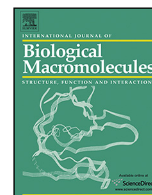
International Journal of Biological Macromolecules 60 (2013) 219–225

Contents lists available at SciVerse ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac



Effects of activating cations and inhibitor on the allosteric regulation of rabbit muscle pyruvate kinase

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ARTICLE INFO

Article history:
Received 16 March 2013
Received in revised form 23 May 2013
Accepted 29 May 2013
Available online 5 June 2013

Keywords:
Pyruvate kinase
Thermodynamics
Allosteric regulation

ABSTRACT

Rabbit muscle pyruvate kinase (RMPK) requires activating cations for activity, but its activity can be allosterically inhibited. In this study, isothermal titration calorimetry (ITC) was used to examine the different effects of activating cations (K^+ or Mg^{2+}) and inhibitor (Phe) on the allosteric regulation of RMPK. The ITC data reveal that the enthalpy change was greater for PEP binding to the active state compared to the inactive state. Meanwhile, the percentage of the active state increased with increasing concentration of K^+ or Mg^{2+}, whereas increasing Phe concentration had the opposite effect. In addition, we hypothesize that the activation of RMPK involves two processes. First, the interaction of Mg^{2+} leads to a more exposed active site of RMPK. The process is rapid and only a small quantity of Mg^{2+} can make RMPK transform to the intermediate state. Second, the subsequent binding of K^+ causes a critical orientation of the active site, which plays a more decisive role for PEP binding to RMPK than Mg^{2+}.

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1. Introduction

Rabbit muscle pyruvate kinase (RMPK) is an important glycolytic regulatory enzyme that catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate in the presence of monovalent and divalent cations [1–3]. RMPK is a homo-tetramer consisting of four chemically identical subunits. The four subunits form two interfaces which are approximately perpendicular; namely, the Y-interface comprising the C and N-terminal domains of monomer pairs 1–2 and 3–4, and the Z-interface comprising all four domains between monomer pairs 1–3 and 2–4 [4,5]. Each folding into four domains: N-terminal domain, domain A, domain B, and domain C. The active site, as well as the residues forming the activating cations and PEP binding sites, is located in the cleft between domains A and B of the same subunit [6,7]. The Phe-binding site, positioned in a deep pocket between domains A and C, is some distance away from the active site [8,9]. The binding of ligands to the active site results in either an "open" or "closed" conformation [4,10], representing the inactive state and active state, respectively [11]. Previous studies found that the allosteric regulatory mechanism of RMPK can be described by the two-state Monod–Wyman–Changeux (MWC) model, which proposed a rapid equilibrium between the active state and inactive state [12,13]. Based on the concerted two-state model, the binding of ligands to the active or inactive state induces conformational changes in the molecule and affects the equilibrium between the active state and inactive state [14]. The ability of effectors (activators and inhibitors) to regulate the enzymatic activity of RMPK is related to their ability to influence the active state ↔ inactive state equilibrium [12].

Various techniques have been used to investigate the effects of effectors on the allosteric regulation of PK [8,15–27]. Fluorescence acrylamide quenching analysis revealed that an interaction with K^+ and Mg^{2+} increases the exposure of the active site [16], resulting in an increased association constant of PEP for RMPK [17]. In contrast, the binding of Phe shifts the Y-interface of the PK molecule to a looser structure [28], reducing the affinity of PK for PEP [17,26]. However, the allosteric regulation of PK by the concentrations of activating cations and inhibitor has remained elusive. In the present study, isothermal titration calorimetry (ITC) was used to probe the different effects of activating cations (K^+ or Mg^{2+}) and inhibitor (Phe) on the allosteric regulation of RMPK.

2. Materials and methods

2.1. Materials

Disodium salt of ADP, tricyclohexylammonium salt of PEP, Phe, NADH, and LDH type II were purchased from Sigma. Tris was obtained from Amresco. KCl and $MgSO_4$ were purchased from Sinopharm Chemical Reagent Co., Ltd.

Abbreviations: PK, pyruvate kinase; ITC, isothermal titration calorimetry; NADH, β-Nicotinamide adenine dinucleotide reduced disodium salt hydrate; LDH type II, L-Lactic Dehydrogenase from rabbit muscle; ADP, adenosine diphosphate; PEP, phosphoenolpyruvate; Phe, phenylalanine.

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http://dx.doi.org/10.1016/j.ijbiomac.2013.05.028

2.2. Protein purification and preparation

Plasmid pET22b containing the wild-type RMPK gene was kindly provided by Dr. George H. Reed at the University of Wisconsin (Madison, WI). Wild-type RMPK was purified from IPTG-induced *E. coli* strain BL21 according to published procedures [29]. The protein purity was assessed by staining SDS-PAGE gels with Coomassie Blue. The PK solution was dialyzed against 50 mM Tris buffer (pH 7.5) overnight to remove glycerol and other additives with three buffer changes before use. The concentration of the PK solution was determined spectrophotometrically by using the extinction coefficient of 0.54 mL/(mg cm) at 280 nm [26]. The molecular weight of RMPK (tetramer) is 237,000 Da [4].

2.3. Enzymatic activity assay

A coupled assay procedure with lactate dehydrogenase in TMK buffer (50 mM Tris, 100 mM KCl, 10 mM $MgSO_4$, pH 7.5) containing 0.3 mM NADH and 3.6×10^{-4} mM LDH was used to determine the enzymatic activity of RMPK [30].

The concentrations of ADP and PEP in the 1.6 mL reaction mixture were both 2 mM. The reaction was initiated by the addition of 10 μL RMPK (0.25×10^{-4} mM) and the final concentration of RMPK was 1.5×10^{-6} mM. In the experiments designed to determine the effects of activating cations on the activity of RMPK with ion concentration as the variable factor, the K^+ concentration-dependent mixture contained 10 mM Mg^{2+} and 0–100 mM K^+ (0, 1, 2, 4, 8, 25, 50, 75, and100 mM), and the Mg^{2+} concentration-dependent mixture contained 100 mM K^+ and 0–10 mM Mg^{2+} (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, 8, and 10 mM). Phe inhibition of RMPK activity occurred in the concentration range of 0 to 60 mM (0, 2, 3, 6, 9 12, 18, 24, 36, 48, and 60 mM) in the presence of 100 mM K^+ and 10 mM Mg^{2+}.

2.4. Isothermal titration calorimetry

All ITC experiments were performed in 50 mM Tris buffer (pH 7.5) using a MicroCal ITC-200 system (GE Healthcare). RMPK, substrates, activating cations, inhibitor, and buffer were thoroughly degassed by stirring under vacuum for 5 min before use. The reaction cell (200 μL) contained 0.2 mM ADP, 4.2×10^{-6} mM RMPK, and activating cations or inhibitor at varying concentrations. The reference cell contained only 50 mM Tris buffer (pH 7.5). In K^+ concentration-dependent experiments, the K^+ concentration ranged from 0 to 100 mM (0, 5, 10, 25, 45, and100 mM) in the presence of a fixed concentration of Mg^{2+} (10 mM). In Mg^{2+} concentration-dependent experiments, the Mg^{2+} concentration ranged from 0–10 mM (0, 0.25, 0.5, 1, 3, and10 mM) with a fixed concentration of K^+ (100 mM). In the presence of 100 mM K^+ and 10 mM Mg^{2+}, the concentration of Phe varied from 0 to 40 mM (0, 4, 8, 16, 36, and 40 mM). The injection syringe (40 μL) was filled with 4 mM PEP and different concentrations of activating cations or inhibitor corresponding with those in the reaction cell in 50 mM Tris buffer (pH 7.5) and rotated at 1000 rpm during temperature equilibration and the experiment. Titration consisted of 20 injections: 0.4 μL for the first injection and 1 μL for each of the following injections. The injection speed was 0.5 μL/s with a 10 min interval between injections. A blank titration of PEP into the buffer was performed to determine the heat of mixing and dilution, which was then subtracted from the heat obtained during the titration of PEP into mixtures. The global calorimetry data were analyzed using a previously published procedure [31,32]. All of the ITC curves were collected automatically by Origin software with subtraction of the basal heat. Free energy change (ΔG) and entropy change (ΔS) were calculated as shown in Eqs. (1) and (2):

$$\Delta G = -RT \ln K \tag{1}$$

$$\Delta G = \Delta H - T\Delta S \tag{2}$$

where K is association constant, R is the gas constant, ΔH is the enthalpy change, and T is the absolute temperature.

3. Results

3.1. Effects of activating cations and inhibitor on enzymatic activity

The enzymatic activity of RMPK was monitored by the decrease in absorbance at 340 nm for 700 s. The reaction rate was measured from the initial linear region of the curve. The reaction rate increased with increasing concentrations of K^+ or Mg^{2+} (Fig. 1). Maximum activity was observed in the presence of 100 mM K^+ and 10 mM Mg^{2+}. The rate of the reaction decreased with increasing Phe concentrations (Fig. 2).

3.2. Thermodynamics monitored by ITC

The thermodynamics of the allosteric regulatory behavior of RMPK induced by changing the concentration of activators and inhibitors was assessed quantitatively by ITC. The ITC curves for the titration of 4 mM PEP into mixtures containing fixed concentrations of ADP (0.2 mM) and RMPK (4.2×10^{-6} mM) in the presence of different concentrations of K^+ and Mg^{2+} are shown in Fig. 3 and Fig. 4, respectively. The ITC curves for PEP titration into mixtures obtained by increasing the concentration of Phe are shown in Fig. 5. The different binding models have been used to fit the ITC data and the results suggest that the analysis of one binding model correspond with the experimental data the best. The sign of heat exchange for the RMPK-catalyzed reaction was positive during PEP titration. All of the thermodynamic parameters of K^+, Mg^{2+}, and Phe

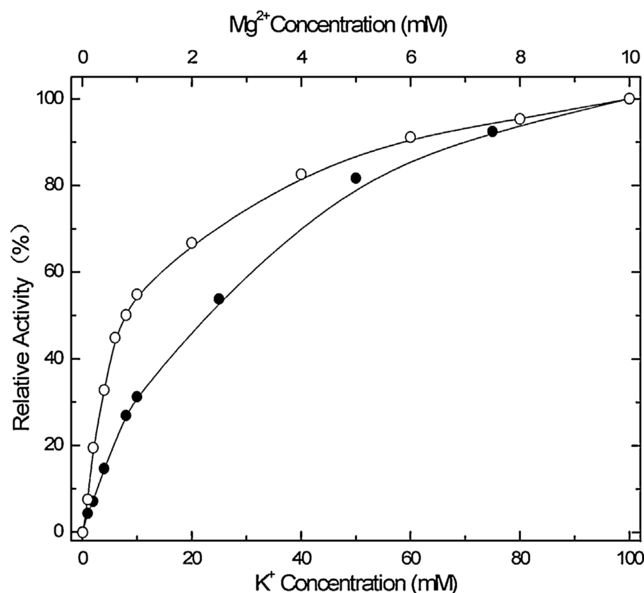

Fig. 1. Activation of rabbit muscle pyruvate kinase by activating cations. Assays were performed in 50 mM Tris at pH 7.5 and 25 °C. The solution composition and symbols are: 2 mM ADP, 2 mM PEP, 0.3 mM NADH, 3.6×10^{-4} mM LDH, 10 mM $MgSO_4$, 0–100 mM KCl in 50 mM Tris buffer at pH 7.5 (●); 2 mM ADP, 2 mM PEP, 0.3 mM NADH, 0.05 mg/mL lactate dehydrogenase, 100 mM KCl, 0–10 mM $MgSO_4$ in 50 mM Tris buffer at pH 7.5 (○).

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