Enzyme and Microbial Technology 43 (2008) 455–459

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

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

Substrate promiscuity of pyruvate kinase on various deoxynucleoside diphosphates for synthesis of deoxynucleoside triphosphates

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ARTICLE INFO

Article history:
Received 15 April 2008
Received in revised form 12 June 2008
Accepted 15 June 2008

Keywords:
Promiscuity
Pyruvate kinase (PK)
Deoxynucleoside triphosphate (dNTP)
Bacillus sp. PK
Zymomonas mobilis PK

ABSTRACT

Directed regulation of the promiscuity of pyruvate kinase (PK) on different deoxynucleoside diphosphate (dNDP) substrates is an effective way to improve the process efficiency of biosynthesis of deoxynucleoside triphosphates (dNTP). The rabbit muscle PK was found to be highly specific on adenosine diphosphate (ADP) substrate, and the activity on dNDP substrates were reduced significantly. It was deduced that the bacteria PKs might have better promiscuity on dNDP substrates because of the less demand for fast energy conversion from ADP to ATP by PK. Two bacteria sourced PK genes from *Bacillus* sp. ATCC 21616 and *Zymomonas mobilis* ATCC 31821 were cloned and expressed in the *E. coli* strain BL21 (DE3). The results showed that the promiscuity of *B.* sp. and *Z. mobilis* PKs on dNDP substrates was improved significantly, which is in agreement with the deduction of bacteria PKs have better substrate promiscuity. The maximum reaction velocities and the Michaelis constants of *Z. mobilis* and *B.* sp. PKs on dNDP substrates were within one order of magnitude difference, respectively, comparing to two order of magnitude difference for rabbit muscle PK. The dNDP conversion experiments showed that the process efficiency was improved when the bacteria PKs were used for the dNTP biosynthesis.

1. Introduction

The phosphorylating reagents deoxynucleoside triphosphates (dNTP) used as the essential precursor subunits for polymerase chain reaction (PCR) include four components: deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP). The recently proposed total biosynthesis of dNTP in our previous reports [1–4] provided an environmentally friendly and cost-effective way to produce these small molecules. This new method required a two-step enzymatic phosphorylation reactions starting from deoxynucleoside monophosphates (dNMPs) to deoxynucleoside diphosphates (dNDPs) in the first step and to the dNTPs in the second step, in which phosphoenolpyruvate (PEP) was used as the phosphate donor. The four dNDP formation reactions from the corresponding dNMP substrates in the first step phosphorylations were catalyzed by four individual kinase enzymes, deoxyadenylate kinase, deoxyguanylate kinase, deoxycytidylate kinase, and deoxythymidylate kinase, respectively, while the four dNTP formation reactions in the second step phosphorylations from dNDPs to dNTPs were catalyzed by one single promiscuous enzyme, pyruvate kinase (PK).

PK is an important allosteric enzyme in glycolysis and also a typical substrate promiscuous enzyme that catalyzes various energy producing reactions, such as ADP to ATP, GDP to GTP, CDP to CTP, UDP to UTP, TDP to TTP, and the corresponding dNDPs to dNTPs [5]. The existence of the nonspecific binding site for various nucleoside diphosphate substrates in PK makes the enzyme very useful in various *in vivo* and *in vitro* enzyme reactions [6]. In the total biosynthesis of the four dNTP components, the commercially available rabbit muscle PK was used to catalyze the second step phosphorylation reactions from dADP to dATP, dGDP to dGTP, dCDP to dCTP, and dTDP to dTTP [4]. However, our previous studies show that this rabbit muscle PK has an extremely high selectivity for different dNDP substrates [1,2,4]. The specific activity of the rabbit muscle PK for the substrates ADP, dADP, dGDP, dCDP, and dTDP were approximately 630.21, 49.67, 7.19, 0.068, and 0.041 μmol/min mg protein, respectively. Comparing to the reaction rate on ADP or dADP substrates, the rabbit muscle PK catalyzed reaction rate on dGDP substrate was slow, and the reactions on dCDP and dTDP substrates were almost ceased. The result indicates that the rabbit muscle PK is a rather specific enzyme although it binds different dNDP substrates and does produce the corresponding triphosphates products.

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doi:10.1016/j.enzmictec.2008.06.004

Nomenclature

A	phosphoenolpyruvate (PEP)
B	dNDP
E	pyruvate kinase (PK)
EA	enzyme substrate complex, PK-PEP
EB	enzyme substrate complex, PK-dNDP
$K_{M,A}$	Michaelis constants of A (PEP) (mmol/L)
$K_{M,B}$	Michaelis constants of B (dNDP) (mmol/L)
V_{max}	maximum reaction rate (μmol/min/unit enzyme)
v	reaction rate in specific activity unit (μmol/min mg prot)

The high substrate selectivity of the rabbit muscle PK significantly restricted the application of this enzyme in the synthetic biochemistry, especially in the complicated enzyme reaction system with multiple substrates, such as the case of the dNTP biosynthesis. The great difference in the substrate selectivity and reaction rate increased amount of enzyme required and reaction time. In such a complicated biosynthetic system for a target product, a promiscuous enzyme with the approximately equal reaction rate on the multiple substrates is highly appreciated for the consideration of process efficiency. The question raised in the unbalanced biosynthesis of different dNTP components droved us to consider the discovery of a more promiscuous PK.

It was deduced that the high selectivity of the rabbit muscle PK probably was the result of the evolutionary process of gaining energy for the adaptation to the cellular environment. The immediate requirement of energy by the animal (rabbit) muscle in the form of ATP conversion required the fast conversion of ATP/ADP, while other form of nucleoside triphosphates (GTP, CTP, TTP, and dNTPs) were not in such an urgent need because these nucleoside triphosphates did not involve in the energy conversion [7]. Based on this deduction, the PK enzymes from microbial sources at the lower level of evolutionary trees were selected. The microorganisms do not require fast energy conversion in their generally low Reynolds number living environments and therefore their PKs are likely more promiscuous for different nucleoside diphosphates substrates. For this purpose, two microorganisms, the Gram-positive *Bacillus* sp. ATCC 21616, and the Gram-negative *Zymomonas mobilis* ZM4 ATCC 31821, were chosen as the sources to obtain the promiscuous PKs. More specifically, the strategies described in this paper were: (1) isolate the genes of PK from the genome of these two organisms, (2) clone the genes into an expression vector plasmid for over-expression of corresponding PKs with different gene sources in *E. coli*, and (3) characterize the kinetic properties of the acquired PKs and test the reaction rate on different nucleoside diphosphates as substrates. The results showed that the proposed strategy worked and the promiscuity of the PKs from the two microorganisms improved significantly. The newly acquired PK can be applied to the biosynthesis of dNTPs and the process efficiency was significantly improved. To our knowledge, this was the first report on the promiscuity of pyruvate kinase for synthesis of dNTPs using the gene cloned from different microorganisms.

2. Materials and methods

2.1. Materials

2′-Deoxyadenosine 5′-diphosphate sodium salt (dADP), 2′-deoxyguanosine 5′-diphosphate sodium salt (dGDP), 2′-deoxycytidine 5′-diphosphate sodium salt (dCDP), 2′-deoxythymidine 5′-diphosphate sodium salt (dTDP), phosphoenolpyruvic acid monopotassium salt (PEP), rabbit muscle PK, and L-lactate dehydrogenase (LDH) from porcine heart were purchased from Sigma–Aldrich (St. Louis, MO, USA). Nicotinamide adenine dinucleotide (NADH-reduced form), and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Shanghai Sangon Co. (Shanghai, China).

2.2. Organisms and plasmids

Bacillus sp. ATCC 21616 and *Z. mobilis* ZM4 ATCC 31821 strains were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). The cloning vector plasmid pUC19 and its host *E. coli* strain DH5α the expression vector pET28a (+) and its host *E. coli* strain BL21 (DE3) were products of Novogen (Madison, WI, USA). The LB medium used for *E. coli* and *B.* sp. culture contains 10 g of peptone, 5 g of yeast extract, and 5 g of sodium chloride per liter deionized H_2O with 100 μg/mL of ampicillin or 50 μg/mL of kanamycin. The YPG medium used for *Z. mobilis* culture contains 10 g yeast extract, 10 g peptone, 20 g glucose per liter of deionized H_2O.

2.3. Gene cloning

The genomic DNA of *B.* sp. and *Z. mobilis* was prepared using Qiagen DNeasy Tissue Kit (Valencia, CA, USA). The concentration of purified genomic DNA obtained was measured using UV spectrophotometer at 260 nm. The genes encoding PK (*Pyk*) were amplified by polymer chain reaction (PCR) using the gene sequence information from NCBI (Web reference [16]). The PCR primers for amplifying *Z. mobilis pyk* were ZPKF (forward): GCTCTAGAAGGAGGTGAAGCTTGACTGAA containing an XbaI site at the 5′-end, and ZPKR (reverse): CACCTCGAGAATTAGGCTTCGATAACATC containing an XhoI site at the 5′-end [8]; and the primers for amplifying *B.* sp. *pyk* were BPKF (forward): GCTCTAGAAGGAGGTGAACAAATGAGAAAAAC containing an XbaI site at the 5′-end, and BPKR (reverse): CACCTCGAGTAATTAAAGAACGCTCGCACG containing an XhoI site at the 5′-end (used to amplify the *B.* sp. *pyk* gene). The PCR reaction was carried out using ABI GeneAmp PCR system 9700 (Foster City, CA, USA) at 5 min denaturation at 94 °C and then 30 cycles of 30 s denaturation at 94 °C, 60 s reannealing at 47 °C and 45 °C for *B.* sp. *pyk* and *Z. mobilis pyk*, respectively, and 90 s elongation at 70 °C using the PCR Kit from Takara (Dalian, China). The PCR products were subcloned into the cloning vector plasmid pUC19 and then transformed into *E. coli* strain DH5α. The *pyk* genes were sequenced using the corresponding PCR primers for the confirmation of the gene sequence PCRed from the *B.* sp. and *Z. mobilis* genomes. Then the gene fragments were cleaved, gel purified, and ligated into the expression vector plasmid pET28a(+). The constructed plasmids were transformed into the host *E. coli* strain BL21(DE3) for over-expression of PK enzymes.

2.4. Expression of PK in E. coli and enzyme assays

0.5 L of fermentation medium was inoculated with 5 mL of overnight culture of freshly transformed *E. coli* strain BL21(DE3) harboring expression plasmids in LB medium with 50 mg/mL of kanamycin. IPTG was added to 0.4 mmol/L at OD_{600} = 0.6 and the cultivation continued for another 4 and 6 h for over-expression of *B.* sp. PK and *Z. mobilis* PK, respectively. The culture temperature was 37 °C and 28 °C for *B.* sp. PK and *Z. mobilis* PK expression, respectively. The same protocols in Bao et al. [1–3] for the recovery of cells, enzyme purification, and enzyme activity assay were followed. The same kinetic model and kinetic assay protocols in Bao et al. [1,2] were followed using a Beckman DU-800 spectrophotometer for determination of kinetic parameters. The working equation for Michaelis constant determination is given in Eq. (1) for the conditions [A] (PEP) = constant and [B] (Mg-dADP, Mg-dGDP, Mg-dCDP, or Mg-dTDP) = constant, respectively.

$$\frac{1}{v} = \frac{K_{M,AB}K_{M,A}}{V_{max}}\left(\frac{1}{[B]} + \frac{1}{K_{M,B}}\right)\frac{1}{[A]} + \frac{1}{V_{max}}\left(\frac{K_{M,AB}}{[B]} + 1\right) \quad (1)$$

2.5. dNDP conversions to dNTP by PK

The dNDP conversion experiments to dNTP by PK were carried out in a 5-mL test tube and the reaction was monitored using high performance liquid chromatography (HPLC, LC-20A, Shimadzu, Kyoto, Japan) with an Intensil ODS-3 column (4.6 mm $D \times 250$ mm L, GL Sciences, Tokyo, Japan). Samples were assayed at 5 min intervals during the reaction. The samples were filtered using Millipore (Bedford, MA, USA) Ultrafree-Centrifugal Filter Units with the molecular weight cut-off of 10,000. The diluted samples were injected into the column and eluted by feeding 20 mmol/L KH_2PO_4–K_2HPO_4 buffer solution containing 5% (by volume) methanol at a flow rate of 0.8 mL per minute at an ambient temperature, while monitoring absorbance at 254 nm.

3. Results

3.1. Cloning and over-expression of pyk genes in E. coli

The expression plasmids harboring *B.* sp. and *Z. mobilis pyk* genes were constructed on pET-28(+) using the two restriction sites XbaI/XhoI and named as pETBPK and pETZPK, respectively. The *pyk*

Table 1
Information of pyruvate kinase gene cloning and expression

Gene sources	*B.* sp.	*Z. mobilis*
Gene length (bp)	1755	1428
Protein MW (kDa)	62.2	51.4
Cultivation temperature (°C)	37	28
Crude enzyme yield (mg/L)	57.6	22.4

gene fragments from *B.* sp. and *Z. mobilis* sources were 1.7 kb and 1.4 kb, respectively. The two plasmids, pETBPK and pETZPK, were transformed into the *E. coli* BL21(DE3) strains for over-expression of PK. The information on gene cloning and expression was summarized in Table 1. The SDS-PAGE gel electrophoresis of the two expressed PK enzymes was shown in Fig. 1. Fig. 1 indicated that the *B.* sp. PK were well over-expressed and recovered as soluble protein. The expression of *Z. mobilis* PK was also over-expressed at 37 °C but was found to be insoluble inclusion body. To obtain the soluble protein of *Z. mobilis* PK, the culture temperature was lowered to 28 °C to let the expression work slowly, and the soluble protein was found in the supernatant as shown in Fig. 1. Fig. 1 also indicated that while the soluble enzyme was obtained under the low culture temperature, the expression level was significantly decreased and the insoluble inclusion body still existed. The purified enzymes were obtained using the Sephadex G-100 column as described by Bao and Ryu [3].

3.2. Substrate specificity to different nucleoside diphosphates (dNDP)

The substrate specificity of the two expressed *B.* sp. and *Z. mobilis* PKs, as well as rabbit muscle PK, on different nucleoside diphosphates substrates were assayed. One unit of PK was defined as the 1 μmol of ADP substrate converted per minute under the condition specified. The specific activity of the PK on the specific (d)NDP substrate was defined as the μmol of (d)NDP converted per minute using 1 unit PK enzyme. The relative specific activity was defined as the ratio of the reaction rate using a defined dNDP substrate to the rate using ADP substrate and the results were shown in Table 2. Table 2 indicated that the *B.* sp. and *Z. mobilis* PK catalyzed reaction rates on dNDP substrates improved significantly comparing to that of the rabbit muscle PK catalyzed reactions.

3.3. Determination of kinetic parameters of PKs on different substrates

For further comparison of the substrate promiscuity of the bacteria PKs on different nucleoside diphosphate substrates, the kinetic parameters of the *Z. mobilis* PK including the maximum reaction velocity and Michaelis constants were determined at 25 °C and pH 7.9. The results are summarized in Table 3. The kinetic parameters of the rabbit muscle PK determined in our previous work [1,2] are also listed in Table 3.

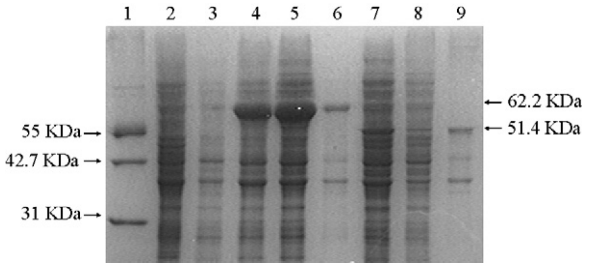

Fig. 1. Expression of pyruvate kinase from *B.* sp. and *Z. mobilis* under IPTG induction. Lane 1, protein MW markers; lane 2, BL21(DE3) control; lane 3, pET28a (+) control; lane 4, total protein expression (PK from *B.* sp.); lane 5, supernatant expression (PK from *B.* sp.); lane 6, protein in precipitation (PK from *B.* sp.); lane 7, total protein expression (PK from *Z. mobilis*); lane 8, supernatant expression (PK from *Z. mobilis*); lane 9, protein in precipitation (PK from *Z. mobilis*). The induction concentration of IPTG is 0.4 mmol/L. Culture temperature is 37 °C and 28 °C, respectively.

Table 2
Relative specific activity of different pyruvate kinases (PK) on various nucleoside diphosphates

Substrates	ADP	dADP	dGDP	dCDP	dTDP
Rabbit muscle PK	1.0	0.099	0.0182	0.00082	0.00035
B. sp. PK	1.0	0.23	0.102	0.005	0.046
Z. mobilis PK	1.0	0.26	0.44	0.029	0.048

Reaction conditions used: 25 °C, 50 mmol/L Tris buffer solution (pH 7.9), 100 mmol/L KCl, 10 mmol/L $MgCl_2$, 1 mmol/L PEP, 1 mmol/L dNDP, 10 U LDH per mL, 10 mg NADH per mL. Initial rates were assayed using Beckman DU-800 spectrophotometer at 340 nm for about 0.5–1 min.

3.4. Time course of dNDP conversion by different PKs

Fig. 2 shows the time course of dADP, dGDP, dCDP and dTDP conversion to the corresponding dNTPs by the three PK enzymes from different organisms: rabbit muscle, *B.* sp., and *Z. mobilis*. All the reactions were carried out under the same conditions except the PK enzyme added was 0.5 unit per mL for the dADP and dGDP conversions, and 5 units per mL for the dCDP and dTDP conversions in all conversion cases. As shown in Fig. 2, the rabbit muscle PK catalyzed reactions worked effectively only for dADP conversion, while the reaction rate was extremely slow for dGDP, dCDP and dTDP conversion reactions. The *B.* sp. PK and *Z. mobilis* PK catalyzed reactions worked effectively for all the four dNDP substrates, especially for the dGDP and dTDP conversion.

4. Discussion

Enzyme promiscuity was a useful synthesis tool that enabled fewer enzymes to perform the multiple functions required by the synthetic system [7,9]. Generally, specialized genes will be adapted to increase the metabolic efficiency during the evolutionary process. A rational deduction suggests that bacteria sourced enzyme would have more chances to be a 'generalist' promiscuity enzyme, while the mammalian animal sourced enzyme would evolve to a 'specialist' adapted to the function of great energy demand [7]. In this work, a promiscuous pyruvate kinase (PK) enzyme was taken as

Table 3
Kinetic parameters determined for PK from *Z. mobilis*

	$K_{M,A}$ (mmol/L)	$K_{M,B}$ (mmol/L)	V_{max} (μmol per minute per unit enzyme)
dADP substrate			
Rabbit muscle PK	0.0458	4.67	0.103
Z. mobilis PK	0.142	1.10	0.541
dGDP substrate			
Rabbit muscle PK	0.112	7.03	0.029
Z. mobilis PK	0.093	0.43	0.774
dCDP substrate			
Rabbit muscle PK	0.0773	9.38	0.0026
Z. mobilis PK	0.217	3.22	0.0529
dTDP substrate			
Rabbit muscle PK	0.0815	13.04	0.00181
Z. mobilis PK	0.127	1.79	0.0643

Reaction conditions used: 25 °C, 50 mmol/L Tris buffer solution (pH 7.9), 100 mmol/L KCl, 10 mmol/L $MgCl_2$, 10 U LDH per mL, 10 mg NADH per mL. One unit of PK was defined as the ADP converted per minute per mg protein under the conditions specified.

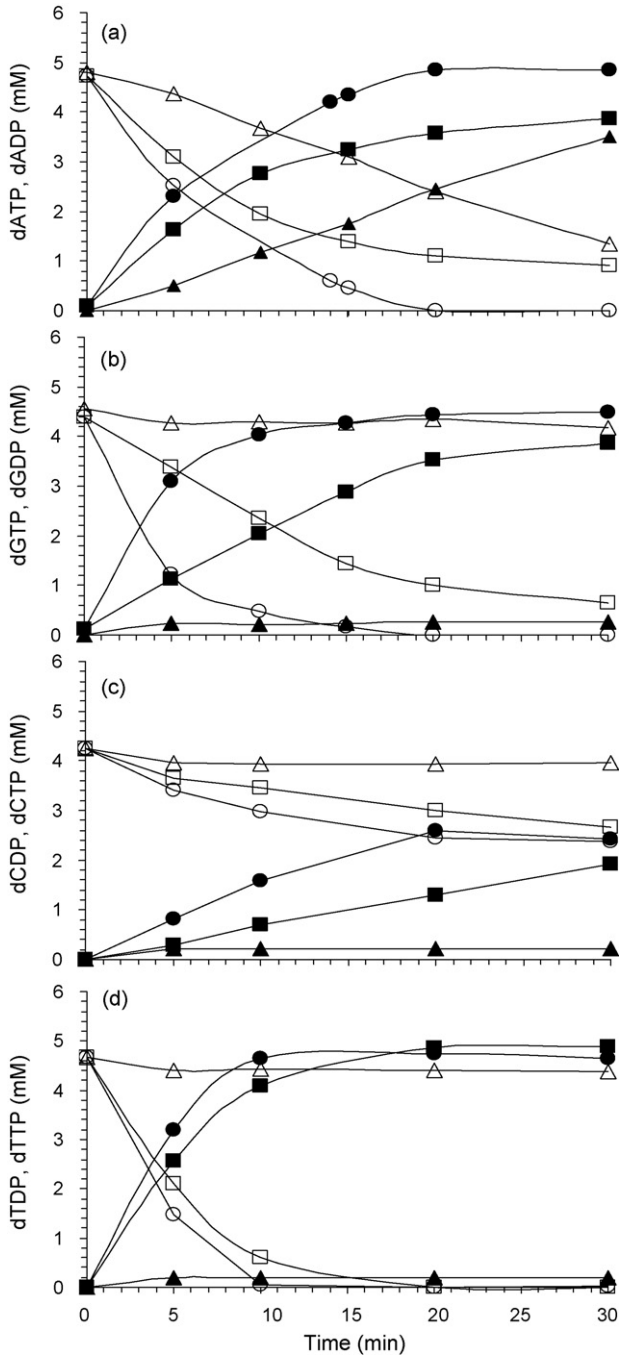

Fig. 2. Time course of dNDP conversion to the corresponding dNTP using different PK enzymes. Each reaction tube contained 50 μL of PK solution, all in 1.0 mL Tris buffer solution. The 50 mmol/L Tris buffer solution (pH 7.9) contained dADP, dGDP, dCDP or dTDP, PEP, Mg^{2+}, K^+ and PEP were incubated in a water bath maintained at 30 °C, the final concentration are 100 mmol/L for K^+, 10 mmol/L for Mg^{2+}, 10 mmol/L for PEP, and 5 mmol/L for dNDP. △ ▲ rabbit muscle PK, □ ■ *B. sp.* PK, and ○ ● *Z. mobilis* PK. Open symbols indicate dNDP substrates, closed symbols indicate dNTP products. (a) dADP conversion. PK 0.5 unit/mL; (b) dGDP conversion. PK 0.5 unit/mL; (c) dCDP conversion. PK 5 units/mL; and (d) dTDP conversion. PK 5 unit/mL.

a model for comparison with the rabbit muscle sourced enzymes. It was deduced that the bacteria sourced PK has more reasons to be a substrate promiscuous enzyme, because the bacteria PK has less energy demand for fast conversion from ADP to ATP by PK, compared to that of the animal muscle PK, and the evolution process will have little effect on modifying its selectivity on different nucleoside diphosphates substrates.

In our previous works [1,2,4], we found that the rabbit muscle PK is a typical specific enzyme with a high selectivity for ADP substrate to ATP, while the activity for other nucleoside diphosphates substrates is rather low. In this work, the Gram-positive *Bacillus* sp. ATCC 21616 and the Gram-negative *Z. mobilis* ZM4 ATCC 31821 were chosen as the two model organisms to obtain the bacteria sourced PK for investigation of their substrate promiscuity. The PK genes (*pyk*) from the Gram-positive bacteria *B.* sp. and Gram-negative bacteria *Z. mobilis* were cloned and over-expression in *E. coli* BL21 (DE3). Similar to Saeki and Ohta's work [10], the soluble *B.* sp. PK was approximately 30% of the total soluble protein after 4 h induction by 0.4 mmol/L IPTG. On the other hand, different from Steiner et al. [8], the *Z. mobilis* PK was expressed mainly in the form of insoluble inclusion body. The soluble PK was obtained when the culture temperature was lowered to 28 °C. In this work, the soluble *Z. mobilis* PK was only approximately 5% of the total soluble protein. The reason for the low recovery yield of the soluble PK might come from the high content of proline residue in its amino residues of the protein composition. There are 31 proline residues among the 475 amino acid residues in *Z. mobilis* PK, compared to 17 proline residues among 585 amino acid residues of *B.* sp. PK. Studies showed that the rates of unfolding and refolding of a polypeptide is affected significantly by the number of proline residues in a protein due to the proline isomerization [11,12].

The expressed *B.* sp. PK and *Z. mobilis* PK showed a significant promiscuity for the dNDP substrates, especially for the dCDP and dTDP substrates. For dADP substrate, the relative specific rate of *B.* sp. and *Z. mobilis* PKs was two folds greater than that of rabbit muscle PK. For dGDP substrate, the relative specific rate of *B.* sp. and *Z. mobilis* PKs was five folds and twenty folds greater than that of rabbit muscle PK, respectively. For dCDP and dTDP substrates, the relative specific rate of *B.* sp. and *Z. mobilis* PKs was one or two orders of magnitude greater than that of rabbit muscle PK. The results indicated that the promiscuity of the *B.* sp. and *Z. mobilis* PK on dNDP substrates were significantly stronger than that of rabbit muscle PK. These results were confirmed by the kinetic data and the dNDP conversion experiments. The Michaelis constant $K_{M,B}$ of the *Z. mobilis* PK for dADP, dGDP, dCDP, and dTDP were 1.10, 0.43, 3.22, and 1.79 mmol/L, respectively, while the $K_{M,B}$ of the rabbit muscle PK were 4.67, 7.03, 9.38, and 13.04 mmol/L, respectively. The maximum reaction rate V_{max} of the *Z. mobilis* PK for dADP, dGDP, dCDP, and dTDP were 0.541, 0.774, 0.0529, and 0.0643 μmol per minute per unit enzyme, respectively, while V_{max} of the rabbit muscle PK were 0.103, 0.029, 0.0026, 0.00181 μmol per minute per unit enzyme, respectively. The V_{max} data indicate that the reaction rate of the *Z. mobilis* PK was about one order of magnitude greater than that of the rabbit muscle PK, and the maximum velocity of *Z. mobilis* PK on dNDP substrates varies in the range of one order of magnitude, while the maximum velocity of rabbit muscle PK for dNDP substrates varies in the range of two orders of magnitude.

The bacteria sourced promiscuous PK was applied to the biosynthesis of dNTP. The dNDP conversions catalyzed by *B.* sp. and *Z. mobilis* PKs worked efficiently for all four dNDP substrates, especially for the dCDP and dTDP conversions. The process efficiency was significantly improved with *B.* sp. PK and *Z. mobilis* PK. The high promiscuity of the bacteria sourced PK for the dADP, dGDP, dTDP and dCDP conversion could lead to a significant cost reduction and improvement of process efficiency for dNTP synthesis in the complicated enzyme reaction system with multiple substrates. In this study, a demonstration of principle is described how one can effectively improve the enzyme specificity, selectivity, and activity and use the enzyme for simultaneous and efficient conversion of multiple substrates to multiple products in a bioreactor.

The catalytic center of pyruvate kinase is composed of three longest loops of the $(\beta/\alpha)_8$-barrel [13]. The ADP/ATP binding

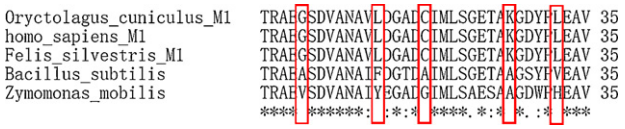

Fig. 3. Alignment of the ADP/ATP binding sequence of PKs from different sources. The origins and accession numbers for these sequences are: *Bacillus subtilis* (D13095) (bacterium); *Zymomonas mobilis* (D13095) (bacterium); *Homo sapiens* M1 (M15465); *Felis silvestris catus* M1 (P11979) (cat); *Oryctolagus cuniculus* M (U09028) (rabbit). The sequences were aligned by the ClustalW program [15].

site lies in domain A, near the domain C. The 34 residues identified as being near the ADP/ATP sites in rabbit muscle PK are: ^{340}TRAEGSDVANAVLDGADCIMLSGETAKGDYPLEA^{373} (rabbit PK numbering) [14]. The protein structure blast result among the human muscle M1, rabbit muscle M1, cat muscle M1, *B. subtilis*, *Z. mobilis* is showed in Fig. 3. The ADP/ATP binding sequences are highly conserved and there is a high degree of identity among them. Most residues are absolutely conserved, except for G344, L352, C357, K366, and L370. The Gly-344 residue is replaced by a Val in *Z. mobilis* PK and an Ala in *B. subtilis* PK. The Leu-352 residue is replaced by a Tyr in *Z. mobilis* PK and an Phe in *B. subtilis* PK. The Cys-357 residue is replaced by a Gly in *Z. mobilis* PK and an Ala in *B. subtilis* PK. The Lys-366 residue is replaced by an Ala in *Z. mobilis* and *B. subtilis* PKs. This residue is also presents in all the plants PKs. Residue Leu-370 is replaced by a His in *Z. mobilis* PK and a Val in *B. subtilis* PK. All these residues replacement may change the substrate specificity.

Acknowledgements

This research was supported by the National Science Foundation of China (Grant No. 20676037), Shanghai Pujiang Program (Grant No. 06PJ14022), and Ministry of Education of China (Grant No. 107123).

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