J. Biochem. **136**, 471–476 (2004)
DOI: 10.1093/jb/mvh152

Possible Structure and Function of the Extra C-Terminal Sequence of Pyruvate Kinase from *Bacillus stearothermophilus*

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Received March 31, 2004; accepted July 12, 2004

The pyruvate kinases from Genus *Bacillus* and a few other bacteria have an extra C-terminal sequence with a phosphoenolpyruvate binding motif composed of about 110 amino acids. To elucidate the possible structure and function of this sequence, the enzyme lacking the sequence was prepared and characterized. The N-terminal sequences of the peptides, which were found only in the lysylendopeptidase digest of the wild enzyme and not in that of the truncated enzyme, were determined. All the determined sequences were found in the extra C-terminal sequence deduced from the DNA sequence. The truncated enzyme showed decreased affinity for phosphoenolpyruvate and the allosteric effector ribose 5-phosphate, and had a reduced thermostability. Other properties, such as tetrameric structure, specific activity, and allosteric characteristics were unchanged. A comparison of the CD spectra of the truncated enzyme and the recombinant enzyme indicated that the structure of the C-terminal sequence should be rich in β-sheet. These findings suggest that the sequence actually exists and that it may form a steady domain interacting with the A-domain and C-domain, which are the catalytic domain and allosteric effector binding domain, respectively.

Key words: domain, extra C-terminal sequence, phosphoenolpyruvate binding motif, pyruvate phosphate dikinase, truncated enzyme.

Abbreviations: ECTS, extra C-terminal sequence; FBP, fructose 1,6-bisphosphate; R5P, ribose 5-phosphate; $S_{0.5}$, the substrate concentration required for half-maximal saturation; TFA, trifluoroacetic acid.

Pyruvate kinase is a key enzyme in glycolysis, catalysing the transphosphorylation from phosphoenolpyruvate (PEP) to ADP to form pyruvate and ATP. Its activity is regulated in various ways. In mammals, four types of isozymes with different kinetic properties are known (*1*). The activity of the liver enzyme is also regulated by phosphorylation-dephosphorylation by a cAMP-dependent protein kinase and a phosphatase (*2*). Almost all pyruvate kinases from single cell organisms are allosteric enzymes that are activated by nucleoside monophosphates or sugar phosphates such as fructose 1,6-bisphosphate (FBP) and inhibited by ATP or inorganic phosphate. The structures of pyruvate kinases are believed to be very similar. Although some exceptions have been reported, they are generally homotetramers with a subunit molecular mass of about 50–62 kDa. As their amino acid sequences are very similar, we are able to align the sequence reasonably (*3*).

The pyruvate kinase from a moderate thermophile, *Bacillus stearothermophilus,* is an allosteric enzyme activated by AMP or ribose 5-phosphate (R5P), but not by FBP (*4*). It is very stable not only at high temperatures, but also at low temperatures over long storage periods, despite the instability of other microbial pyruvate kinases. The gene for the enzyme has been cloned and sequenced (*5*, *6*). A characteristic feature of the deduced amino acid sequence is that the enzyme has a long extra C-terminal sequence (ECTS) composed of about 110 amino acid residues. A part of the sequence is highly homologous to the phosphoenolpyruvate binding motif observed in phosphoenolpyruvate: sugar phosphotransferase system enzyme I, pyruvate phosphate dikinase and phosphoenolpyruvate synthase (*5*, *7*). In the center of the motif is a histidine residue that is conserved in all above enzymes, and which is phosphorylated in the course of the enzymatic reaction. Since its discovery, this ECTS have been found in enzymes from three *Bacilli*, one *Lactobacillus*, and one cyanobacterium (*3*, *8–10*). All have the PEP binding motif. Interestingly, the histidine residue that plays an essential role in the above enzymes is also conserved in all ECTS of pyruvate kinases. Nguyen and Staier predicted that the ECTS can bind phosphoenolpyruvate and should have some catalytic property (*11*). As the amino acid sequence deduced from one of the initial reports of the *B. steraothermophilus* pyruvate kinase gene lacked the ECTS (*6*), even the presence of the ECTS is not fully verified. In this paper, we present clear evidence for the presence of the ECTS and some characteristics of an enzyme lacking the sequence to elucidate the role of the ETCS.

EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains—Pig muscle lactate dehydrogenase, tricyclohexylammonium phosphoenolpyruvate, R5P and ADP were purchased from Roche Biochem-

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icals. NADH was a product of Oriental Yeast Co. Ltd., Japan. Butyl-Toyopearl 650S was obtained from Toyosoda, Japan. A Mono-Q HR10/10 column was a product of Amersham Biotechnology Inc. The lysylendopeptidase was product of Wako Chemicals, Japan. *Esherichia coli* MV1184 [*ara* Δ(*lac-proAB*) *rpsL thi* ϕ*80lacZ*Δ*M15* Δ(*srl-recA*)306::Tn*10*(*Tc*^{r})/F′: *traD36 proAB lac*I^q *lacZ*ΔM15], MV1190 [Δ(*lac-proAB*) Δ(*srl-recA*)306::Tn*10*(*Tc*^{r}) *supE thi* /F′: *traD36 proAB lac*I^q *lacZ*ΔM15], XL1-Blue [*recA lac endA1 gyrA96 thi hsdR17 supE44 relA1*/F′ *proAB lac*I^q *lacZ*ΔM15 Tn*10*(*Tc*^{r})], and BW313 [HfrKL16PO/45 [*lys*(61–62)/*dut*1] *ung*1 *thi*-1 *relA*1] were employed for the production of the enzyme and site-directed mutagenesis.

Site-Directed Mutagenesis and Related Techniques—Expression vectors for pyruvate kinase, pKH501, and its derivative with 5 unique restriction enzyme recognition sites, pKH510, were prepared as described elsewhere. The amount of pyruvate kinase produced by *E. coli* cells harboring pKH501 was about 40% of total soluble protein after 16 h cultivation under isopropyl-1-thio-β-D-galactopyranoside induction (0.5 mM) at 37°C in Luria-Bertani broth (1% bacto-tryptone, 0.5% bacto-yeast extract and 0.5% NaCl). Site-directed mutagenesis was carried out by the method of Kunkel (*12*) using U-containing single-stranded DNA prepared from *E. coli* BW313 cells harboring pKH501. The DNA sequence was determined by the dideoxy chain termination method (*13*). Truncation of the ECTS of pyruvate kinase was achieved by changing the AAA codon of lysine 480 to a TAG amber nonsense codon using oligonucleotide 5′-CCTTGCCCCTAGGCGAGA-AGA-3′. The introduction of the amber codon was confirmed by the concomitant introduction of a *Sty*I site, and the plasmid thus obtained was designated pKH511. The entire coding regions of the pyruvate kinase mutants were sequenced, confirming the introduction of the mutation and the integrity of the remaining sequence.

Enzyme Purification—One percent inoculi of *E. coli* MV1184 cells harboring plasmid pKH510 or pKH511 were cultivated overnight at 37°C in Luria-Bertani broth containing 50 μg/ml of ampicillin. Four hours after inoculation, isopropyl-1-thio-β-D-galactopyranoside was added to 0.5 mM and the cultivation was continued for 16–18 h. The cells were harvested by centrifugation, suspended in 3 volumes of 25 mM Tris-HCl buffer, pH 7.5, containing 50 mM KCl and 10 mM $MgCl_2$, and disrupted with a Sonifier ultrasonic disruptor (Branson, Connecticut). Debris was removed by centrifugation, and solid ammonium sulfate was added to the supernatant to 0.35 saturation. The solution was centrifuged to remove insoluble materials, and applied onto a Butyl-Toyopearl column equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 0.35 saturated ammonium sulfate. The enzyme was eluted by a decreasing linear gradient of ammonium sulfate. The fractions containing the enzyme were collected and dialyzed against 25 mM Tris-HCl buffer, pH 7.5, and applied onto a MonoQ HR10/10 column. The enzyme was eluted by a linear gradient of NaCl (0–0.5 M). The eluted enzyme was concentrated and the buffer was replaced with 25 mM Tris-HCl buffer, pH 7.5, using a centricon-30 (Amicon, Danvers, MA). The proteins obtained from *E. coli* cells harboring pKH510 and pKH511 are designated as the recombinant enzyme and truncated enzyme, respectively, in this paper.

N-Terminal Sequence Analysis of Peptides in the ECTS—Pyruvate kinase from *B. stearothermophilus* cells was purified according to the method described in Ref. *4* and designated as the wild enzyme. One nanomole each of the wild and truncated enzymes in 100 μl of 50 mM Tris-HCl, pH 8.0, was denatured by boiling and digested at 37°C for 6.5 h with 1:200 (w/w) lysylendopeptidase. The digests were reduced by the addition of EDTA and β-mercaptoethanol to final concentrations of 2.7 mM and 70 mM, respectively, for 4 h at room temperature. The reduced peptides were carboxymethylated by the addition of 1 M of iodoacetic acid to a final concentration of 70 mM and incubated at room temperature for 30 min in the dark. The peptides were separated on a C8 HPLC column, Senshu-Pak FP-308-1252, eluted by a linear gradient from 0.1% TFA to 0.07% TFA containing 80% CH_3CN. The N-terminal amino acid sequences of the peptides found only in the wild enzyme were determined by an Applied Biosystem Model 470A protein sequencer.

Enzymatic Assay—The activity of the enzyme was measured by a coupling assay method using lactate dehydrogenase (*4*). During purification, the enzymatic activity was measured at 2 mM PEP and 4 mM unpurified ADP. In the kinetic measurements, commercial ADP was further purified using Dowex 1X-2 to remove contaminating AMP (*4*). Protein was measured by the method of Lowry (*14*) using bovine serum albumin as the standard. The molecular activity of the enzyme is expressed as the number of PEP molecules converted to pyruvate by a tetrameric enzyme in one second. The molecular masses of the wild enzyme and the truncated enzyme are assumed to be 249,268 Da and 205,644 Da, respectively. The kinetic data were analyzed using KaleidaGraph (Synergy Software, Reading, PA).

PAGE with and without SDS—PAGE with and without SDS were carried out by the methods of Laemmli (*15*) and Davis (*16*), respectively. Gels were stained with Coomassie Brilliant Blue R-250.

CD Spectra—The CD spectra of the recombinant and truncated enzymes were measured by JASCO J-720 spectropolarimeter at concentrations of 0.04 and 0.08 mg/ml protein in 10 mM Na-phosphate buffer, pH 6.4, in a 1 mm light path cell. The secondary structure contents were calculated according to mode2 (In this mode, the sum of the secondary structure content is fixed at 100%.) of the program supplied by the maker and based on Yang *et al.* (*17*).

RESULTS

Introduction of an Amber Mutation at Lysine 480—The AAA codon for lysine residue at position 480 was converted to an amber nonsense codon by site-directed mutagenesis to truncate the ECTS. *E. coli* cells MV1184 (*sup*$^+$) and MV1190 (*supE*) were transformed by pKH510, and mutated plasmids and the crude cell extracts were analyzed by SDS- and native PAGE (Fig. 1). *E. coli* cells harboring pKH510, which did not carry the amber mutation, produced large amounts of protein with an apparent molecular mass of 62 kDa. This was the case whether the host cells carried the *supE* mutation or not, and the molecular mass was identical to that of the pyruvate kinase from *B. stearothermophilus* (*4*). On the other

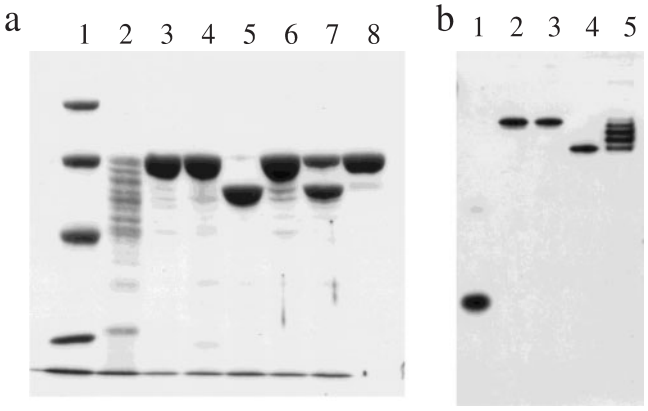

Fig. 1. **SDS and native PAGE of crude extracts of *E. coli* cells harboring pKH510 and pKH511.** (a) SDS-PAGE of crude extracts of *E. coli* cells harboring various plasmids at a gel concentration of 10%. Lane 1, molecular mass markers from top to bottom: phosphorylase *b*, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor; 20.1 kDa. Lane 2. *E. coli* XL1-Blue (pBluescript II SK$^+$); Lane 3, *E. coli* XL1-Blue (pKH501); Lane 4, E. coli MV1184 (pKH510); Lane 5, *E. coli* MV1184 (pKH511); Lane 6, *E. coli* MV1190 (pKH510); Lane 7, *E. coli* MV1190 (pKH511); Lane 8, purified pyruvate kinase from *Bacillus stearothermophilus*. (b) Native PAGE of *E. coli* cells harboring various plasmids at a gel concentration of 7%. Lane 1, bovine serum albumin as an electrophoretic mobility marker; Lane 2, *E. coli* XL1-Blue (pKH501); Lane 3, *E. coli* MV1184 (pKH510); Lane 4, *E. coli* MV1184 (pKH511); Lane 5, *E. coli* MV1190 (pKH511).

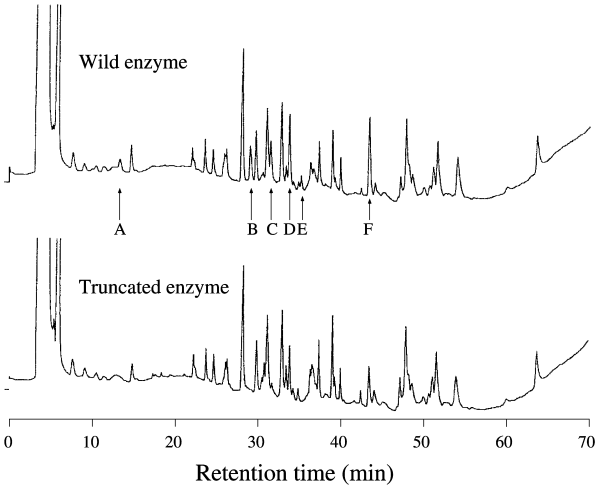

Fig. 2. **Elution profiles of lysylendopeptidase digests of wild and truncated enzymes from a C8 HPLC column.** Proteins were digested with lysylendopeptidase and separated as described in the text. The elution profiles of fragments of the wild enzyme (upper) and those of the truncated enzyme (lower) are presented. The peaks found only in the wild enzyme digests are indicated (A to F).

hand, *E. coli* MV1184 cells harboring pKH511, into which the amber mutation was introduced, produced large amounts of truncated protein with an apparent molecular mass of 52 kDa. This value is in good agreement with the calculated molecular mass of the truncated enzyme (51,411 Da) deduced from the DNA sequence. When the host cells carried the *supE* mutation, 62 kDa protein as well as 52 kDa protein were produced. This may be due to the partial suppression caused by the production of large amounts of enzyme. On native PAGE, the crude extracts of MV1184 harboring pKH510 and pKH511 showed single bands of enzyme with different mobilities. On the other hand, the crude extract from MV1190 cells harboring pKH511 showed additional 3 bands with intermediate electrophoretic mobilities. This suggests that the truncated enzyme is a tetramer, as is the case of the wild enzyme (*4*). The molecular masses of the recombinant and truncated enzymes were estimated by native PAGE, at varying gel concentrations (*18*) as 223 kDa and 186 kDa, respectively. This also supports the idea that the truncated enzyme is a tetramer.

Determination of N-Terminal Amino Acid Sequences of the Peptides in the ECTS—The HPLC patterns of the carboxymethylated lysylendopeptidase digests of the wild and truncated enzymes are presented in Fig. 2. Peptides that appear only in the wild enzyme must be derived from the ECTS. Some such peptides are indicated in Fig. 2 (A to F). The N-terminal amino acid sequences of peaks A, B, and C were determined as SAFGK, VHVISDLLAK, and DGQEITVD, respectively. Peak F contained a small amount of other peptides and was purified further to give the sequence AAAIITEEGGLTSHAAVVGLSL. Those sequences are found in the ECTS amino acid sequence deduced from the DNA sequence of the gene (*5*) as ^{488}SAFGK, ^{471}VHVISDLLAK, ^{566}DGQEITVD, and ^{528}AAAIITEEGGLTSHAAVVGLSL. This clearly indicates the presence of the ECTS in the *Bacillus stearothermophilus* pyruvate kinase.

Comparison of the Kinetic Properties of the Truncated and Recombinant Enzymes—The kinetic properties of the truncated and recombinant enzymes with respect to PEP in the absence and presence of 0.1 mM AMP are summarized in Table 1. The molecular activity of the truncated enzyme, taking into consideration the difference in molecular mass, is essentially identical to that of the recombinant enzyme. Both enzymes exhibited sigmoidal saturation curves for PEP with essentially identical Hill coefficients. They also exhibited hyperbolic saturation curves upon the addition of AMP. The truncated enzyme showed a 1.5 fold larger $S_{0.5}$ value for PEP in both the absence and presence of AMP.

Table 1. **Kinetic parameters of recombinant and truncated pyruvate kinases with respect to phosphoenolpyruvate.**

	Recombinant pyruvate kinase		Truncated pyruvate kinase	
Parameters	No addition	+0.1 mM AMP	No addition	+0.1 mM AMP
k_{cat} (/s)	926 ± 62	1399 ± 52	1085 ± 59	1729 ± 54
$S_{0.5}$ (mM)	0.96 ± 0.09	0.28 ± 0.05	1.46 ± 0.10	0.56 ± 0.06
Hill coefficient	2.24 ± 0.37	1.0	2.31 ± 0.25	1.0

The enzymatic activity is assayed using 1 mM purified ADP.

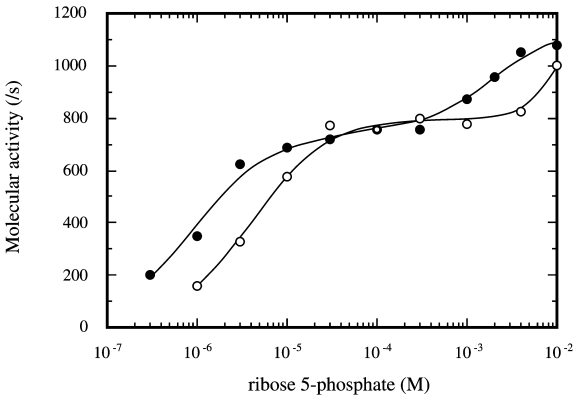

Fig. 3. **Activation profile of recombinant and truncated pyruvate kinases by R5P.** Pyruvate kinase activity was measured employing 1 mM purified ADP and 0.5 mM phosphoenolpyruvate at varying R5P concentrations. Recombinant pyruvate kinase (solid circles); truncated pyruvate kinase (open circles).

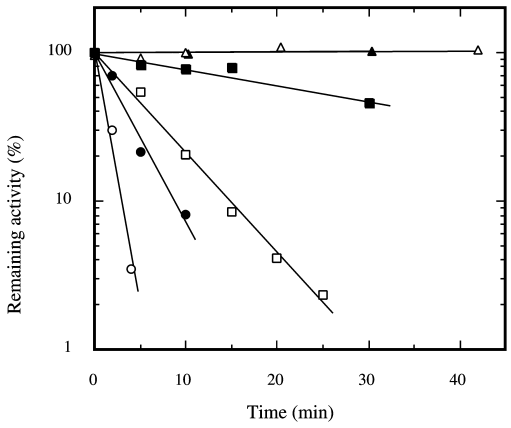

Fig. 4. **Thermostability of the recombinant and truncated pyruvate kinases.** The recombinant and truncated enzymes were incubated in 40 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 5 mM $MgCl_2$, at enzyme concentrations of 0.17 mg/ml (recombinant enzyme) and 0.15 mg/ml (truncated enzyme). Aliquots (1 μl) were withdrawn at the indicated times and the remaining activity was assayed at 30°C in 50 mM imidazole-HCl, pH 7.2, containing 50 mM KCl, 7 mM $MgCl_2$, 2 mM PEP, 4 mM ADP, 0.12 mM NADH, and 20 μg/ml pig muscle lactate dehydrogenase. The recombinant enzyme was incubated at: 60°C (solid triangles); 65°C (solid squares); and 70°C (solid circles). The truncated enzyme was incubated at: 60°C (open triangles); 65°C (open squares); and 70°C (open circles).

Activation Profile by R5P—R5P, another allosteric activator, changes the saturation curves of both enzymes for PEP to hyperbolic, as was seen in the presence of AMP (data not shown). The activation of the pyruvate kinase from *B. stearothermophilus* by R5P is characteristic in its two-stage activation (*19*). The activation profile of the pyruvate kinase by R5P is compared to that of the recombinant enzyme and presented in Fig. 3. The recombinant enzyme was activated by R5P in a two-stage manner, with half maximal concentrations of 0.87 ± 0.13 μM and 2.05 ± 0.88 mM, respectively. Those values are essentially identical to those of the wild enzyme (*19*). The truncated enzyme was also activated by R5P, with a half maximal activation about 5.4 fold larger then that of the recombinant enzyme. In the presence of more than a few millimolar R5P, the truncated enzyme is likely to be further activated and should have a lower affinity for the activator than the recombinant enzyme.

PEP Hydrolysis at High Concentrations of Enzyme—The pyruvate kinase reaction does not proceed in the absence of ADP. Therefore, when the enzyme is incubated in the presence of K^+, Mg^{2+}, and PEP in the absence ADP, no pyruvate is formed. If the PEP binding motif is phosphorylated by PEP alone, however, the formation of stoichiometric amounts of pyruvate is expected. So, to an assay mixture without ADP, recombinant enzyme was added to 1 mg/ml and the reaction was followed at 340 nm. A very slow decrease in absorbance was observed, and the decrease exceeded the expected value of stiochiometry (about 0.1). No initial rapid decrease in absorbance was observed. The same results were obtained with the truncated enzyme. This slow decrease in the absorbance at 340 nm may be due to small amounts of contaminating phosphatase or PEP hydrolytic activity, which is possessed by pyruvate kinase *per se* (*20*).

Thermostability of the Truncated Enzyme—The inactivation of each enzyme at various temperatures is presented in Fig. 4. The truncated enzyme was inactivated significantly faster than the recombinant enzyme. The temperatures at which the remaining activity was 50% of the initial activity after 10 min incubation were 67.2°C for the recombinant enzyme and 62.6°C for the truncated enzyme.

CD Spectra of the Recombinant and Truncated Enzymes—CD spectra in far UV region of the recombinant and truncated enzymes are shown in Fig. 5. The truncated enzyme shows a slightly deeper trough at 208–225 nm than the recombinant enzyme. Although very noisy, the difference spectrum is also presented in the figure. The calculated secondary structure contents are presented in Table 2 and indicate that the ECTS is rich in β-sheet.

DISCUSSION

Presence of the ECTS in Pyruvate Kinase—The evidence presented here clearly indicates the presence of a long ECTS in *B. stearothermophilus* pyruvate kinase as pre-

Table 2. **Secondary structure contents calculated from CD spectra.**

	α-helix	β-sheet	Turn	Random	Total
Recombinant	25.0 (147)	52.7 (309)	3.1 (18)	19.2 (113)	100 (587)
Truncated	32.0 (153)	42.3 (203)	6.4 (31)	19.3 (92)	100 (479)
ECTS	1.5 (2)	76.0 (82)	0.0 (0)	22.4 (24)	100 (108)

Values in parentheses are numbers of residues. Values for the ETCS were calculated from the numerical difference spectrum of the recombinant and truncated enzyme.

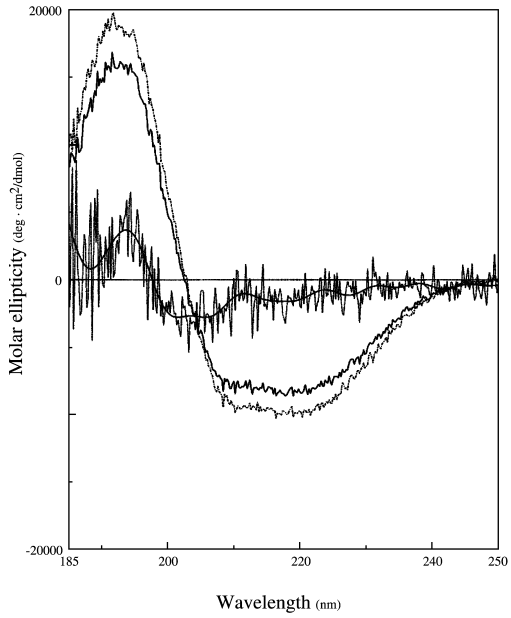

Fig. 5. **CD spectra of the recombinant and truncated enzymes.** The CD spectrum of the recombinant enzyme (bold line) and that of the truncated enzyme (dotted line) are presented. The difference spectrum, which should be attributable to the ECTS, is also shown (thin line).

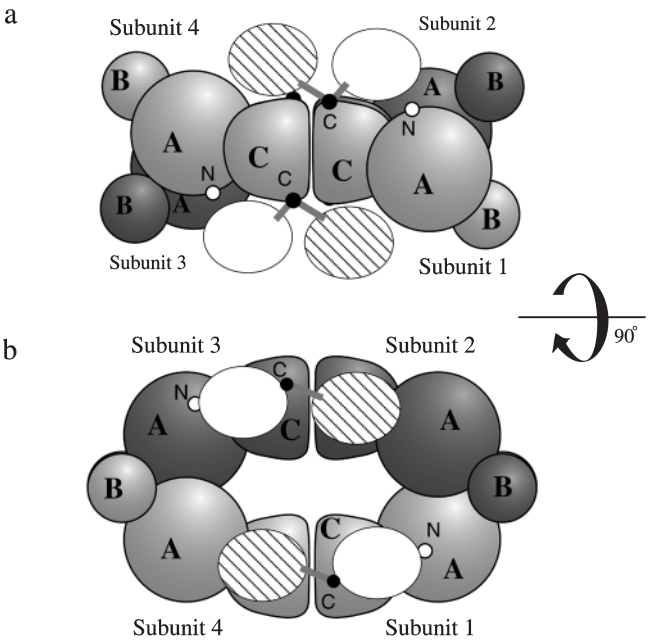

Fig. 6. **Schematic representation of the location of the ECTS domain in the pyruvate kinase structure.** Pyruvate kinase is composed of four identical subunits (1 to 4), each subunit comprising three domains, the A-, B-, and C-domains. Bacterial enzymes lack the N-terminal domain. The N-terminal (white circle) and C-terminal (black circle) amino acids are located in the A-domain and C-domain, respectively. Subunit 1 interacts with subunit 4 through the C-domain in a tail-to-tail manner, and with subunit 2 through the A-domain. The active site is located at the interface of the A-domain and B-domain, and the effector binding site is believed to be located in the C-domain. One possible location for the ECTS domain is indicated by the white ovals. In this orientation, the ECTS occupies a position that corresponds closely to that occupied by the N-terminal domain of mammalian enzymes and can interact with the A- and C-domains in the same subunit. Another possible location is indicated by the hatched ovals. In this case, the ECTS domain is slightly away from the C-domain, but can interact with the A- and C-domains of the neighboring subunit. a, side view; b, top view.

dicted from the genomic sequence (*5*). The sequence reported by Walker *et al.* (*6*) may contain some errors.

Structure of the ECTS—The CD spectra of the recombinant and truncated enzymes suggest that the ECTS is rich in β-sheet. The ECTS should not be a flexible loop nor stretch, but provide a steady domain. The phosphohistidine domain of pyruvate phosphate dikinase, which has a phosphoenolpyruvate binding motif resembling that of the ETCS, is composed of 115 amino acid residues and consists of two 4-stranded orthogonal β-sheets covered by three helices (*21*). Its β-sheet content was calculated to be 48%, a value compatible with our results if the ECTS has a similar structure to that of the phosphohistidine domain.

Location of the ECTS Domain—The first three dimensional structure of cat muscle pyruvate kinase indicated that the subunit consists of N, A, B, and C domains and that the active site is located between the A and B domains (*22*). The sequence homology of pyruvate kinases known so far enables us to propose that these enzymes share an essentially identical three dimensional structure (*3*). The N-terminal domain, composed of about 40 amino acid residues, forms an integral part of the intersubunit contact, and is completely lacking in bacterial enzymes. As the N- and C-termini are closely located (*22*), the ECTS may occupy a region of the N-terminal domain to stabilize the inter-domain interaction. The fact that *E. coli* pyruvate kinases lack both the N-terminal domain and the ECTS (*23*), however, strongly suggests that it is not necessary to fill the region occupied by the N-terminal domain with the ECTS. The C-domain of the enzyme is thought to be essential for subunit interaction (*24*), and the allosteric site is located in C-domain (*25*). The $S_{0.5}$ value for PEP and the concentration of R5P required for half maximal activation are increased 1.5 fold and 5.4 fold, respectively (Table 1). This indicates some possible interaction between the ECTS and A-domain and between the ECTS and C-domain. Some possible locations of the ECTS domain enabling such interactions are presented in Fig. 6.

Possible Function of the ECTS—As the ECTS was first found in a thermophile, we were interested in the thermostability of the truncated enzyme. Although the thermostability is significantly reduced, the truncated enzyme is still thermostable compared to the muscle enzyme. Moreover, the pyruvate kinase from a psychrophile also has an ECTS (*8*), making it unlikely that the ECTS plays any essential role in the thermostability of the thermophilic enzyme. The truncated enzyme exhibited essentially identical kinetic properties to those of the recombinant enzyme as summarized in Table 1, indicating that the ECTS is not essential for the activity or the allosteric nature of the enzyme. The truncated enzyme shows a sigmoidal saturation curve for PEP and is activated by AMP and R5P. This indicates that the

homotropic and heterotropic cooperativity of the allosteric enzyme is unaffected by removing the ECTS.

Why does the pyruvate kinase from *B. stearothermophilus* have an ECTS and why is there a PEP binding motif in the sequence? The evidence shown in this paper indicates that the sequence is not essential for the maintenance of the tetrameric structure, the enzymatic activity, the allosteric nature or the thermostability. The possibility that the conserved histidine residue in the PEP binding motif is phosphorylated by PEP seems unlikely. There seems to be no apparent function for the ECTS. Although the ECTS has a weak interaction between the A-domain and C-domain, it is unlikely to have been evolutionary advantageous for *Bacillus* pyruvate kinase. Recently a new activity of the muscle M2 isozyme was reported (*26*). The PEP binding motif may participate in some as yet unknown enzymatic reaction.

It is known the genes for some enzymes that catalyze reverse reactions or metabolically related reactions fuse and form multifunctional enzymes (*27*). Once pyruvate kinase might have been a bifunctional enzyme with pyruvate phosphate dikinase or another enzyme having a PEP binding motief. During evolution, many pyruvate kinases may have lost their partner. However, the *Bucillus* enzyme might still retain a vestigial sequence of its alumnus. Another idea is that the presence of the ECTS represents the beginning of an evolutionary change in pyruvate kinase. The functions of many proteins seem to be actualized by the assembly of domains with various functions. A protein may obtain a new function by acquiring some domains, and these domains can move from one protein to another.

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