DIFFERENTIATION OF TWO CATALYTIC SITES ON PHOSPHORYLASE KINASE FOR PHOSPHORYLASE *b* AND TROPONIN T PHOSPHORYLATION

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1. Introduction

Troponin which regulates the actomyosin ATPase (reviewed [1]) has been shown to exist in phosphorylated and non-phosphorylated forms. The subunit, T, which mediates the binding of the holotroponin complex to tropomyosin can be preferentially phosphorylated by the Ca^{2+}-dependent phosphorylase kinase (EC 2.7.1.38) [2–4] and only to a very small extent by the cAMP-dependent kinase [2,5].

Phosphorylase kinase, a large oligomer of the composition $\alpha_4\beta_4\gamma_4$ [6,7] or $\alpha'_4\beta_4\gamma_4$ [8,9] mol. wt 1.25×10^6), phosphorylates the troponin subunit, T, ca. 300 times slower than phosphorylase *b* [3]. This could be due to a lower substrate specificity of phosphorylase kinase for the T subunit or to a contamination of this enzyme with another protein kinase as proposed [10]. Alternatively, phosphorylase kinase may be a double or multiheaded enzyme which is composed of several kinases.

It will be shown here that phosphorylase kinase and troponin T kinase activity are enriched together. By eluting the enzyme from a DEAE column the activity ratio is not constant which might be correlated with a small change in the ratio of the subunits $(\alpha + \alpha') : \beta : \gamma$. Antibodies against phosphorylase kinase inhibit both, the troponin and phosphorylase kinase activity. However, the inhibition pattern differs with both substrates. In addition troponin does not inhibit the conversion of phosphorylase *b* to *a*. It is concluded that troponin and phosphorylase are phosphorylated by two different catalytic centers. These may be situated on the same holoenzyme.

2. Materials and methods

Phosphorylase kinase from rabbit skeletal muscle was prepared as in [6], modified [11]. Phosphorylase *b* was crystallized as in [12] and holotroponin (TI_2C) purified as in [13], modified [14].

Antibodies against phosphorylase kinase were prepared as in [15]. Phosphorylase kinase activity with the substrate phosphorylase *b* was determined on a Technicon auto-analyzer as in [16]. Activity with 4.3 mg troponin/ml was measured as the incorporation of ^{32}P into the substrate with the filter paper method [17]. One unit kinase activity represents the transfer of 1 nmol phosphate to the protein substrate/min. Protein was determined on an auto-analyzer as in [18]. Polyacrylamide gel electrophoresis in the presence of SDS was performed as in [8,19]. Kinetic measurements in fig.2, 3 and 4 were fitted to a hyperbola according to [20] on a Telefunken TR 440 computer and then plotted double reciprocally.

3. Results

Table 1 shortly summarizes the purification of phosphorylase kinase and troponin T kinase for which the procedures in [6,11] were employed. Both activities are enriched ca. 75-fold from a crude extract up to the homogeneous enzyme (see below). In all fractions the phosphorylase kinase activity at pH 8.2 is 400–800-fold higher than the troponin T kinase activity (table 1). The discarded fractions show the same activity ratio in each step (not shown).

Table 1
Purification of phosphorylase *b* kinase and troponin T kinase

Fraction	Purification (*x*-fold)		Activity ratio U/mg (Phos. *b* kinase) / U/mg (Troponin T kinase)
	Phos. *b* kinase	Troponin T kinase	
Crude extract	1	1	787
Acid precipitation	1.1	2.5	574
30 S	7.5	14.8	397
$(NH_4)_2SO_4$ precipitation	58	40	540
Gel filtration on Sepharose 4B	65	61	375
DEAE cellulose chromatography	75	76.5	791

The fractions were tested with both substrates at pH 8.2. The purification procedure [6,11] was scaled up to allow the preparation from 4 kg rabbit muscle. The yields of phosphorylase *b* kinase and troponin T kinase were 9.5% and 9.3%, respectively

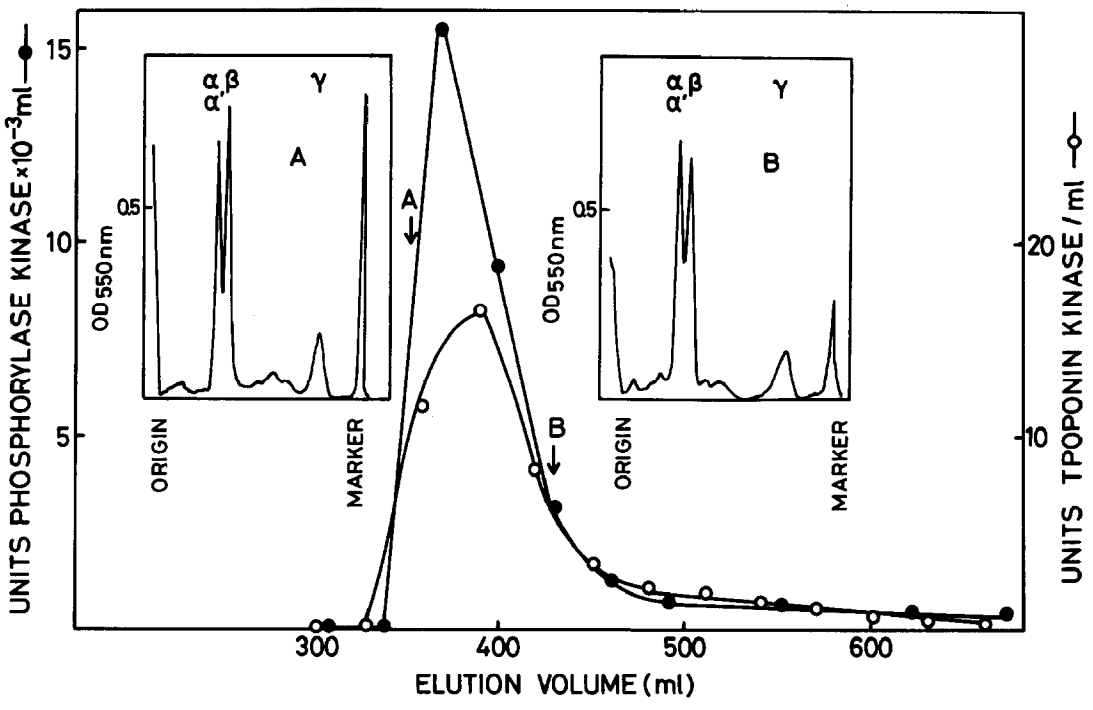

Fig.1. Elution profile of phosphorylase kinase and troponin kinase from a DEAE-cellulose column. The fraction (520 ml) obtained from the Sepharose 4B gel filtration step (see table 1) was applied to a DEAE-cellulose column (5 cm i.d. × 25 cm). The enzyme was eluted with a linear NaCl gradient from 0–500 mM (total vol. 1200 ml) in 50 mM Na^+-β-glycerophosphate/ 2 mM EDTA/1 mM 1,4-dithioerythritol (pH 7.0). Flow rate ~ 130 ml/h. Fractions were collected and tested for kinase activities at pH 8.2 with troponin and phosphorylase *b* as substrates (see section 2). Samples (A, B) were removed, denatured in SDS and separated by polyacrylamide–SDS gel electrophoresis. The densitometric traces of these gels are shown in the insets A and B.

Both the phosphorylase and the troponin T kinase activity are eluted from a Sepharose 4B column with the same V_e/V_0 ratio 1.47. Identical ionic strength elutes both kinase activities from DEAE-cellulose (fig.1). The molar ratio of the phosphorylase kinase subunits $(\alpha + \alpha') : \beta : \gamma$ varies to some extent in the different fractions. The insets in fig.1 show that in the ascending part the ratio of $(\alpha + \alpha') : \beta : \gamma$ = 0.8 : 1.0 : 1.1 (A) and in the descending part is 1.0 : 1.0 : 1.0 (B). In the ascending part of the curve in fig.1 the ratio of the phosphorylase *b* to troponin T kinase activity is ca. 2-fold higher than in the descending part.

The progress curves for the troponin T phosphorylation are linear at pH 6.8 and 8.2 (insets fig.2). Kinetic analysis shows that the V_{max} at pH 8.2 (10.8 U/mg) is 2.5-fold higher than at pH 6.8 (4.4 U/mg) whereas the K_m value shows no significant difference (pH 8.2, 35.6 μM; pH 6.8, 24.7 μM) (fig.2).

The addition of anti-phosphorylase kinase apparently increases the K_m value for troponin ca. 3-fold (61.3 μM without and 181 μM with antibody) and influences very weakly the V_{max} (14 U/mg without and 22.4 U/mg with antibody) (fig.3). In contrast the same antibody decreases the K_m value for phosphorylase *b* ca. 2-fold (166 μM without and 70 μM with antibody) as well as the V_{max} ca. 3-fold

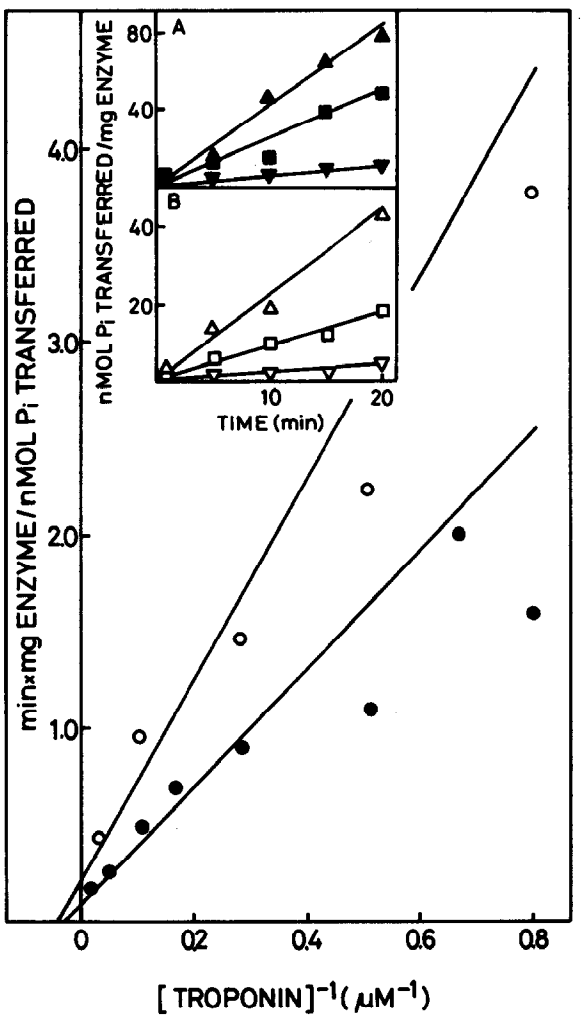

Fig.2. Double reciprocal plot of the initial velocity versus troponin concentration at pH 8.2 (•) and pH 6.8 (○). The enzyme concentration was 40 μg/ml at pH 8.2 and 144 μg/ml at pH 6.8, respectively. The insets show the progress curves for troponin phosphorylation at pH 8.2 (A) and pH 6.8 (B). Troponin concentrations were: 18.7 μM (▲), 9.35 μM (■) and 1.25 μM (▼) (A); 23.7 μM (△), 9.35 μM (□) and 1.25 μM (▽) (B).

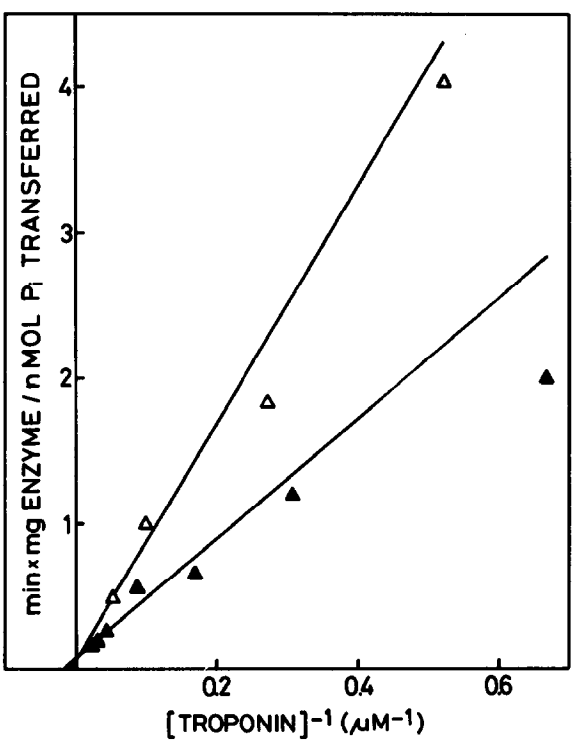

Fig.3. Inhibition of troponin phosphorylation by anti-phosphorylase kinase at pH 8.2. Double reciprocal plots of the initial velocities versus troponin concentration are shown without (▲) or with (△) 5 mg anti-phosphorylase kinase/ml. The enzyme concentration was 40 μg/ml.

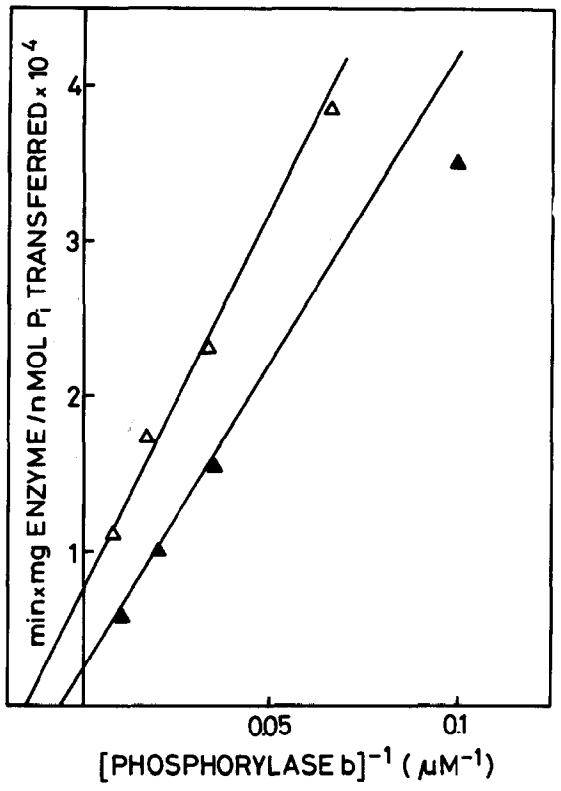

Fig. 4. Inhibition of phosphorylase *b* phosphorylation by anti-phosphorylase kinase at pH 8.2. Double reciprocal plots of the initial velocities versus phosphorylase *b* concentration are shown without (▲) or with (△) 0.01 mg anti-phosphorylase kinase/ml. The enzyme concentration was 0.08 μg/ml.

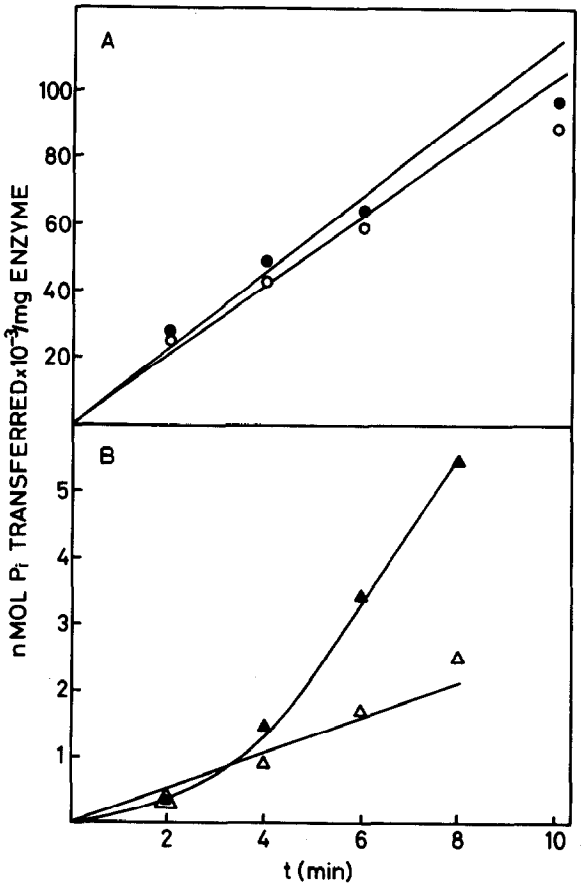

Fig.5. Phosphorylase *b* to *a* conversion in the presence of troponin at pH 8.2 (A) and pH 6.8 (B). Open symbols (○, △) show the phosphorylase *b* to *a* conversion without troponin at a substrate concentration of 4 mg phosphorylase *b*/ml, closed symbols (●, ▲) in presence of 2.2 mg troponin/ml. The enzyme concentration in A was 0.06 μg/ml and in B 3.8 μg/ml.

(41 700 U/mg without and 13 500 U/mg with antibody) (fig.4).

Figure 5 shows that phosphorylase *b* to *a* conversion is not inhibited but rather accelerated at pH 6.8 by troponin; at pH 8.2 neither inhibition nor activation is observed.

4. Discussion

Purification of phosphorylase kinase with the described procedure yields an essentially homogeneous protein containing both isoenzymes in a *w/r* ratio of ca. 10/1 [6,8,9]. The parallel purification of the phosphorylase *b* and troponin, T, phosphorylating activity, indicates that there is only one enzyme which catalyzes both reactions (see table 1). In addition the elution of both activities with identical elution volumes from a Sepharose 4B column and the desorption with an identical salt concentration from DEAE cellulose leads to the conclusion that the same holoenzyme carries both these activities (see fig.1). If troponin kinase would be a contaminating activity of phosphorylase kinase this former activity cannot be separated by these methods. A contamination with the cAMP dependent protein kinase cannot be responsible for the troponin phosphorylating activity described here. The presence of ca. 10 μM free Ca^{2+} in all tests inhibits the cAMP-dependent phosphorylation of troponin [14].

However, a ca. 2-fold change in the ratio of both activities in the elution profile from the DEAE-cellulose column suggests that these two activities are separable. This separation might be correlated with a very small shift in the subunit composition of the protein. As shown in fig.1 (insets) the molar ratio of the subunits $(\alpha + \alpha') : \beta : \gamma$ is not constant in all fractions. Either the shift of the subunit composition or of the isoenzyme ratio could be responsible for the change in the ratio of both kinase activities. An exchange of the subunit $(\alpha + \alpha')$ and β versus γ must occur in the cell, since it was shown that synthesis and degradation of the $(\alpha + \alpha')$ and β subunits is 1.6-fold faster than that of the γ-subunit [21].

Kinetic experiments with variable troponin concentrations show that the pH 6.8/8.2 activity ratio is ca. 0.4 (cf. fig.2) which is 8-fold higher than that of 0.05 observed under standard test conditions for phosphorylase *b* phosphorylation [22].

Antibodies against phosphorylase kinase inhibit both the phosphorylase *b* and troponin, T, phosphorylation. Comparison of fig.3 and 4 shows that the inhibition pattern of phosphorylase *b* phosphorylation resembles an uncompetitive whereas troponin, T, phosphorylation a competitive type of inhibition. The substrates are probably not the cause for this observed difference but rather the presence of different antibodies in the γ-globulin fraction. In agreement with this result it was shown that anti-phosphorylase kinase contains antibodies against each subunit α, β and γ [15]. Therefore, antibodies which might bind to different sites on the protein could cause the variance in the inhibition pattern; it therefore allows the conclusion that these two substrates are phosphorylated by two catalytic sites. The existence of two catalytic sites on phosphorylase kinase was also suggested by the Ca^{2+} activation of this enzyme which in presence of high concentrations of Mg^{2+} occurs in two steps [23]. If it is assumed that both substrates are phosphorylated by the same catalytic center competitive inhibition of phosphorylase *b* phosphorylation by troponin is expected since at pH 8.2 the K_m value for troponin is ca. 3-fold lower than for phosphorylase *b* (fig.3 and 4). In presence of 40 μM phosphorylase *b*, 24 μM troponin should inhibit the initial phosphorylase *b* to *a* conversion rate 35% as can be calculated if the K_m for troponin is used as a K_i value. The experiment in fig.5 shows no inhibition of phosphorylase *b* to *a* conversion by troponin at pH 8.2. In comparison, at pH 6.8 the troponin addition accelerates the phosphorylase *b* to *a* conversion which might be explained by an acceleration of the autocatalytic phosphorylation as discussed for other peptides [24]. Nevertheless it shows that the troponin phosphorylation does not interfere with phosphorylase phosphorylation which yields the strongest evidence for the nonidentity of the catalytic centers.

Acknowledgements

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