THE EFFECT OF A VARYING Mg^{2+}_{free} CONCENTRATION UPON THE KINETIC BEHAVIOUR OF HUMAN LIVER L-TYPE PYRUVATE KINASE

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1. Introduction

We have proposed [1,2] negative cooperativity for human liver L-type pyruvate kinase, a phenomenon also observed in [3–7]: Lineweaver-Burk plots V^{-1} versus [phosphoenolpyruvate]$^{-1}$ were straight lines at low phosphoenolpyruvate concentrations, and bent downward near the y axis (high phosphoenolpyruvate concentrations). Our conclusions were drawn from experiments performed at Mg^{2+}_{total} = 12 mM or 20 mM [1,2]. However, phosphoenolpyruvate has been proposed to bind Mg^{2+}_{free} with a K_{eq} = 5.5×10^{-3} M^{-1} at 25°C [8]. So, at high phosphoenolpyruvate concentrations Mg^{2+}_{free} would be removed from the reaction mixture. We investigated the hypothesis that the phenomenon interpreted as negative cooperativity [1,2] is in fact due to removing inhibitory Mg^{2+}_{free} from the reaction mixture at high phosphoenolpyruvate concentrations. Our results are in favour of this hypothesis.

2. Materials and methods

NADH (disodium salt), ADP (disodium salt), fructose-1,6-diphosphate (trisodium salt), phosphoenolpyruvate (tricyclohexylammonium salt) and lactate dehydrogenase (rabbit muscle, 550 IU/mg) were obtained from Boehringer, Mannheim. Dithiotreitol and bovine serum albumin were from Sigma, St Louis. All reagents were of the highest purity available.

Human liver L-type pyruvate kinase was purified by the method in [9]. The preparation had spec. act. 360 IU/mg, and was stored as an $(NH_4)_2SO_4$-precipitate at −80°C. Prior to use the enzyme was dialyzed extensively at +4°C, against the buffer used in the kinetic experiments, containing 0.5 M sucrose, 1 mM dithiotreitol, and 1 mg/ml bovine serum albumin for stabilisation.

Pyruvate kinase activity was measured either at 25°C or 37°C by the method in [10]. Two buffer systems were used. Buffer A (the buffer used in [1,2]) contained 0.1 M triethanolamine–HCl (pH 7.5) at 37°C, 0.1 M KCl, 20 mM $MgCl_2$, 0.1 M NADH, 10 IU lactate dehydrogenase (not dialysed), 1 mM dithiotreitol and 0.1 mM fructose-1,6-diphosphate. Buffer B contained 0.01 M Tris–HCl (pH 8.0) at 25°C, 0.05 M KCl, 0.1 mM NADH, 10 IU lactate dehydrogenase (dialysed against the assay buffer), 1 mM dithiotreitol and 0.1 mM fructose-1,6-diphosphate. In buffer B either Mg^{2+}_{total} was kept constant at 20 mM, or Mg^{2+}_{free} was kept constant at 1, 10 or 20 mM using K_{eq} = 5.5×10^{-3} M^{-1} (25°C) for the $MgPEP^-$ complex [9] and K_{eq} = 3.39×10^{-4} M^{-1} (25°C) for the $MgADP^-$ complex (11). $[Phosphoenolpyruvate]_{total}$ was 0.02–10 mM; ADP_{total} was kept at 3 mM. The enzyme was preincubated in the reaction mixture containing all ligands except ADP for 5–10 min; the reaction was initiated by the addition of ADP.

3. Results and discussion

When a Lineweaver-Burk plot was made in buffer A (Mg^{2+}_{total} = 20 mM) at 25 or 37°C, a linear relationship was observed at low phosphoenolpyruvate concentrations ($PEP_{total} \leq 0.5$ mM). However, at high phosphoenolpyruvate concentrations the curve bends

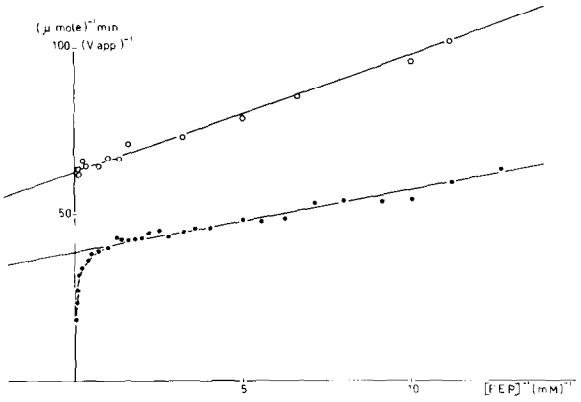

Fig.1. Lineweaver-Burk (L-B) plots V^{-1} versus [phosphoenolpyruvate]$^{-1}$ of human liver L-type pyruvate kinase. (●——●) L-B plot in buffer A at 20 mM Mg^{2+}_{total} at 25°C. (○——○) L-B plot in buffer B at Mg^{2+}_{free} = 10 mM at 25°C. The difference in V_{max} is due to the use of different enzyme concentrations.

downward, a phenomenon that we [1,2] interpreted as negative cooperativity. A similar phenomenon was observed in buffer B when Mg^{2+}_{total} was kept at 20 mM (fig.1). However, when a Lineweaver-Burk plot was made in buffer B at Mg^{2+}_{free} = 1, 10 or 20 mM (at 25°C), a linear relationship was found at all phosphoenolpyruvate concentrations tested (fig.1).

These results suggest that the phenomenon interpreted as negative cooperativity [1,2] is in fact due to removing inhibitory Mg^{2+}_{free} from the reaction mixture at high phosphoenolpyruvate concentrations. In order to check whether indeed phosphoenolpyruvate binds Mg^{2+}_{free} in our assay system, we made V versus [Mg^{2+}_{total}] plots at 37°C of human liver L-type pyruvate kinase in buffer A (20 mM Mg^{2+}_{total}), at a few different phosphoenolpyruvate concentrations. At phosphoenolpyruvate = 1 mM (the enzyme is fully saturated with this substrate), we find a maximum in the plot at ~1.5 mM Mg^{2+}_{total}. At phosphoenolpyruvate = 20 mM, we find a maximum in the plot around 10 mM Mg^{2+}_{total}. Because the true substrates of human liver L-type pyruvate kinase are not known, a full interpretation of these results cannot be given. However it is clear, that the shift in position of the maximum can only be caused by an interaction between Mg^{2+} and phosphoenolpyruvate. Because in both cases the enzyme is fully saturated with phosphoenolpyruvate, this interaction cannot be mediated through the enzyme, and is therefore probably simply an ionic binding process in solution. So, for example, at phosphoenolpyruvate = 10 mM, in the buffer system with Mg^{2+}_{total} = 12 mM, the concentration of the inhibitor Mg^{2+}_{free} is reduced by a factor of ~2, compared to in the absence of phosphoenolpyruvate, resulting in a more active enzyme.

For the L-type pyruvate kinase forms from human erythrocytes, negative cooperativity would also be an artefact of a varying Mg^{2+}_{free} concentration, just as for the liver enzyme. So, in kinetic studies on L-type pyruvate kinase, Mg^{2+}_{free} must be kept carefully under control.

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