Kinetic analysis of cooperativity of phosphorylated L-type pyruvate kinase

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*Dedicated to Academician Ülo Lille, Pofessor Emeritus
at Tallinn University of Technology, on his 75th birthday*

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Abstract. Kinetics of L-type pyruvate kinase (EC 2.7.1.40) catalysed reaction between phosphoenolpyruvate (PEP) and ADP was analysed under steady-state conditions and the interaction of both substrates with the enzyme was characterized proceeding from bi-substrate kinetic mechanism of this process. Cooperative regulation of the rate of this process by one of the substrates, PEP, was taken into consideration by using a sequential ligand binding model. It was found that two PEP molecules may bind with similar affinity with the tetrameric enzyme ($K = 30$ mM), while the effectiveness of the binding of the next two substrate molecules is enhanced through cooperative interaction between the enzyme subunits, which decreases the dissociation constant of the enzyme–substrate complex approximately 10 times.

INTRODUCTION

Pyruvate kinase (ATP-pyruvate 2-O-phosphotransferase, EC 2.7.1.40, further denoted as PK) catalyses the final step of glycolysis, transferring the phosphoryl group from phosphoenolpyruvate (PEP) to ADP and producing pyruvate and ATP [1]:

$$PEP + ADP \xrightarrow[\text{PK}]{\text{Mg}^{2+}, \text{K}^+} \text{pyruvate} + ATP. \tag{1}$$
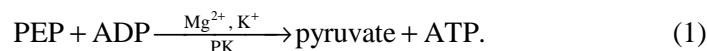
In tissues of vertebrates this metabolically crucial reaction is catalysed by four isoenzymes, denoted as M1, M2, L, and R forms of PK, which all are tetramers

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and consist of similar subunits [2]. Three of these isoenzymes, M2, L, and R, show allosteric properties for binding PEP under physiological conditions [3, 4]. This means that the binding of a substrate molecule at one site of multimeric enzyme may affect ligand binding at other sites. Such communication between the enzyme subunits provides a sensitive way of the regulation of the enzyme activity [5] and this property plays obviously a significant role also in the regulation of the glycolytic pathway.

The second substrate of this reaction, ADP, is not involved in the cooperative regulation of PK activity [1, 6], although the cooperative changes induced by PEP may also alter its interaction with the appropriate binding sites on the enzyme. Surprisingly, this aspect of the catalytic process, as well as the inter-relationship between properties of cooperatively regulated substrate binding sites for PEP on PK, has not been discussed in detail.

In this report we analyse simultaneous interaction of both substrates, PEP and ADP, with the phosphorylated form of the L-type PK of rat liver [7, 8], and characterize the affinity changes of the enzyme caused by the cooperative interactions. The enzyme was expressed in *Escherichia coli* [9] and thereafter stoichiometrically phosphorylated by the catalytic subunit of cAMP-dependent protein kinase [10]. Further it is denoted as L-PK in this paper.

MATERIALS AND METHODS

Enzymes and chemicals

By using cDNA from rat liver L-PK was expressed in *E. coli* and the protein was purified to homogeneity as described in [9]. The enzyme was stoichio-metrically phosphorylated in the presence of the catalytic subunit of the cAMP-dependent protein kinase (Biaffin GmbH & Ko, Kessel, Germany) and ATP. Details of this procedure and molecular properties of the phosphorylated L-type PK (L-PK) were described in [10]. The concentration of the enzyme solution was determined spectrophotometrically, taking into consideration extinction of Trp, Tyr, and Cys at 280 nm, as described by Aitken & Learmonth [11]. The amount of these amino acids is known from the primary structure of L-PK: Trp – 3, Tyr – 10, Cys – 6 [7]. On the basis of this amino acid composition the extinction coefficient $30\,590\,M^{-1}\,cm^{-1}$ was calculated for the L-PK subunit. L-Lactate dehydrogenase from rabbit muscle (LDH), bovine serum albumin (BSA) fraction V, PEP tricyclohexylammonium salt, and adenosin-5'-diphosphate disodium salt were purchased from Boehringer Mannheim GmbH (Germany). Dithiothreitol, nicotinamide adenine dinucleotide reduced form disodium salt (NADH) and tris(hydroxymethyl)-aminomethane (TRIS) were from Sigma-Aldrich (USA). Magnesium chloride and potassium chloride were from Acros (Fisher Scientific International Inc., USA). The enzyme stock solutions were diluted with 50 mM TRIS buffer (pH 7.4), containing 0.1% BSA. The Milli-Q deionized water was used in all experiments.

Assay of L-PK activity

Activity of L-PK was measured spectrophotometrically, adopting the procedure described earlier [12]. The assay is based on the following sequence of reactions. First, L-PK catalyses the formation of pyruvate and ATP (Eq. 1). Secondly, the pyruvate formed in this reaction is used by LDH to form L-lactate converting simultaneously NADH into NAD^+. The latter change can be followed spectrophotometrically, as absorbance of the solution strongly decreases if NADH is converted into NAD^+ ($\Delta\varepsilon = 6220$ L/cm mol, $\lambda = 340$ nm). Applicability of this reaction sequence assumes that the rate of the latter process is very fast if compared with the first reaction. We have proved this by using different LDH concentrations under similar conditions of the L-PK catalysis. In all these cases the apparent velocity of the L-PK catalysed reaction was similar.

Kinetic measurements were made in 50 mM TRIS/HCl buffer (pH 7.4, 30 °C), containing 0.2 mM NADH, 0.002 mg/mL (1.5 units/mL) LDH, 100 mM KCl, 10 mM $MgCl_2$, 0.1% BSA, 0.1 mM DTT, and 1.5 nM L-PK. Substrate concentrations varied from 0.01 up to 10 mM in the case of PEP and from 0.01 to 6 mM in the case of ADP.

The reaction mixture (0.960 mL) was composed in 1 cm thermostatted quartz cells and the reaction was initiated by addition of 40 µL of L-PK solution. The initial velocity of the catalysis was monitored during 1–3 min ($\lambda = 340$ nm, UV–VIS spectrophotometer Unicam UV300, ThermoSpectronic, USA, integration time 0.25 s, sampling interval 1 s) and the initial velocities (v) were calculated from the time course of the absorbance (Fig. 1). The relationship between the initial velocity and L-PK concentration was linear, pointing to the fact that the change of the optical density of the assay mixture was caused by the enzymatic reaction.
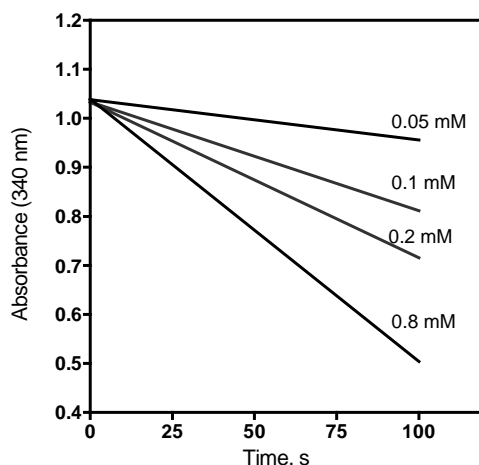
Fig. 1. Spectrophotometric assay of L-PK activity at 1 mM ADP and different PEP concentrations. Conditions of the reaction as described in text.

Data processing

All data were analysed by non-linear least-squares regression analysis using the GraphPad Prism version 4.0 (GraphPad Software Inc., USA), SigmaPlot 9.0 (Systat Software Inc., USA), and Microsoft Excel XP (Microsoft Corporation, USA). The values reported are given with standard errors.

RESULTS

Asymmetric cooperativity in L-PK-catalysed bi-substrate reaction

The results of the present kinetic study are in good agreement with the generally accepted understanding that the rate of the L-PK catalysed reaction is cooperatively regulated only by one of the substrates, PEP, while the interaction of the other substrate, ADP, with the enzyme follows the common Michaelis–Menten type kinetics [1, 6]. This asymmetry in cooperativity of the bi-substrate reaction is illustrated in Fig. 2, where the plots of the initial rate vs. ADP and PEP concentrations are shown. In these experiments the concentration of one of the substrates was kept constant and therefore the simple Hill rate equation (2) could be used for data processing:

$$v = \frac{V[\mathrm{S}]^n}{K_{0.5}^n + [\mathrm{S}]^n},\qquad(2)$$

where S stands for the variable substrate, V is the maximal velocity observed under the conditions used, n is the cooperativity parameter (Hill coefficient), and the constant $K_{0.5}^n$ denotes the substrate concentration at which the reaction rate is half of the maximal rate. To demonstrate asymmetry of cooperativity in the L-PK
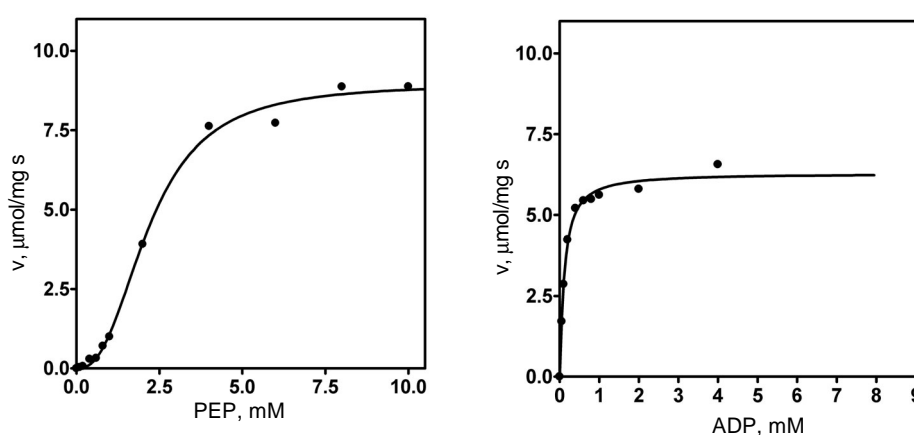
Fig. 2. Influence of PEP at 1 mM ADP and ADP at a constant PEP concentration (2 mM) on the rate of the L-PK catalysed reaction in 50 mM TRIS/HCl buffer, pH 7.4, 30 °C.

catalysed reaction, the same equation was used to analyse the data shown in Fig. 2 and the following results were obtained.

At variable PEP concentration and 1 mM ADP concentration:
$V = 9.0 \pm 0.2$ μmol/mg s,
$K_{0.5} = 2.2 \pm 0.1$ mM,
$n = 2.5 \pm 0.2$.

At variable ADP concentration and 2 mM PEP concentration:
$V = 6.3 \pm 0.2$ μmol/mg s,
$K_{0.5} = 0.11 \pm 0.01$ mM,
$n = 1.1 \pm 0.2$.

Indeed, the Hill parameter n is significantly different for PEP and ADP, and in the latter case this value is not distinguishable from unity. This means that no cooperative regulation of the reaction rate by ADP can be observed, and the rate equation (2) reduces to the common Michaelis–Menten rate equation for this substrate.
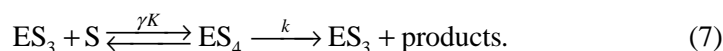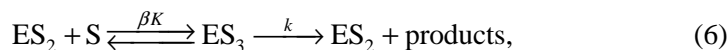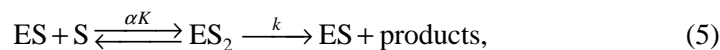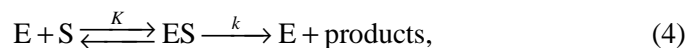
$$v = \frac{V[S]}{K_m + [S]}.\tag{3}$$

The V values calculated from the two data sets in Fig. 2 are different. This is not surprising as the rate of this bi-substrate reaction is determined by the affinity–concentration ratio of the substrates, and this ratio is obviously not similar. It is also important to emphasize that the constant $K_{0.5}^n$ in Eq. (2) reflects simultaneous interaction of several substrate molecules with the enzyme and thus cannot be directly compared with the Michaelis constant K_m in Eq. (3).

Sequential interaction model for cooperative effect of PEP

The idea of asymmetric appearance of cooperativity in the catalytic activity of L-PK was extended to develop a more explicit kinetic model, describing sequential interaction of PEP molecules with the enzyme. This model assumes that the interaction of the substrate molecule with its binding site on one subunit affects binding properties of the remaining ("free") subunits of the multimeric enzyme [13]. Therefore, maximally four different levels of L-PK affinity for PEP can be defined, which correspond to the four possible levels of enzyme saturation with this substrate. For simplification of the model and reduction of the number of variables, we assumed that the presence of another substrate, ADP, has no effect on PEP binding.

The four levels of the enzyme saturation with substrate S (S stands for PEP in this particular case) can be presented by the following schemes, where the substrate interaction with the first subunit is quantified by the dissociation constant K, affinity for the second substrate molecule is quantified by αK, and affinity for the third and fourth substrate molecules by βK and γK, respectively [14]. Formally this situation can be presented by the following reaction schemes, assuming the sequential substrate interaction with the enzyme [14].

$$E + S \underset{}{\overset{K}{\rightleftharpoons}} ES \xrightarrow{k} E + products, \tag{4}$$

$$ES + S \underset{}{\overset{\alpha K}{\rightleftharpoons}} ES_2 \xrightarrow{k} ES + products, \tag{5}$$

$$ES_2 + S \underset{}{\overset{\beta K}{\rightleftharpoons}} ES_3 \xrightarrow{k} ES_2 + products, \tag{6}$$

$$ES_3 + S \underset{}{\overset{\gamma K}{\rightleftharpoons}} ES_4 \xrightarrow{k} ES_3 + products. \tag{7}$$

It is important to consider that the overall rate of the process depends on the total concentration of the enzyme–substrate complexes. However, the formation of these complexes is determined besides the equilibriums shown in schemes (4)–(7) also by the probability factors. So, in the case of the tetrameric enzyme there are four ways to form an ES complex from E, six ways to form the complex ES_2 from ES, four ways to form ES_3 from ES_2, and one way to form ES_4 from ES_3. Taking into account these probability factors and assuming that all complexes are in equilibrium, the following rate equation can be obtained for the cooperatively functioning tetrameric enzyme (details see in [14]):

$$v = V \frac{\dfrac{[S]}{K} + \dfrac{3[S]^2}{\alpha^2 K^2} + \dfrac{3[S]^3}{\beta^3 K^3} + \dfrac{[S]^4}{\gamma^4 K^4}}{1 + \dfrac{4[S]}{K} + \dfrac{6[S]^2}{\alpha^2 K^2} + \dfrac{4[S]^3}{\beta^3 K^3} + \dfrac{[S]^4}{\gamma^4 K^4}}. \tag{8}$$

In this equation the maximal reaction rate V is determined by the total amount of the active sites participating in catalysis, which is four times greater than the analytical concentration of the enzyme:

$$V_{MAX} = 4K[E]_{total}. \tag{9}$$

Following the basic idea of stepwise formation of the enzyme–substrate complexes, the constraints that α, β, and γ must be equal or greater than 1 were used in processing the experimental data by Eq. (8). Under these conditions, the best fit of the experimental data for PEP was achieved at:
$V = 10$ μmol/mg s,
$K = 30$ mM,
$\alpha = 1.0$,
$\beta = 0.1$,
$\gamma = 0.1$.

It should be mentioned that variation of each of these parameters within 10% limit of their value clearly changed the goodness of the fit, characterized by the sum of the squares of the deviations between the experimental and predicted values as well as by the multiple correlation coefficient ($R_i = 0.998$ for the set of parameters above).

Secondly, the goodness of the fit was significantly reduced when the complex ES_4 alone, or both complexes ES_4 and ES_3, were omitted from the analysis by taking $\gamma = 0$ or $\beta = \gamma = 0$, respectively. Thus, the kinetic data agree with the

tetrameric structure of the enzyme and point to the fact that all the four different levels of the complex formation are necessary for modelling the positive cooperative regulation of the reaction velocity.

However, keeping in mind the bi-substrate nature of the reaction, it should be emphasized that for the catalytic step the presence of ADP in the enzyme–substrate complex is needed. Therefore, if the enzyme is not saturated by this second substrate, the constant K in Eq. (8) does not necessarily quantify affinity of L-PK for PEP. On the other hand, α, β, and γ characterize the cooperative interactions between the enzyme subunits and therefore should be independent of the reaction conditions.

Similarly, the constant V, if calculated from one-parameter plots presented by Eqs (2), (3), or (8), is not a characteristic value for the bi-substrate reaction in general, but also depends on the affinity/concentration ratio of the second substrate. This cross-effect of substrate concentration is clear from the results above where the V values calculated from the rate versus concentration plots for ADP and PEP are different. Therefore, for further insight into the mechanism of the L-PK catalysed reaction, the following attempt was made to consider the bi-substrate nature of this reaction.

Bi-substrate kinetic model with an empirical Hill coefficient

Due to obvious complications in explicit kinetic modelling of a cooperatively regulated bi-substrate reaction catalysed by tetrameric enzyme, the kinetic data were analysed proceeding from the rate equation for a non-cooperative bi-substrate random-order enzymatic reaction [14], where the reactive complex involves the enzyme, ADP, and PEP. However, as far as the formation of this ternary complex is affected only by PEP interaction with other subunits of L-PK, we introduced the Hill parameter n into the conventional rate equation for the bi-substrate reaction (see in [14], pp. 274–276), and the following kinetic equation was obtained:

$$v = \frac{\left(\dfrac{V[\mathrm{ADP}]}{K_A + [\mathrm{ADP}]}\right)[\mathrm{PEP}]^n}{\left(\dfrac{K_A K_B + K_B[\mathrm{ADP}]}{K_A + [\mathrm{ADP}]}\right)^n + [\mathrm{PEP}]^n}. \tag{10}$$

In this equation V stands for the maximal velocity, K_A and K_B characterize the interaction of ADP and PEP with the enzyme, and n is the Hill coefficient for PEP. Processing the kinetic data by the two-parameter equation (10) provided the following results:

$V = 9.6 \pm 0.7$ μmol/mg s,

$K_A = 0.10 \pm 0.05$ mM (for ADP),

$K_B = 2.2 \pm 0.3$ mM (for PEP),

$n = 2.5 \pm 0.3$ (for PEP).
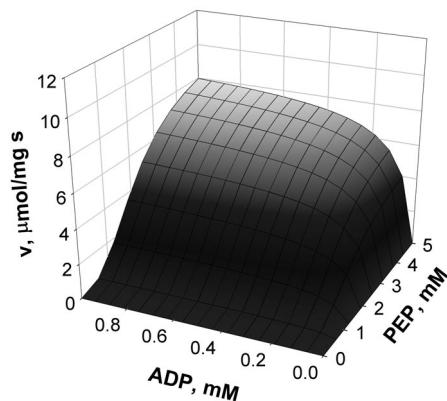
Fig. 3. Illustration of the bi-substrate kinetic model for the L-PK catalysed reaction with PEP and ADP.

For illustration, the 3D dependence of the reaction rate on the concentration of PEP and ADP is shown in Fig. 3. It is obvious that in this case the constant V is independent of the concentration of any of the substrates and thus represents the maximal rate of the overall reaction. Analogously, the constant K_A should explicitly characterize the interaction of ADP with the enzyme, as far as the influence of PEP on the reaction rate is taken into consideration by separate terms in Eq. (10) and characterized by the constants n and K_B. The values of these constants are in good agreement with the results of data processing by the single-parameter equation (2), and the physical meaning of these parameters should be discussed separately.

DISCUSSION

Although the kinetic properties of L-PK, isolated in its phosphorylated form from various vertebrates, have been studied over several decades, the affinity of this enzyme for its substrates, as well as the extent of alteration of this affinity through cooperative interactions, has not been thoroughly discussed taking into account the bi-substrate nature of the reaction. Therefore we have undertaken a thorough kinetic analysis of this metabolically significant reaction, with an attempt to estimate the affinity of the enzyme against both substrates, ADP and PEP, and to model its cooperative behaviour. Fortunately, this analysis can be simplified proceeding from the generally recognized fact that L-PK reveals cooperativity only for PEP, while no cooperativity is observed with the other substrate, ADP.

Taking into account this phenomenon of asymmetric cooperativity, the conventional bi-substrate kinetic model was adopted for the L-PK catalysed reaction and the values of the maximal rate (V) as well as K_A and K_B were

calculated from Eq. (10). As the simultaneous influence of both substrates on the reaction rate was taken into account by this model, these parameters do not depend on the concentration of either of the two substrates. Therefore the constant K_A should explicitly characterize the interaction of ADP with the enzyme, while the meaning of K_B depends on the mechanism of cooperativity. In other words, the bi-substrate kinetic model agrees with the assumption that ADP does not affect the L-PK interaction with PEP, and on the contrary, PEP does not affect the L-PK interaction with ADP. This conclusion is important for the applicability of the sequential model of cooperativity, used in this study to analyse the mechanism of this phenomenon.

In the presence of 1 mM ADP, routinely used in kinetic experiments, the majority of the L-PK binding sites (approx. 90%) are in complex with this substrate. This means that the parameter K, calculated from Eq. (8), should be a rather good estimate for the true substrate constant (K_s) for PEP, characterizing the affinity of the binding of the first substrate molecule with the tetrameric enzyme. Surprisingly, the affinity of this substrate is much lower compared to the affinity of ADP. On the other hand, however, the binding of PEP with L-PK is controlled through the cooperativity of the enzyme, decreasing the substrate concentration that is necessary for its catalytic activity.

Following the sequential model, the cooperativity of the enzyme is characterized by parameters α, β, and γ. These parameters, also called "interaction factors", compare affinities of the first binding step with each of the following binding steps [14]. Therefore, if $\alpha = 1$, there is no difference between the enzyme–substrate dissociation constants on the first and on the second step. In other words, the binding of the first substrate molecule has no effect on the binding of the second molecule. This is the case observed for L-PK. The affinity of the enzyme for the third substrate molecule is increased as $\beta = 0.1$. This means that the dissociation constant of the enzyme–substrate complex ES_3 is approx. 3 mM. A similar value of the dissociation constant can be obtained also for the last complex ES_4, as again, the interaction factor γ is equal to 0.1. Thus, the affinities of L-PK for the last two PEP molecules are similar. Most likely this means that the binding of substrate molecules with two subunits of L-PK triggers off the conformational transition that changes the substrate binding sites on the two remaining subunits. These changes should be similar to provide similar affinity for PEP.

To sum up, the cooperative regulation of the activity of the phosphorylated form of L-type PK seems to occur by a rather simple mechanism, which involves only one structural transition of the enzyme subunits. The binding of two substrate molecules with the tetrameric enzyme triggers off this transition, and it changes the binding properties of the remaining two subunits. This change of the binding properties is quantified by a 10-fold decrease of the enzyme–substrate complex dissociation constants, which is sufficient for the generation of cooperativity of the enzyme. In terms of the substrate–protein interactions, this change is quite realistic and can be achieved by addition of one or two weak

interactions between the substrate molecule and its binding site. As a result of these additional interactions small changes in the concentration of PEP may have a far more significant effect on the rate of the process compared with the effect of the substrate concentration on the velocity according to the Michaelis–Menten type kinetics. It seems to be important to stress that this cooperativity model is basically different from the classical models of cooperative ligand binding introduced in the 1960s, i.e. the two-state concerted model [15] and the sequential model [16], which both view cooperativity of tetrameric proteins as a result of interaction of four similar subunits.

Although a more detailed molecular mechanism of L-PK cooperativity cannot be discussed in the light of the present kinetic data, it is interesting to mention that a new model of cooperativity was relatively recently proposed for human hemoglobin tetramer interaction with oxygen [17, 18]. In this model the tetrameric hemoglobin structure is treated as "dimer of dimers", capable of revealing asymmetric distribution of cooperativity effects in a cascade of ligand binding events. In other words, the cooperativity phenomenon is ascribed to the coupling effect of dimers, not monomers. A similar situation has been demonstrated in the present paper for L-PK.

ACKNOWLEDGEMENT

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Fosforüleeritud L-tüüpi püruvaadi kinaasi kooperatiivsuse kineetiline analüüs

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On uuritud L-tüüpi püruvaadi kinaasi (EC 2.7.1.40) poolt katalüüsitud fosfoenoolpüruvaadi (PEP) ning ADP vahelise reaktsiooni kineetikat ja protsessi on kirjeldatud bi-substraatse reaktsiooni mudeli järgi, milles on arvestatud täiendavalt ühe substraadi (PEP) kooperatiivset toimet reaktsiooni kiirusele. Reaktsioonil avalduva kooperatiivse toime mehhanismi kirjeldamiseks on kasutatud ligandi järjestikulise sidumise mudelit. On leitud, et ensüümi afiinsus PEP suhtes ei muutu esimese ning teise substraadimolekuli seostumisel ja see protsess on kirjeldatav dissotsiatsioonikonstandiga 30 mM. Kolmanda ja neljanda molekuli seostumisel ilmneb aga kooperatiivne efekt, mis on sarnane substraadi seostumisel kolmanda ning neljanda ensüümi alaühikuga ja mis vähendab dissotsiatsioonikonstanti 10 korda.