FRUCTOSE-1,6-DIPHOSPHATE BINDING BY TWO FORMS OF PYRUVATE KINASE PURIFIED FROM HUMAN ERYTHROCYTES

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1. Introduction

Although kinetic properties of human erythrocyte pyruvate kinase (EC 2.7.1.40) have been extensively studied [1,2] little data about the binding of its ligands are available. Previous work showed that fructose-1,6-diphosphate binding was enhanced by phosphoenolpyruvate [3]. The number of sites, however, remained unknown since the enzyme was not pure. We have recently described a new purification method [4] characterized by its rapidity and good yield. This method enabled us to separate two forms of red cell pyruvate kinase, as described in another paper [5]. The first form, designated as red cell pyruvate kinase I, is composed of 4 identical L-type subunits. The other (red cell pyruvate kinase II) is a heterotetramer made from 2 L-type subunits and 2 subunits called 'L', having a molecular weight slightly higher than that of the L-type subunits. We have demonstrated that the enzyme 'L_4' (i.e., L-type pyruvate kinase from liver or red cell pyruvate kinase I) had specific activity twice that of the enzyme '$L_2 L'_2$' (i.e., red cell pyruvate kinase II). A mild proteolytic digestion, using trypsin, resulted in an increase of the enzyme activity and in a transformation of L' into L-type subunits such that the trypsin-treated red cell enzyme II could no longer be distinguished from the enzyme I.

The purpose of this paper is to study the number and the properties of the fructose-1,6-diphosphate binding sites for both forms of human erythrocyte pyruvate kinase.

2. Material and methods

[U-^{14}C]Fructose-6-phosphate (spec. act. 288 mCi/mmol) was purchased from Le Commissariat à l'Energie Atomique, France, [U-^{14}C]Fructose-1,6-diphosphate was prepared by incubation with ATP and fructose-6-phosphate kinase [3]. Radioactive fructose-1,6-diphosphate was isolated by ion-exchange chromatography. Purity was checked by column and paper chromatography.

Red cell pyruvate kinase I and II were stored at −70°C as an ammonium sulfate precipitate. After centrifugation, ammonium sulfate precipitate was dissolved in the dialysis buffer (see below) and ammonium sulfate was removed by chromatography on a Sephadex G-25 fine column equilibrated with the dialysis buffer.

Protein assay was made by the method of Lowry et al. [6]. The molecular weights of the different forms of pyruvate kinase are given in another paper [5].

Equilibrium dialysis experiments were performed as previously described [3], during 15 h at 25°C, in triethanolamine buffer, pH 7.0, containing 5 mM EDTA, 50 mM KCl, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol and 300 mM sucrose. One compartment contained various fructose-1,6-diphosphate concentrations, while the other contained about 9 μg pure pyruvate kinase. The total volume of each compartment was 0.3 ml. After dialysis, aliquots of each compartment were counted by liquid scintillation with Uni-

solve I (Koch-Light). Albumin (1 mg/ml) was present on each side on the membrane to avoid Gibbs-Donnan effect. The absence of fructose-1,6-diphosphate fixation by albumin has been verified. Enzyme activity remained constant during dialysis. Degradation of fructose-1,6-diphosphate during the dialysis experiment has been eliminated by chromatography on a Dowex-2-column, according to Bartlett [7].

3. Results

Fructose-1,6-diphosphate binding was studied on both pyruvate kinases I and II in the presence of 0.5 mM phosphoenolpyruvate.

With pyruvate kinase I the binding curve was hyperbolic. Scatchard plot was linear and showed a single class of sites with a dissociation constant of 4×10^{-8} M (fig.1). The extrapolation on the x-axis gave 3.4 binding sites for fructose-1,6-diphosphate. Since pyruvate kinase I is a tetramer [5], we may conclude that there are four binding sites/tetramer, that is to say, one/subunit.

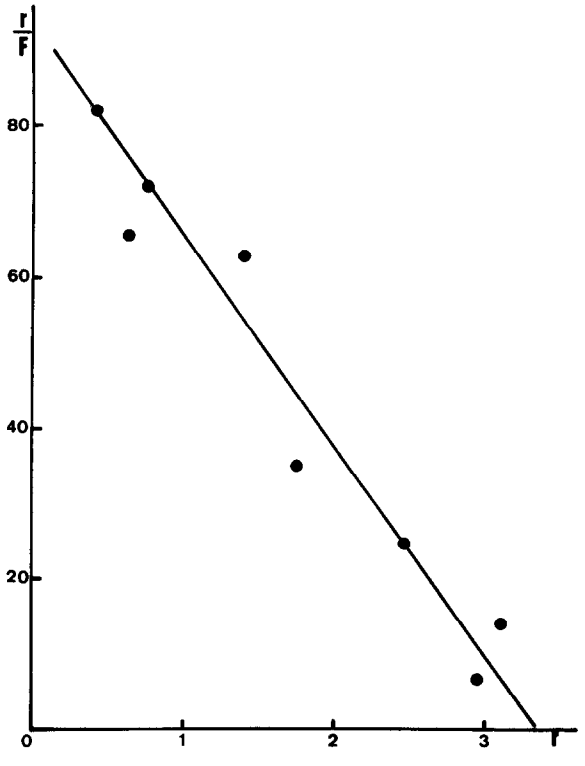

Fig.1. Binding of fructose-1,6-diphosphate by erythrocyte pyruvate kinase I in the presence of 0.5 mM phosphoenol pyruvate. Data are expressed as r/F versus r, where r is the number of moles of fructose-1,6-diphosphate bound/mole tetramer (mol. wt 240 000), and F is free fructose-1,6-diphosphate concentration (10^{-6} M). Concentration of tetramer is 1.10^{-7} M.

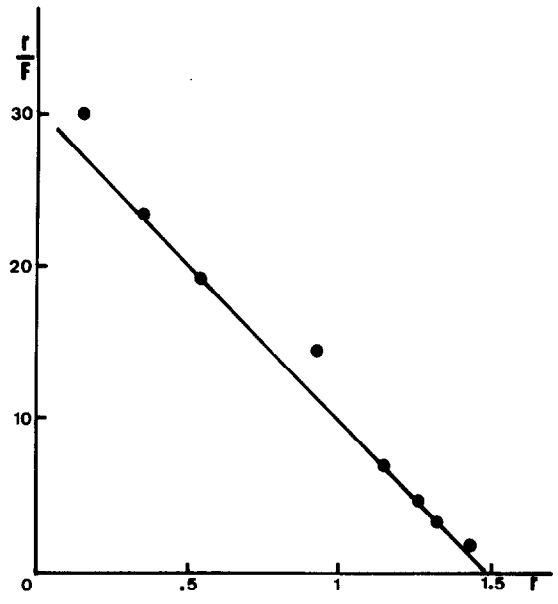

Fig.2. Binding of fructose-1,6-diphosphate by erythrocyte pyruvate kinase II in the presence of 0.5 mM phosphoenol pyruvate. Data are expressed as r/F versus r. Concentration of tetramer is 1.25×10^{-7} M.

With pyruvate kinase II fructose-1,6-diphosphate binding was non-cooperative, as for pyruvate kinase I. There was only one class of sites. The dissociation constant was 3.7×10^{-8} M (fig.2). The extrapolation of the Scatchard plot on the x-axis gave 1.5 sites. Since red cell pyruvate kinase II, as well as pyruvate kinase I, is a tetramer [5], we may conclude that there are only two sites/tetramer and that only two subunits are able to bind fructose-1,6-diphosphate.

4. Discussion

These results agree with binding data previously reported on partially purified enzyme [3]. The dis-

sociation constant for fructose-1,6-diphosphate is the same for the two forms of pyruvate kinase. This dissociation constant value, 4×10^{-8} M, is similar to the kinetic association constant previously reported, 5×10^{-8} M [8]. For both forms, I and II, no cooperativity in the binding of fructose-1,6-diphosphate is evident in the presence of phosphoenolpyruvate. This finding is consistent with kinetic properties [1] and is easily explained in terms of the allosteric model proposed by Monod et al. [9]: in the presence of high concentrations of the substrate phosphoenolpyruvate, allosteric equilibrium is shifted towards the *R*-state and the binding of the activator fructose-1,6-diphosphate is hyperbolic.

The main result is that there are four sites on pyruvate kinase I and only two sites on pyruvate kinase II. For pyruvate kinase I each subunit has one binding site for fructose-1,6-diphosphate. If we assume, according to Monod et al. [9], that oligomeric proteins are symmetric, we may postulate that each subunit of pyruvate kinase I possesses one catalytic site.

For pyruvate kinase II there are only two sites for fructose-1,6-diphosphate with a value of 3.7×10^{-8} M, similar to the value for pyruvate kinase I. Perhaps other sites of lower affinity exist. We have checked that, with increased concentrations of phosphoenol pyruvate (up to 1×10^{-2} M), fructose-1,6-diphosphate binding is not enhanced, either with pyruvate kinase I or with pyruvate kinase II. We have shown in the preceding paper [5] that form II is a heterotetramer $L_2L'_2$ and has a specific activity half that of form I. We may suppose that the L′ subunits have neither fructose-1,6-diphosphate binding site, nor efficient catalytic site. The absence of efficient catalytic site would easily explain the half specific activity.

Marie et al. [5] have shown that in vitro, a mild tryptic attack is able to transform pyruvate kinase II into pyruvate kinase I. The heterotetramer '$L_2L'_2$' is converted into a homotetramer 'L_4'. This conversion is accompanied by a 1.6-times enhancement of the specific activity. Two hypotheses could be put forward to explain such data: either the peptide removed by trypsin could mask both the fructose-1,6-diphosphate binding and the catalytic sites, or hydrolysis of this peptide could promote a transconformation of the L′ subunits from a form without affinity for fructose-1,6-diphosphate and catalytically poorly or not active towards a form with high affinity for fructose-1,6-diphosphate and catalytically fully active.

In conclusion, the demonstration that the precursor form '$L_2L'_2$' contained two binding sites for fructose-1,6-diphosphate and had only half the specific activity of the 'L_4' enzyme seems to indicate that a hypothetical 'L'_4' native form would be unable to fix fructose-1,6-diphosphate and would be catalytically poorly or not active. Thus, proteolytic activation would be indispensable in order that erythrocyte pyruvate kinase reaches its maximum catalytic efficiency.

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