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The kinetic effects of in vitro phosphorylation of rabbit muscle enolase by protein kinase C

A possible new kind of enzyme regulation

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Rabbit muscle enolase was found to be phosphorylated in vitro by calcium-activated phospholipid-dependent protein kinase. The extent of incorporation (about 0.6 mol/mol subunit) and the apparent K_m for the reaction (3.0 μM subunit) were determined. Kinetic studies on the unphosphorylated and phosphorylated enzymes displayed an unexpected effect of phosphorylation resulting in activation of the forward reaction and inhibition of the backward one.

Enolase; Phosphorylation; Protein kinase C; Enzyme regulation; (Rabbit muscle)

1. INTRODUCTION

Enolase (EC 4.2.1.11) catalyses the dehydration of D-glycerate 2-phosphate to give phospho*enol*pyruvate. In literature on the regulation of metabolic pathways, enolase is generally considered not to be regulated to a very high extent [1–4]. The reasons for this are several.

For any single enzyme to have a regulatory significance for a whole pathway, it is necessary that this enzyme imposes a restriction on metabolism, that it constitutes a bottleneck in this particular pathway. This property can be tested for, by comparing the apparent equilibrium constant for the reaction with the actual mass-action ratio in the cell [5]. Performing this for enolase in mammalian tissues shows that the two figures are close, and thus the enolase reaction is close to equilibrium [1]. A reaction of a pathway being allowed to reach equilibrium is an indication that the relative amount of enzyme catalysing this reaction is higher than those for the preceding and following reactions. Thus, according to that test, the enolase reaction would not be considered as a rate-limiting step.

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The fact that enolase is a bidirectional enzyme could also be used as an argument against its being a regulated enzyme, since any regulation would be expected to affect both forward and backward reactions, thus favoring neither glycolysis nor gluconeogenesis [3].

Although the isolated enzyme has been studied for many years and a substantial body of information on its molecular and kinetic properties has been collected [6,7], no sign of an allosteric regulation has yet been found. As to covalent modification, enolase and two other glycolytic enzymes have been shown to be phosphorylated at tyrosine residues in Rous sarcoma virus (RSV)-transformed cells [8,9]. However, phosphorylation is slow, far from stoichiometric (about 5%) and kinetic effects do not seem very coherent; no change in K_m but a slight decrease in V_{max} accompanied by a 3–5-fold

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increase in the amount of cytosol enolase protein [9]. This does not exclude a regulatory role for this phosphorylation, e.g. by affecting partitioning of the enzyme or association to other proteins.

We here present the finding that rabbit muscle enolase is phosphorylated in vitro by calcium-activated phospholipid-dependent protein kinase (protein kinase C). We also report that this phosphorylation affects the kinetics of the enzyme in opposite ways for the forward and backward reactions, respectively. Such a phenomenon has hitherto not been reported for any enzyme.

2. MATERIALS AND METHODS

Crystalline rabbit skeletal muscle enolase from both Sigma and Boehringer Mannheim was used. The purity of the enzyme was confirmed by the absence of any contaminant bands on polyacrylamide gel electrophoresis. The enzyme was dialysed against 50 mM Mes-NaOH buffer, pH 6.0, containing 0.2 mM EDTA and 0.1 mM dithiothreitol. In calculations of molar concentrations, a subunit M_r of 41000 was assumed [4].

1,2-Diolein and L-α-phosphatidyl-L-serine from Sigma were treated as in [10].

Calcium-activated phospholipid-dependent protein kinase was purified according to Parker et al. [11]. One unit of protein kinase transferred one pmol [^{32}P]phosphate per min from 0.1 mM ATP into histone H1 (Sigma type III-S, 1.0 mg/ml) under the conditions specified below.

[^{32}P]ATP was purchased from NEN.

The phosphorylation experiments were conducted at 30°C and pH 6.0 in 75 μl incubation mixtures, unless otherwise specified, containing Mes-NaOH (43 mM), EDTA (0.053 mM), EGTA (0.067 mM), magnesium acetate (5 mM), calcium acetate (0.67 mM), [^{32}P]ATP (0.1 mM, 50 cpm/pmol), phosphatidylserine (60 μg/ml), diolein (1.1 μg/ml) and substrates as indicated.

The reaction was interrupted by the application of 50 μl of the mixture to a small Whatman 3MM paper rectangle, which was washed and dried as in [10]. The incorporated radioactivity was then measured as Čerenkov radiation. In all experiments a parallel series of tests without enolase was run in order to determine endogenous incorporation into protein kinase. The incorporation figures presented are thus net values.

Enolase concentrations were calculated using an absorbancy coefficient of 0.9 at 280 nm for 1 mg enolase per ml solution [12].

Enolase activity was measured with the direct assay suggested by Warburg and Christian [6], observing the appearance or disappearance of phospho*enol*pyruvate as the change in A_{240}. The assay was performed at 25°C, pH 6.8, and the 1 ml mixture contained 49.5 mM imidazole-HCl, 0.5 mM Mes-NaOH, 400 mM KCl, 6.0 mM $MgCl_2$ and substrates as indicated.

3. RESULTS

Rabbit muscle enolase was found to be phosphorylated by protein kinase C. The maximal extent of phosphorylation was about 0.6 mol per

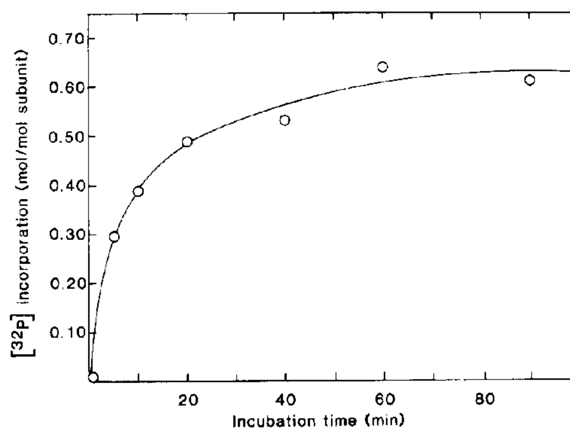

Fig.1. Time course of phosphorylation of rabbit muscle enolase (160 μg/ml) by protein kinase C (1650 U/ml). Conditions as described in section 2.

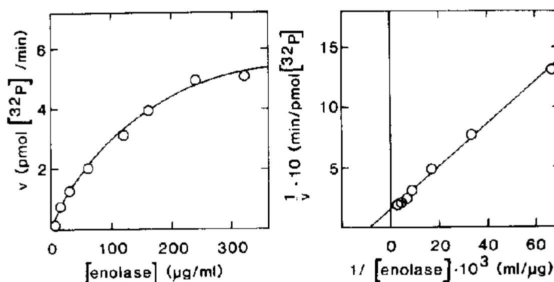

Fig.2. K_m determination for phosphorylation of rabbit muscle enolase by protein kinase C. (Left) Direct substrate saturation plot. (Right) Double-reciprocal plot of the same data. General conditions as described in section 2; kinase activity was 1320 U/ml and incubation time 10 min.

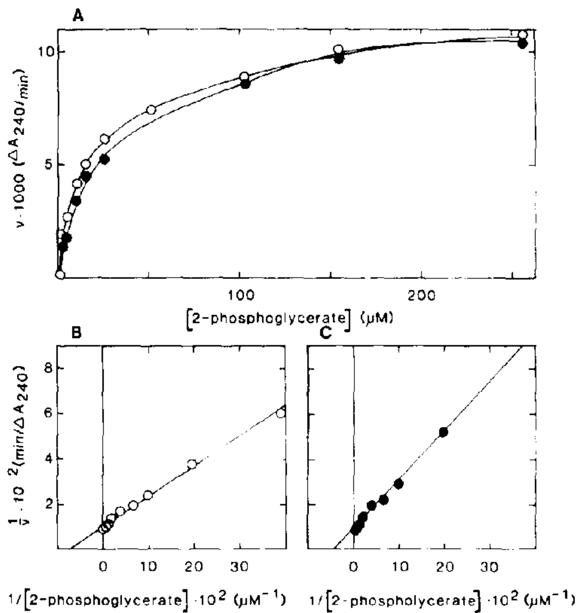

Fig.3. K_m determination for enolase and phosphoenolase catalysing the forward (glycolytic) reaction. (A) Direct substrate saturation plots. (B) Double-reciprocal plot for phosphoenolase. (C) The same for the unphosphorylated enzyme. (○) Phosphorylated enzyme; (●) unphosphorylated enzyme.

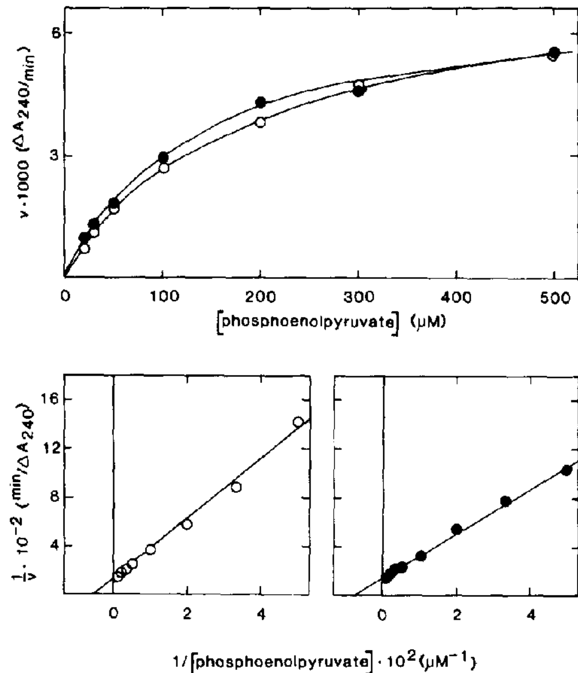

Fig.4. K_m determination for enolase and phosphoenolase catalysing the backward (gluconeogenetic) reaction. (A) Direct substrate saturation plots. (B) Double-reciprocal plot for phosphoenolase. (C) The same for the unphosphorylated enzyme. (○) Phosphorylated enzyme; (●) unphosphorylated enzyme.

mol subunit (fig.1). In the absence of Ca^{2+} and lipids no significant incorporation was obtained.

The apparent K_m for the phosphorylation reaction was found to be about 120 μg/ml (3.0 μM) (fig.2).

Kinetics were studied for both the forward (D-glycerate 2-phosphate as substrate) and backward (phospho*enol*pyruvate) reactions. For the forward reaction, a lowering of the apparent K_m upon phosphorylation was observed; a value of 16 μM for the maximally phosphorylated enolase rather than the 27 μM for the unphosphorylated enzyme (fig.3). In contrast, the backward reaction attained a greater K_m (164 vs 107 μM) as a result of phosphorylation (fig.4).

4. DISCUSSION

The experimental results seem to increase the possibility of enolase being a regulatory enzyme. The calcium-activated phospholipid-dependent protein kinase occurs in various tissues, including skeletal and heart muscle [13,14]. The K_m value observed for the phosphorylation reaction (3.0 μM subunit) is somewhat higher than those found for the hitherto most effective substrates for this protein kinase, i.e. histone H1, myelin basic protein and troponin T (0.13–0.3 μM) [15,16]. Still, the value observed is clearly below the physiological concentration of enolase in muscle of about 50–100 M (calculated from [12,17]). These facts indicate that the phosphorylation might also take place in the living tissue. Yet, in vivo studies still remain to establish a physiological significance. It will also be of great interest to investigate enolase from other tissues as well as the isolated isoenzymes as substrates for protein kinase C.

The kind of kinetic effect observed seems interesting. The reversal of metabolic flow by simultaneous activation of the forward reaction and inhibition of the backward reaction is not a rare phenomenon, a classical example being the regulation of glycogen synthesis and degradation.

It now seems possible that the same principle might apply when both reaction directions are catalysed by a single enzyme, as in the case of the enolase reaction. No such case has been reported earlier, and no bidirectional enzyme whatsoever has been found to be regulated through phosphorylation. However, the observed effects of enolase phosphorylation indicate that this kind of regulation is possible and that the same kind of mechanism might be involved in the regulation of other enzymes.

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