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MOLECULAR HETEROGENEITY OF RABBIT HEART PHOSPHORYLASE KINASE

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Summary

Phosphorylase kinase (ATP: phosphorylase-*b* phosphotransferase, EC 2.7.1.38) from rabbit heart, when submitted to electrophoresis on Pevikon, separates into two discrete peaks A and B. The two peaks have been analyzed using re-electrophoresis, chromatography on DEAE-cellulose, thermal stability, inactivation by EGTA (ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid) and reaction with an anti-muscle phosphorylase kinase antiserum. It can be concluded that rabbit heart extracts contain two isozymes of phosphorylase kinase. The more negatively charged isozyme seems to be identical with the muscle enzyme. The other isozyme resembles the liver enzyme but differs from the major fraction of the latter by its charge. It is likely that there exist at least three molecular types of phosphorylase kinase.

Introduction

In a previous paper [1] we have shown that mammalian phosphorylase kinase (ATP: phosphorylase-*b* phosphotransferase, EC 2.7.1.38) exists in several molecular forms. Two types could be defined, called "muscle" and "liver" types by analogy to phosphorylase. This conclusion was based on genetic, immunologic and physico-chemical evidence.

The properties of phosphorylase kinase from heart are intermediary between these two types.

In the strain of mice ICR/IAN, which carries a genetic deficiency in muscle phosphorylase kinase, the activity of the heart enzyme is about half normal [1,2]. In addition, the heart phosphorylase kinase is only partially inactivated by an antimuscle phosphorylase kinase antiserum [1]. We made the hypothesis

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that the heart contains both the muscle type and another type of phosphorylase kinase. In the present work we made an attempt to investigate the possible heterogeneity of the heart enzyme, using techniques based on the charge of the protein, combined with immunological methods. We have been able to separate two forms of heart phosphorylase kinase which differ in several properties.

Material and Methods

Tissue extracts. Hearts from freshly killed rabbits were used. Frozen tissue gave inconsistent results. Minced heart tissue was extracted in a Potter-Elvehjem apparatus with the following solutions:

Glycerophosphate buffer (50 mM sodium glycerophosphate, 2 mM EDTA, 1 mM dithiothreitol pH 7.8) for chromatographic separation on DEAE-cellulose.

TEB buffer (50 mM Tris/6 mM EDTA/ 20 mM boric acid, pH 7.8) for electrophoretic analysis.

The homogenate was centrifuged for 10 min at 20 000 × *g* and the supernatant was used for chromatography or electrophoresis.

Enzyme assays and methods of separation. Phosphorylase kinase activity was assayed as previously described [1] at pH 8.2 with or without added calcium. The preparation and assay of the antiserum against muscle phosphorylase kinase, and the chromatography on DEAE-cellulose, have been described [1].

Electrophoresis. Several attempts were made to characterize the enzymes directly on cellulose acetate and polyacrylamide gels by specific staining. The results were not conclusive. We then tried an electrophoretic support which allows quantitative elution of the enzyme prior to its determination. Pevikon [3] allows both migration of big molecules like phosphorylase kinase and complete elution.

380 g of Pevikon were suspended in 500 ml of TEB buffer. The Pevikon was allowed to settle and the supernatant buffer was decanted. This operation was performed twice. The Pevikon thus washed was mixed with 100 ml of the same buffer and layered on a plexiglass tray (25 × 27 cm).

After partial drying three slots (0.3 × 3 cm) were cut 7 cm from the cathodic side. The samples were mixed with a small amount of Pevikon and were poured into the slots. Migration was effected at 4°C under 20 mA for 4 h or overnight under 5 mA using TEB as electrophoresis buffer. After migration the Pevikon was cut from each sample departure point in 1 × 3-cm portions which were eluted with 0.3 ml of water and 1 mM mercaptoethanol.

Phosphorylase kinase activity was then determined unless stated otherwise at pH 8.2 in the presence of $CaCl_2$.

Results

(1) Electrophoresis of rabbit tissue extracts on Pevikon (Fig. 1). Upon electrophoresis heart extracts showed two activity peaks of phosphorylase kinase (peaks A and B). In most experiments the activity of peaks A and B was nearly equal, peak B being generally narrower and higher than peak A. Under the same conditions a muscle extract displayed only one peak of activity at the position

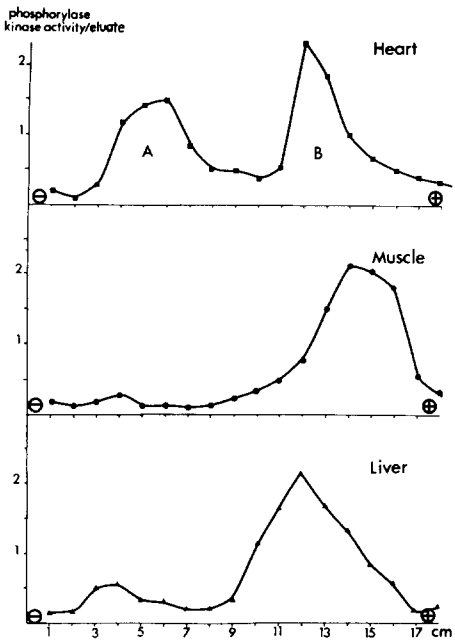

Fig. 1. Electrophoresis of rabbit tissue extracts on Pevikon. Heart: ■———■; muscle: ●———●; liver: ▲———▲. Each sample contains 20—40 phosphorylase kinase units expressed in term of units phosphorylase *a* formed.

of cardiac peak B. A liver extract showed two activity peaks: the first one of low activity migrated like cardiac peak A, and a second containing most of the activity migrated like cardiac peak B or slightly less towards the anode.

(2) Re-electrophoresis on Pevikon. Fig. 2 shows that each eluate gave only one single activity peak, the migration of which was the same as in the first run. A mixture of peaks A and B restored the result obtained with the initial heart

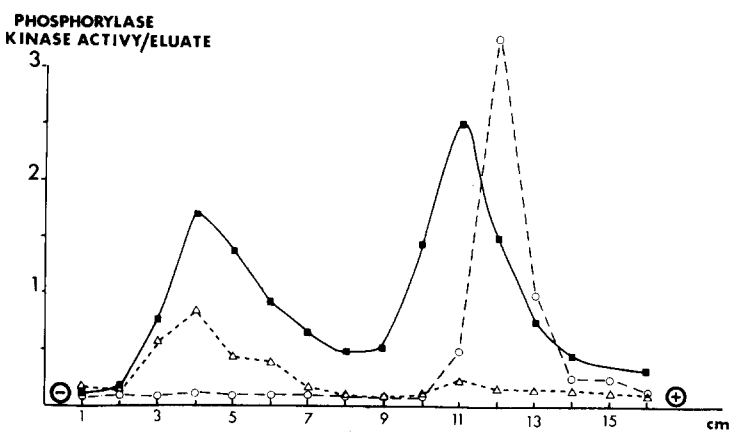

Fig. 2. Peaks obtained after Pevikon electrophoresis were concentrated separately and submitted to a second electrophoresis. Total heart extract: ■———■; peak A: △- - - - -△; peak B: ○- - - - -○.

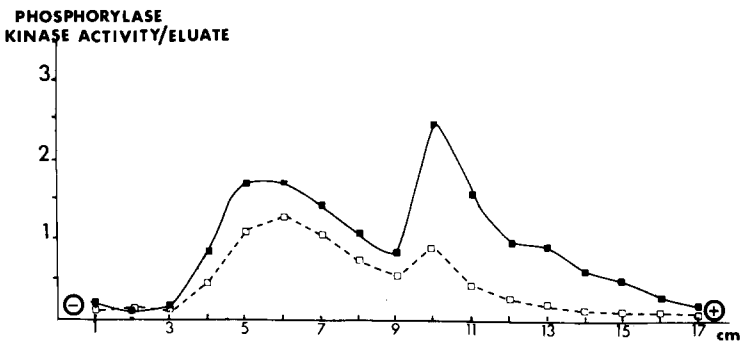

Fig. 3. Electrophoresis of heart extract before and after 15-min heating at 45°C. Heart extract before heating: ■———■; heart extract after heating: □- - - - -□.

extract. These results showed the reproducibility of the separation and eliminated the possibility of an interconversion equilibrium between the two forms.

(3) Thermostability of peaks A and B (Fig. 3). The heart extract was kept at 45°C for 15 min and then electrophoresed. The loss of activity was greater for peak B (65% loss) than for peak A (33% loss). Peak B seems to be more thermolabile than peak A.

(4) Existence of active and inactive forms (Fig. 4). As shown by Krebs et al.

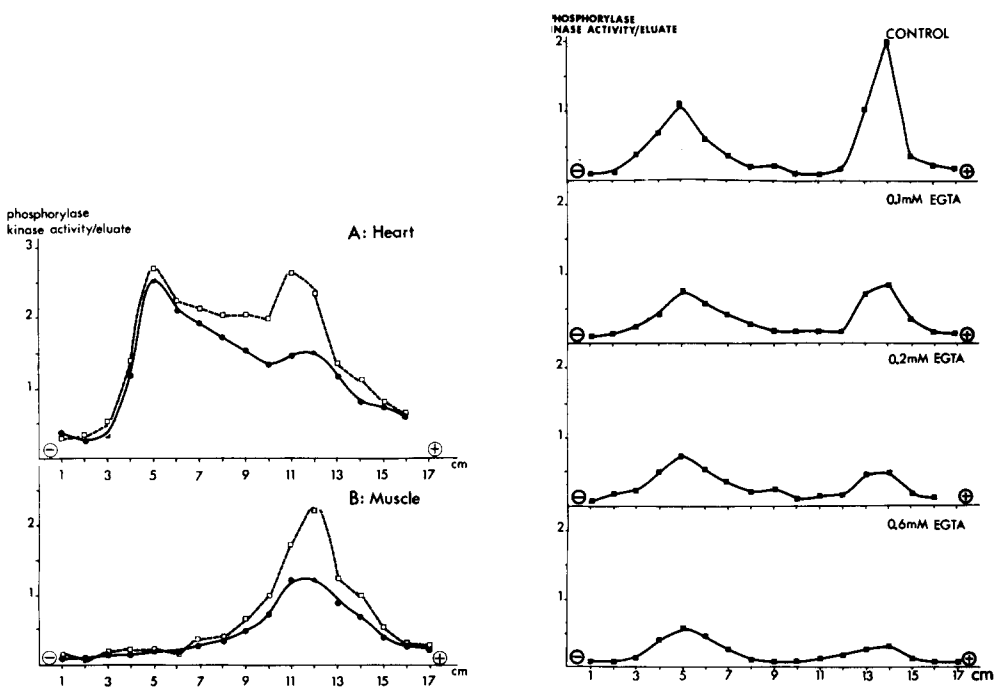

Fig. 4. A: comparative activity of heart peaks at pH 6.8 and 8.2: (without added Ca^{2+} in the assay mixture). B: comparative activity of muscle peak at pH 6.8 and 8.2. pH 6.8: ●———●; pH 8.2: □- - - - -□.

Fig. 5. Effect of increasing concentrations of EGTA on the two heart activity peaks. The control is effected in presence of added $CaCl_2$.

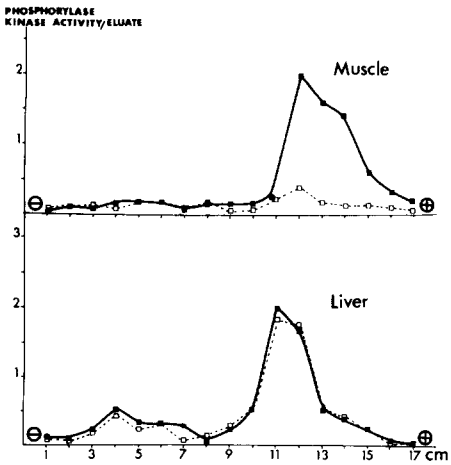

Fig. 6. Effect of EGTA, 0.2, mM on the muscle and liver peaks obtained on Pevikon. ■———■, presence of $CaCl_2$ (0.15 mM) in the assay mixture; □- - - - -□; presence of EGTA (0.2 mM) in the assay mixture.

[4] muscle phosphorylase kinase exists in an active and an inactive form. Only the active form is phosporylated. The non-phosphorylated form shows little activity at pH 6.8 but is active at pH 8.2.

Thus the activation of the enzyme can be monitored by following the increase of the activity at pH 6.8 compared to that at pH 8.2.

The two peaks observed in the heart extract could correspond to active or inactive forms of phosphorylase kinase. To test this hypothesis we submitted the heart extract to electrophoresis, then the eluates were assayed at pH 6.8 and at pH 8.2 in a 0.1 M sodium glycerophosphate, 0.1 M Tris buffer without added calcium chloride.

The results are shown in Fig. 4. Peak A was only slightly more active at pH 8.2 than at pH 6.8. By contrast peak B which was little active at pH 6.8 was strongly activated at pH 8.2. The same experiment made on a muscle extract showed a strong activation at pH 8.2; muscle phosphorylase kinase is known to be in its inactive form in crude extracts [4,5]. Cardiac peak A, therefore, seemed to be in an active form while peak B, like the muscle enzyme, was in an inactive form in crude extracts.

(5) Calcium activation and EGTA (ethyleneglycol-bis(β-aminoethyl ether)-N, N'-tetraacetic acid) inactivation (Figs. 5 and 6). Phosphorylase kinase requires calcium ions for optimal activity. This has been shown for the enzyme from rabbit skeletal muscle [6—9] cardiac muscle [8], guinea pig brain [10], human platelets [11] and rat liver [12]. Since the requirement for calcium is in the micromolar range, it is necessary to add EGTA to abolish all Ca^{2+} activity. In addition Khoo and Steinberg [12] have reported than this requirement is absolute for the muscle enzyme, which is completely inhibited by EGTA, but not for the liver enzyme which keeps at least 25—40% of its activity in the absence of Ca^{2+}.

Fig. 5 shows that peak B was markedly more inhibited than peak A when we increased the EGTA concentration from 0.1 mM to 0.6 mM in the assay mix-

ture. In the same conditions at 0.1 mM EGTA (Fig. 6) muscle activity was strongly inhibited, by contrast liver enzyme was little inactivated.

(6) Immunologic study of the cardiac peaks. We have tested each cardiac peak against an antimuscle phosphorylase kinase serum. The conditions used were those described in ref. 1 which gave us a 70% inhibition of phosphorylase kinase activity of heart extract.

Under these conditions 15% of peak A activity was inhibited while 90% of the peak B activity was inhibited.

If the heart extracts were preincubated before migration with normal or antimuscle phosphorylase kinase serum (Fig. 7) peak B activity disappeared when the antiserum concentration went up. By contrast peak A was not at all inhibited.

(7) Comparison of the results given by electrophoresis and chromatography on DEAE-cellulose. A heart extract was chromatographed on a DEAE-cellulose column (45 × 2 cm) equilibrated in 50 mM sodium glycerophosphate/2 mM EDTA/1 mM dithiothreitol buffer, pH 7.8, and eluted with a 0—0.5 M NaCl gradient; two elution peaks of phosphorylase kinase were obtained (Fig. 8A).

Each activity peak was concentrated separately and the concentrates were electrophoresed on Pevikon. Fig. 8B, C show that the first peak from the

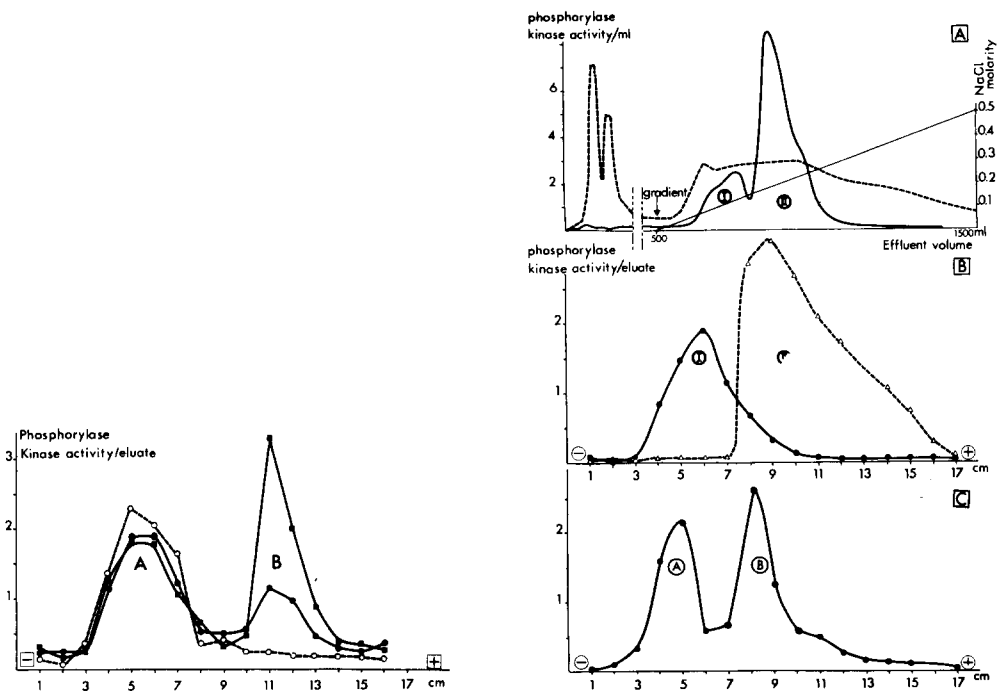

Fig. 7. Effect of increasing concentrations of antimuscle phosphorylase kinase serum when incubated before migration with a heart extract. Heart extract was incubated with antiserum or normal serum before migration for 3 h at ambient temperature and centrifuged 15 min at 20 000 × *g*. ■———■, control incubated with normal serum; ●———●, antiserum dilution 1/16; ○- - - - -○, antiserum dilution 1/8.

Fig. 8. Comparison of the results given by electrophoresis and chromatography on DEAE-cellulose. A, Chromatography of heart extract on DEAE-cellulose. Two elution peaks, I and II, of phosphorylase kinase were obtained. B, migration of peaks I and II upon Pevikon electrophoresis compared to control migration (C) of a total heart extract.

column migrated at the position of peak A, while the second peak migrated at the position of peak B. Separation of the two forms, therefore, is identical with electrophoretic and chromatographic techniques.

(8) Is cardiac peak A a proteolytic activated form of phosphorylase kinase? Some properties of cardiac peak A are similar to those of "activated phosphorylase kinase": a limited proteolytic attack of muscle phosphorylase kinase by trypsin is known to give an altered phosphorylase kinase which is catalytically active [4, 14]; its ratio of activity at pH 6.8/8.2 approaches unity [15—17].

To verify that cardiac peak A is not the product of a proteolytic attack occurring during the extraction, we have made control experiments in which the tissues were extracted in the presence of diisopropylfluorophosphate (10^{-3} M) and EGTA (10^{-3} M) to inhibit an eventual Ca^{2+}-dependent phosphorylase kinase activator [14]: the results (not shown) were identical in the presence or absence of inhibitors.

These results made unlikely the possibility of an artifactual degradation of inactive cardiac phosphorylase kinase; nethertheless cardiac peak A could correspond to an in vivo proteolytic degradation of cardiac peak B which is very probably identical with the muscle enzyme.

Purified muscle phosphorylase kinase was submitted to a limited tryptic proteolysis as described by Hayakawa et al. [18] and then to Pevikon electrophoresis. A crude muscle extract, purified muscle phosphorylase kinase, and purified muscle phosphorylase kinase activated by trypsin gave the same electrophoretic picture (not shown), showing only one peak at the position of cardiac peak B.

It is therefore unlikely that peak A corresponds to a proteolytic activated form of phosphorylase kinase.

Discussion

Our experiments show that the phosphorylase kinase activity of rabbit heart extracts can be separated into two discrete peaks upon Pevikon electrophoresis. The separation can also be obtained by chromatography on DEAE-cellulose. The peak first eluted from the column corresponds to the less negatively charged electrophoretic peak A, and the second chromatographic peak to the more negatively charged electrophoretic peak B. No interconversion has been found between these two peaks; electrophoresis of each of the separated fractions gives only one activity peak. Analogous results have been found with mouse heart extracts (unpublished results).

The two forms differ from each other in numerous properties: (a) Peak A has only slightly greater activity at pH 8.2 than at pH 6.8: this active kinase could be a phosphorylated form or a proteolytic activated phosphorylase kinase. This last hypotheses seems to be very unlikely since the addition of diisopropylfluorophosphate and EGTA to the extraction medium did not alter the electrophoretic pattern. By contrast, peak B is much more active at pH 8.2 than at ph 6.8 and is probably non-phosphorylated. The results could in theory be due to a single partially phosphorylated protein. This, however, is unlikely

since one would expect the phosphorylated form to carry greater negative charge, which is not the case.

(b) Peak A is more thermostable than peak B.

(c) Peaks A and B differ in their Ca^{2+} dependence; fraction B is calcium-dependent (strongly inhibited by EGTA) while fraction A is much less calcium-dependent.

(d) The sensitivity to an antiserum raised against muscle phosphorylase kinase is different: fraction B is much more inhibited by the antiserum than fraction A.

These results provide strong evidence that peak B obtained after Pevikon electrophoresis and the muscle enzyme are the same enzyme. Identical migration, presence in crude extracts in inactive form, calcium dependence, and sensitivity to antimuscle enzyme antiserum, all suggest that these are identical species.

By contrast, peak A shows properties in common with the liver enzyme: activity at pH 6.8 in crude extracts [14]; preservation of a residual activity in the absence of calcium ions [12]; relative insensitivity to antimuscle phosphorylase kinase antiserum [1]. The electrophoretic mobility, however, is very different. Only a minor fraction of liver extract activity migrates at the position of peak A. The major fraction migrates at a more anodic position.

In conclusion, from the data presently available to us, the existence of at least three isozymes of phosphorylase kinase in rabbit tissues is likely: a muscle type, which is also present in the heart, a liver type, and a heart type, the properties of which are more similar to those of liver type than to those of muscle type. These results raise the problem of the specificity of one or two of the three subunits of phosphorylase kinase in each tissue.

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