++. Purified lobster phosphorylase b, 140 units, was incubated at 35° with 2.4 units of kinase in 0.005 M cysteine and 0.02 M glycerol phosphate at pH 7.5. Total volume was 1.0 ml. Phosphorylase b content was assayed after various intervals of incubation. (a) incubation mixture supplemented with MnCl₂ to 0.065 M at 5 minutes (no ATP); (b) incubation mixture supplemented with ATP to 0.605 M at 5 minutes (no Mn++); (c) incubation mixture supplemented with both ATP and MnCl₂ to 0.005 M at 5 minutes. 0.005 M activated the enzyme to 30% of the activity attained in the presence of the same concentration of Mn++. An experiment was designed to eliminate the possibility that the conversion of phosphorylase b to a depicted in Fig. 4 might be merely an activation of phosphorylase b by ATP or its possible degradation product, AMP. In this experiment, 1450 units of purified phosphorylase b were incubated with phosphorylase kinase, Mn++, and ATP. After 90 minutes, the phosphorylase assay indicated that 71% of the phosphorylase b had been converted to phosphorylase a. The phosphorylase of the reaction
mixture was precipitated with ammonium sulfate (to 0.5 saturated). The precipitated phosphorylase was dissolved in 0.05 M glycerol phosphate of pH 6.8 and dialyzed extensively against the latter buffer. (This treatment would remove any ATP or AMP.) Upon assay for phosphorylase, it was found that the enzyme was still 72% phosphorylase a and the recovery of phosphorylase was 1000 units or 70% of the 1450 units of phosphorylase b used in the experiment.

Earlier observations (7) indicated that rabbit muscle phosphorylase b was converted to phosphorylase a upon incubation with crude lobster muscle extracts. Subsequent tests with the partially purified lobster phosphorylase kinase described in this paper did not show any capacity of this enzyme to convert purified rabbit phosphorylase b to a. The rabbit phosphorylase b for these latter tests had been freed of traces of rabbit phosphorylase kinase by the method of Krebs and Fischer (8). It is quite possible that rabbit phosphorylase b used in the earlier experiments (7) contained small amounts of rabbit phosphorylase kinase which could account for the observed formation of phosphorylase a.

SUMMARY

An enzyme was extracted and extensively purified from lobster muscle, which catalyzed the conversion of lobster muscle phosphorylase a to b. It was activated by such metal ions as Mn++ or Mg++ and was completely inhibited by 0.1 M sodium fluoride. This lobster enzyme also could convert rabbit muscle phosphorylase a to b.

A second enzyme from lobster muscle was capable of converting lobster muscle phosphorylase b to a. This enzyme required cysteine, Mn++ and adenosine triphosphate for activity.

REFERENCES

The Conversion of Lobster Muscle Phosphorylase $a$ to $b$ and Phosphorylase $b$ to $a$

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