Construction of new forms of pyruvate carboxylase to assess the allosteric regulation by acetyl-CoA

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The single polypeptide chain of *Bacillus thermodenitrificans* pyruvate carboxylase (PC) is composed of the biotin carboxylase (BC), carboxyl transferase (CT) and biotin carboxyl carrier protein (BCCP) domains from the amino terminus. This polypeptide chain was divided into two between the CT and BCCP domains. The resulting proteins, PC-(BC + CT) and PC-(BCCP), were expressed in *Escherichia coli* separately, purified to homogeneity and characterized. PC-(BC + CT) was 4% active as native PC in the carboxylation of pyruvate with PC-(BCCP) as substrate with a $K_m$ of 39 μM. Moreover, acetyl-CoA stimulated the carboxylation of PC-(BCCP) about 3-fold, whereas it was without effect in the corresponding reaction with free biotin. In addition to these engineered proteins, another form of enzyme was also constructed in which the BC domain of *B.thermodenitrificans* PC was replaced with the BC subunit of *Aquifex aeolicus* PC, whose activity is independent of acetyl-CoA. The resulting chimera was about 7% active as native PC, but its activity was independent of acetyl-CoA. The basis of these observations, the mechanism by which acetyl-CoA regulates the reaction of PC is discussed.

**Keywords**: acetyl-CoA/biotin/chimera/pyruvate carboxylase

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**Introduction**

Pyruvate carboxylase (PC) (EC 6.4.1.1) is a biotin-dependent enzyme and is involved in gluconeogenesis by mediating carboxylation of pyruvate into oxalacetate (Utter and Kech, 1960). PC is distributed in many eukaryotes and also in some prokaryotes in two different forms (Barden et al., 1960). The PC reaction is believed to proceed in two steps, just like those of other biotin-dependent carboxylases such as acetyl-CoA carboxylase (ACC): enzyme-bound biotin is carboxylated first by bicarbonate and ATP and the carboxyl group bound temporarily on biotin is subsequently transferred to pyruvate (Wood and Barden, 1977; Attwood, 1995):

$$\text{ATP} + \text{HCO}_3^- + \text{Enz-biotin} \rightleftharpoons \text{Enz-biotin-Co}_2^- + \text{ADP} + \text{Pi} \quad (1)$$

$$\text{Enz-biotin-Co}_2^- + \text{pyruvate} \rightleftharpoons \text{Enz-biotin} + \text{oxalacetate} \quad (2)$$

Thus, PC carries at least three functional domains: biotin carboxyl carrier protein (BCCP), biotin carboxylase (BC), which mediates the first partial reaction (Equation 1) and carboxyl transferase (CT), which catalyzes the second partial reaction (Equation 2). The BC domain is located at the amino terminus of the single polypeptide chain of PC, followed by the CT domain with the biotin-carrying domain residing in the carboxyl terminus (Lim et al., 1988). In the subunit-type PC, the polypeptide chain is divided between the BC and CT domains (Mukhopadhyay et al., 1998). Acetyl-CoA and aspartate modulate the activity of the former class of PC allosterically, but the latter class of PC is insensitive to acetyl-CoA (Cazzulo and Stoppani, 1968; Ashman et al., 1972; Libor et al., 1978; Mukhopadhyay et al., 1998; Jitrapakdee and Wallace, 1999). Because of the lack of three-dimensional structural information, the detailed mechanism of carboxylation by PC and its allosteric regulation remain largely obscure. Hence, protein engineering approaches would be useful to unravel the mechanism of reaction and regulation of this enzyme.

Here, PC from *Bacillus thermodenitrificans* was engineered in such a way as to divide the single polypeptide chain into two at the boundary of the CT and BCCP domains (Figure 1). The resulting two proteins, PC-(BC + CT) and PC-(BCCP), were purified and characterized. Together with a chimeric PC was also constructed by replacing the BC domain of *B.thermodenitrificans* PC with the BC subunit of *Aquifex aeolicus* PC, whose activity is independent of acetyl-CoA. On the basis of the kinetic properties of these engineered proteins, the mechanism of acetyl-CoA activation of PC is discussed.

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**Materials and methods**

**Materials**

Inorganic salts and common organic chemicals were obtained from commercial sources. Avidin was purchased from ProZyme (San Leandro, CA) and acetyl-coenzyme A from Wako Pure Chemicals (Osaka, Japan). Reagents for genetic engineering such as restriction enzymes were purchased from Takara (Kyoto, Japan) and oligonucleotides were custom synthesized by Hokkaido Science (Sapporo, Japan). The TOPO TA cloning kit was supplied by Invitrogen (Carlsbad, CA).

**Construction of over-expression plasmids for PC-(BC + CT) and PC-(BCCP)**

The *B.thermodenitrificans* PC gene cloned in pBluescript vector (pPC) was the source of engineering of this enzyme (Kondo et al., 1997). Based on the reasoning described in the Results section, the gene was divided at the *MluI* site present within 3123–3128 bp from the 5′-terminus of the open reading frame (ORF). The polypeptide chain was separated into two at this site by introducing a stop codon or an initiation codon for the expression of BC plus CT and BCCP,
Construction of an over-expression plasmid for chimeric PC

The β subunit (aaBC: MW 50 kDa) of *A. aeolicus* PC was amplified by PCR in a way identical with that described previously (Kondo et al., 2004). The PCR product was TA cloned and sequenced. The insert was cut out of the vector and cloned into the pTrc99A vector to yield a recombinant plasmid, pPC-(aaBC), which served as the source of the BC domain of chimeric PC. Thus, an ~950 bp C-terminal fragment of *A. aeolicus* BC gene was PCR-amplified with pPC-(aaBC) as template and oligonucleotides BC1 and BC2 as primers: BC1, 5'-CCGGGG-GGCGTTGGTGGTA-3', primes on the coding strand and contains a SacII site (underlined); BC2, 5'-GGTACCGTGATACGTCGCGGAAGACCCGG-3' primes on the non-coding strand and carries a KpnI site (underlined) in place of the stop codon. The resulting PCR product was TA cloned and sequenced. The insert was excised from the plasmid and recloned into the SacII/KpnI sites of pPC-(aaBC), to give pPC-(aaBC1). Likewise, an ~400 bp N-terminal fragment of the CT domain of *B. thermodesulfuricans* PC was amplified with pPC-(CT + BCCP) (Sueda et al., 2004) as template using the following primers: CT1, 5'-GGTACCGACGCACCGAAAGACCCGG-3' and CT2, 5'-CCGATCTCCACGGATCTCTTATAAAAGCG-3' (restriction enzyme sites are underlined). The forward primer, CT1, harbored a KpnI site present for the original GTC codon and the reverse primer, CT2, harbored a BamHI site present on the PC-(CT + BCCP) gene. It is noted that the KpnI site was placed at the codons for the last two residues of BC so as to change the amino acids. The resulting fragment was TA cloned and sequenced, then recloned into the KpnI/BamHI sites of pPC-(aaBC1) to yield pPC-(aaBC1 + CT1). The C-terminal fragment of PC, excised from the plasmid pPC-(CT + BCCP) with BamHI/PstI, was recloned into the same sites of pPC-(aaBC1 + CT1) to yield a recombinant plasmid, pchPC for chimeric PC. The number of amino acid residues and the calculated molecular mass of the chimeric protein prepared were 1158 residues and 129 685 Da, respectively.

Construction of an over-expression plasmid for birA

The coding region of the birA gene (966 bp) of *E. coli* was amplified by PCR in one step with the following primers: birA1, 5'-CATATGAAGGATAACACCGTCACCTG-3'; birA2, 5'-CTCGAGATTATTATTCGCACTACGCCAGG-3' (restriction enzyme sites are underlined). PCR conditions were the same as those described above. The PCR product was purified by agarose gel electrophoresis before ligation into pCR2.1-TOPO. After confirming the correct DNA sequence, the coding region was excised from the plasmid and cloned into the NdeI/HindIII sites of pET-24a to produce pBirA.

Protein expression and purification

*Escherichia coli* JM109 transformed with one of the over-expression plasmids was grown in LB medium supplemented with 50 μg/ml ampicillin and 1 μg/ml n-octylglucoside, which is a soya-bean binding domain was present. The two proteins, PC-(BCCP) and BirA, were expressed in *E. coli* BL21(DE3) (Novagen, Madison, WI) separately or simultaneously, following transformation with one or two of the plasmids prepared above. Transformants were grown in 1 l of LB medium in the presence of ampicillin (50 μg/ml) or kanamycin (30 μg/ml) or both. A fresh overnight culture (10 ml) from a single colony...
was used to inoculate 1 l of medium. The cultures were grown at 37°C for 8–10 h, then isopropyl β-thiogalactoside (IPTG) was added to a final concentration of 1 mM and the cultures were incubated for an additional 8–10 h. The cells were harvested by centrifugation at 5000 r.p.m. (4200 g) for 10 min at 4°C. Proteins were purified according to the procedures previously described (Sueda et al., 2004; Yong-Biao et al., 2004). In brief, the harvested cells were disrupted by sonication and centrifuged. The proteins were purified by ammonium sulfate fractionation and N,N-diethyldiaminethyl (DEAE)-cellulose chromatography (2×10 cm, Whatman, Maidstone, UK) and proteins were eluted with a linear gradient of 0–500 mM NaCl in 20 mM potassium phosphate (KPi), pH 7.0. The pooled fractions were concentrated and then subjected to gel filtration chromatography on Superdex 200 (Amersham, Piscataway, NJ). Chimeric PC and PC-(BCCP) were purified finally by monomeric avidin Sepharose affinity chromatography (Jitrapakdee et al., 1999). Other proteins were purified by anion-exchange chromatography on Mono Q HR 5/5 (Amersham). The protein concentration was determined spectrophotometrically from the amino acid composition.

In vitro and in vivo biotinylation of BCCP

In vitro biotinylation was carried out according to the procedures reported previously (Chapman-Smith et al., 1994, 1999). Unless stated otherwise, the reaction mixture contained 50 mM Tris–HCl (pH 8.0), 3 mM ATP, 5 mM MgCl₂, 100 mM KCl, 0.5 mM biotin, a sufficient amount of apo-BCCP and biotin protein ligase, also called BirA. The reaction was initiated by the addition of biotin protein ligase and incubated at 37°C overnight, then the reaction mixture was applied to a monomeric avidin Sepharose affinity column and finally eluted with buffer containing 1 mg/ml biotin. In vivo biotinylation was performed by the co-expression of acceptor protein and biotin protein ligase in the presence of free biotin (1 µg/ml) (Chapman-Smith et al., 1994).

Pyruvate carboxylase assays

Pyruvate carboxylase was assayed in the direction of oxalacetate formation by coupling the reaction with malate dehydrogenase as coupling enzymes to monitor MgADP formation. The reaction mixture contained the following components, unless stated otherwise: 100 mM Tris–HCl (pH 8.0), 2 mM ATP, 5 mM MgCl₂, 100 mM KCl, 100 mM NaHCO₃, 0.1 mM acetyl-CoA, 0.5 mM phosphoenolpyruvate, 0.15 mM NADH, 5 units of lactate dehydrogenase, 5 units of pyruvate kinase and 10–40 µg of PC-(BC + CT) or chimeric PC or 200 µg of PC-(aaBC) in 1 ml. For PC-(BC + CT) assay, 100 mM biotin or 100 µM BCCP was added to the above reaction mixture and for PC-(aaBC) assay, 100 mM biotin was added. The kinetic parameters, Kₘ and Vₘₐₓ values, of PC-(BC + CT) for biotin were determined by varying the biotin concentration from 0 to 100 mM with fixed concentrations of ATP (2 mM) and bicarbonate (100 mM) and the Kₘ and Vₘₐₓ for BCCP were determined by varying the BCCP concentration from 0 to 175 µM at fixed concentrations of ATP (2 mM) and bicarbonate (100 mM). Again, the kinetic parameters, Kₘ and Vₘₐₓ, for the ATPase reaction of PC-(aaBC) were determined according to the procedure described previously (Sueda et al., 2004).

To assess the binding of acetyl-CoA to PC-(BC), PC-(aaBC) and chimeric PC, their ATP cleavage activity was determined by varying the acetyl-CoA concentration from 0 to 200 µM with fixed concentrations of ATP (2 mM) and bicarbonate (100 mM). Free biotin (100 mM) was also present in the reaction mixture in the PC-(BC) and PC-(aaBC) systems.

Oxamate-induced oxalacetate decarboxylase assays

Oxalacetate decarboxylase activity of PC-(BC + CT) and chimeric PC was measured with oxamate as stimulant according to the procedures reported previously (Attwood and Cleland, 1986). The reactions were monitored by measuring the formation of pyruvate, which was then reduced to lactate by lactate dehydrogenase and the concomitant oxidation of NADH was monitored at 340 nm. All assays were performed at 30°C and the reaction mixture contained the following components, unless stated otherwise: 100 mM Tris–HCl (pH 8.0), 5 mM MgCl₂, 100 mM KCl, 0.2 mM oxalacetate, 0.1 mM acetyl-CoA, 1 mM oxamate, 0.15 mM NADH, 5 units of lactate dehydrogenase and 30–60 µg of PC-(BC + CT) or chimeric PC in 1 ml. For PC-(BC + CT) assay, 100 mM biotin or 100 µM BCCP was added to the above reaction mixture. The reactions were started by the addition of PC-(BC + CT) or chimeric PC but, prior to the addition, a background rate of oxalacetate decarboxylation was established and this was subtracted from the rate in the presence of enzyme.

The kinetic parameters of PC-(BC + CT) for biotin were determined by varying its concentration from 0 to 100 mM at fixed concentrations of oxamate (1 mM) and oxalacetate (0.1 mM). Again, the kinetic parameters for BCCP were determined by varying its concentration from 0 to 200 µM at fixed concentrations of oxamate (1 mM) and oxalacetate (0.1 mM).

Results

Engineering of the pyruvate carboxylase gene

Although it is difficult to know exactly the boundary of the CT and BCCP domains of \textit{B.thermocellurum} PC

\textbf{ATP cleavage assays in the absence of pyruvate}

These assays were carried out at 30°C as described previously (Attwood and Graneri, 1992) using pyruvate kinase and lactate dehydrogenase as coupling enzymes to monitor MgADP formation. The reaction mixture contained the following components, unless stated otherwise: 100 mM Tris–HCl (pH 8.0), 2 mM ATP, 5 mM MgCl₂, 100 mM KCl, 100 mM NaHCO₃, 0.1 mM acetyl-CoA, 0.5 mM phosphoenolpyruvate, 0.15 mM NADH, 5 units of lactate dehydrogenase, 5 units of pyruvate kinase and 10–40 µg of PC-(BC + CT) or chimeric PC or 200 µg of PC-(aaBC) in 1 ml. For PC-(BC + CT) assay, 100 mM biotin or 100 µM BCCP was added to the above reaction mixture and for PC-(aaBC) assay, 100 mM biotin was added. The kinetic parameters, Kₘ and Vₘₐₓ values, of PC-(BC + CT) for biotin were determined by varying the biotin concentration from 0 to 100 mM with fixed concentrations of ATP (2 mM) and bicarbonate (100 mM) and the Kₘ and Vₘₐₓ for BCCP were determined by varying the BCCP concentration from 0 to 175 µM at fixed concentrations of ATP (2 mM) and bicarbonate (100 mM). Again, the kinetic parameters, Kₘ and Vₘₐₓ, for the ATPase reaction of PC-(aaBC) were determined according to the procedure described previously (Sueda et al., 2004).

To assess the binding of acetyl-CoA to PC-(BC), PC-(aaBC) and chimeric PC, their ATP cleavage activity was determined by varying the acetyl-CoA concentration from 0 to 200 µM with fixed concentrations of ATP (2 mM) and bicarbonate (100 mM). Free biotin (100 mM) was also present in the reaction mixture in the PC-(BC) and PC-(aaBC) systems.
(1147 residues), it seemed certain that the CT domain ends before amino acid number around 940. For one thing, sequence homology is hardly seen in this region among PCs from various sources. Hence this PC gene was divided into two ends before amino acid number around 940. For one thing, sequence homology is hardly seen in this region among PCs from various sources. Hence this PC gene was divided into two.

The pyruvate carboxylase activity of PC-(BC + CT) and chimeric PC
It was found that the divided protein, PC-(BC + CT), is as capable as native PC of mediating pyruvate carboxylation in the presence of free d-biotin or holo-BCCP, suggesting that its three-dimensional structure remains largely intact even in the absence of the BCCP domain. The enzymic activity of PC-(BC + CT) determined at 30°C in the presence of various concentrations of pyruvate, ATP, bicarbonate and biotin or BCCP is shown in Figure 3. The Michaelis constants, K_m, for the three substrates in the carboxylation of pyruvate by PC-(BC + CT) with free biotin and by chimeric PC were determined. The K_m values for pyruvate of PC-(BC + CT) and chimeric PC were 0.28 ± 0.03 and 0.25 ± 0.02 mM, respectively, which were virtually identical with those of native PC (Sueda et al., 2004), but the K_m for bicarbonate of chimeric PC (3.09 ± 0.46 mM) decreased 10-fold from that of native PC, presumably because the BC subunit of A.aeolicus PC was used (see below). The K_m value of PC-(BC + CT) for bicarbonate was 22.1 ± 1.9 mM, which was nearly identical with that of native PC. In the kinetic analysis for ATP of PC-(BC + CT), substrate inhibition was manifest at high ATP concentrations just like in native PC and two kinds of data analysis were therefore exploited. The K_m values of PC-(BC + CT) thus obtained for ATP with or without substrate inhibition taken into account were 1.01 ± 0.09 and 0.64 ± 0.06 mM, respectively, which were nearly identical with those of native PC in either of the data analyses (Sueda et al., 2004). Interestingly, substrate inhibition at high ATP concentrations was not evident with chimeric PC and the K_m value (0.25 ± 0.01 mM) decreased to half.

The pyruvate carboxylase activity of native PC and PC-(BC + CT) was modulated by acetyl-CoA sigmoidally, as shown in Figure 4A, providing evidence for the allosteric nature of this modulation. These data were analyzed by the Hill equation to yield apparent n values of 3.0 and 3.2 for native PC and PC-(BC + CT), respectively (Figure 4B). In other words, cooperative binding of acetyl-CoA does not change significantly between the two enzymes. By contrast, the dissociation constant, K_D, for acetyl-CoA of native PC, 44.0 μM, was considerably smaller than that of PC-(BC + CT), 56.2 μM. The PC activity of PC-(BC + CT) with BCCP as substrate was enhanced about 3-fold upon addition of acetyl-CoA.
Bicarbonate was the variable substrate (0.5–100 mM) with 10 mM pyruvate, 2 mM ATP and 100 mM biotin; the $V_{\text{max}}$ whereas those determined from the data from 0 to 5.0 mM on the basis of considering substrate inhibition were parameters determined with the data from 0 to 1.0 mM on the basis of the simple Michaelis–Menten equation were $K_m$ for pyruvate was $0.28 \pm 0.03$ mM and $V_{\text{max}}$ $24.4 \pm 0.74$ min$^{-1}$. (B) ATP was the variable substrate (0–5 mM) with 10 mM pyruvate, 100 mM bicarbonate and 100 mM biotin. In the kinetics for ATP, substrate inhibition was evident and therefore two different kinds of analysis were made for the data obtained. Kinetic parameters determined with the data from 0 to 1.0 mM on the basis of the simple Michaelis–Menten equation were $K_m$ 0.64 ± 0.06 mM and $V_{\text{max}}$ 36.0 ± 1.8 min$^{-1}$, whereas those determined from the data from 0 to 5.0 mM on the basis of considering substrate inhibition were $K_m$ 1.01 ± 0.09 mM and $V_{\text{max}}$ 51.9 ± 3.2 min$^{-1}$. (C) Bicarbonate was the variable substrate (0.5–100 mM) with 10 mM pyruvate, 2 mM ATP and 100 mM biotin; the $K_m$ for bicarbonate was 22.1 ± 1.9 mM and $V_{\text{max}}$ 29.0 ± 0.9 min$^{-1}$. (D) Biotin was the variable substrate (0–100 mM) with 10 mM pyruvate, 100 mM bicarbonate and 2 mM ATP; the $K_m$ for biotin was 23.2 ± 1.4 mM and $V_{\text{max}}$ 28.3 ± 0.6 min$^{-1}$. (E) BCCP was the variable substrate (0–175 μM) with 10 mM pyruvate, 100 mM bicarbonate and 2 mM ATP; the $K_m$ for BCCP was 0.039 ± 0.003 mM and $V_{\text{max}}$ 60.6 ± 1.7 min$^{-1}$. In each case, the kinetic parameters and their standard errors were determined by non-linear regression analysis.

whereas it was without effect in the same reaction with free biotin as substrate (Table I). Again, the PC activity of chimeric PC was determined by varying the acetyl-CoA concentration, but it was independent of the ligand over the concentration range studied (0–200 μM) (Table I). It is, nevertheless, noteworthy that the activity of chimeric PC in the absence of acetyl-CoA was about 20 times higher than that of native PC.

**ATP cleavage activity of engineered proteins**

PC is known to catalyze the ATP cleavage reaction in the absence of pyruvate (Attwood and Graneri, 1992) and hence it is possible to study the reaction of BC (Equation 1) independently of the CT reaction (Equation 2) by measuring this activity. The ATPase activity of PC-(BC + CT), determined under essentially the same conditions as the complete reaction except for the omission of pyruvate, also increased about 2.5-fold with BCCP in the presence of acetyl-CoA from that in its absence, but the activity was virtually the same with free biotin, regardless of the presence or absence of acetyl-CoA (Table II). The ATP cleavage reaction of chimeric PC did not change with acetyl-CoA concentration over the range adopted (0–200 μM), just like that of its overall reaction (Tables I and II). Again, the ATP cleavage activity of PC-(BC) and PC-(aaBC) with free biotin was not affected by acetyl-CoA over the concentration range adopted (0–200 μM).

According to the proposed reaction mechanism for BC (Knowles, 1989; Attwood, 1995), the ATPase activity of PC-(aaBC) was dependent on three substrates and the activity in the absence of biotin is ~5% of the maximum in its presence. The $K_m$ value for bicarbonate was 2.88 ± 0.36 mM, which was nearly identical with that for the complete reaction of chimeric PC, and the $K_m$ value for biotin was 17.2 ± 1.1 mM. For ATP, substrate inhibition was observed just like in PC-(BC + CT) and the $K_m$ values evaluated by analysis of the data without and with substrate inhibition were 0.044 ± 0.001 and 0.058 ± 0.004 mM, respectively, which were considerably smaller than that of chimeric PC. As expected from the property of subunit type PCs (Cazzulo and Stoppani, 1968; Ashman et al., 1972; Libor et al., 1978), the ATPase activity of PC-(aaBC) was virtually unchanged in the presence and absence of acetyl-CoA (Table II).

In our previous study (Sueda et al., 2004), an engineered protein PC-(BC) was prepared by dividing the single polypeptide chain of *B. thermosdenitrificans* PC at the boundary of the BC and CT domains and the ATPase activity of the resulting protein, determined with free biotin as substrate, was independent of acetyl-CoA (Table II). When studied again here but with BCCP as substrate by varying acetyl-CoA concentration from 0 to 200 μM, acetyl-CoA dependence was observed. The Hill coefficient, apparent $n$ value, calculated by the Hill equation for acetyl-CoA and PC-(BC)
was 2.0, which is considerably smaller than that of native PC and PC-(BC + CT). The $K_D$ value for acetyl-CoA of PC-(BC) with BCCP as substrate was 56.6 µM, which is identical with that of PC-(BC + CT) but considerably larger than that of native PC. The ATP cleavage activity of PC-(BC) with BCCP as substrate in the presence of the saturated concentration of acetyl-CoA was 4.3 times higher than that in its absence (Table II).

Oxamate-induced oxalacetate decarboxylase activity of PC-(BC + CT) and chimeric PC

It was reported that oxamate stimulates the decarboxylation of oxalacetate by PC (Attwood and Cleland, 1986). Hence it is possible to study the CT reaction (Equation 2) of PC separately from the BC reaction (Equation 1) with this assay (Attwood and Cleland, 1986). The oxamate-induced decarboxylation of oxalacetate by PC-(BC + CT) with BCCP, investigated by measuring the oxalacetate decarboxylase activity in the presence of a saturating concentration of oxamate (1 mM) at 30°C, was about 30 times higher than that in its absence (Table III), as in native PC. Such a phenomenon was also observed with free biotin as the substrate. Likewise, the oxalacetate decarboxylase activity of chimeric PC increased about 6-fold on addition of oxamate. The oxalacetate decarboxylase activity of PC-(BC + CT) and chimeric PC increased with increase in oxamate concentration up to 1 mM, followed by substrate inhibition. The effect of oxalacetate concentration on the decarboxylation reaction of these enzymes was also determined, where substrate inhibition was manifest at high concentrations of oxalacetate. Such a phenomenon was observed also for native PC (Sueda et al., 2004) and PC from chicken liver and was accounted for by competitive substrate inhibition (Attwood and Cleland, 1986). It is noted that the decarboxylation activity of PC-(BC + CT) and chimeric PC was virtually the same in the presence and absence of acetyl-CoA (Table III); therefore, the catalytic reaction of the CT domain appears to be independent of acetyl-CoA.

Table II. Effect of 0.1 mM acetyl-CoA on ATP cleavage reaction at pH 8.0 and 30°C

<table>
<thead>
<tr>
<th>Protein</th>
<th>Substrate</th>
<th>Enzymic activity (min⁻¹)ᵃ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With acetyl-CoA</td>
</tr>
<tr>
<td>Native PCᵇ</td>
<td>–ⁿ</td>
<td>2.84 ± 0.21</td>
</tr>
<tr>
<td>PC-(BC + CT)</td>
<td>BCCP</td>
<td>42.5 ± 1.4</td>
</tr>
<tr>
<td>PC-(BC + CT)</td>
<td>Biotin</td>
<td>9.56 ± 0.70</td>
</tr>
<tr>
<td>PC-(BC)</td>
<td>BCCP</td>
<td>4.79 ± 0.25</td>
</tr>
<tr>
<td>PC-(BC)ᵇ</td>
<td>Biotin</td>
<td>1.02 ± 0.06</td>
</tr>
<tr>
<td>PC-(aaBC)</td>
<td>Biotin</td>
<td>4.93 ± 0.21</td>
</tr>
<tr>
<td>Chimeric PC</td>
<td>–ⁿ</td>
<td>4.98 ± 0.62</td>
</tr>
</tbody>
</table>

ᵃThe values are the means ± SD from three separate experiments.
bData were taken from Sueda et al. (2004).
ⁿBiotin was not necessary owing to the presence of endogenous biotin in the native and chimeric PC.

Substrate activity of BCCP in the reactions of PC-(BC + CT)

The kinetic parameters for the reactions of pyruvate carboxylation, ATP cleavage and oxalacetate decarboxylation of PC-(BC + CT) with BCCP or biotin as substrates are summarized in Table IV. The maximum velocity ($V_{max}$) of pyruvate carboxylation with BCCP as substrate was about two times greater than that with biotin as substrate. More significantly, the $K_m$ for BCCP is 590-fold lower than that for free biotin. As a result, BCCP is 1270-fold more efficient as substrate than biotin in terms of the catalytic efficiency ($V_{max}/K_m$). Again, the maximum velocity of ATP cleavage reaction with BCCP was 4.5 times greater than that with biotin, whereas the $K_m$ for BCCP was 620 times smaller than that for biotin. Hence the catalytic efficiency of ATP cleavage with BCCP as substrate was 2810 times.
Oxalacetate decarboxylation

ATP cleavage

at pH 8.0 and 30°C

Pyruvate carboxylation

Table IV. Kinetic parameters for pyruvate carboxylation, ATP cleavage and oxalacetate decarboxylation reactions of PC-(BC + CT) in the presence of BCCP or free biotin at pH 8.0 and 30°C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( V_{\text{max}} ) (min(^{-1}))</th>
<th>( K_m ) (mM)</th>
<th>( V_{\text{max}}/K_m ) (min(^{-1}).mM(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate carboxylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCCP</td>
<td>60.6 ± 1.7</td>
<td>0.039 ± 0.003</td>
<td>1550</td>
</tr>
<tr>
<td>Biotin</td>
<td>28.3 ± 0.6</td>
<td>23.2 ± 1.4</td>
<td>1.22</td>
</tr>
<tr>
<td>ATP cleavage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCCP</td>
<td>53.9 ± 2.7</td>
<td>0.033 ± 0.005</td>
<td>1630</td>
</tr>
<tr>
<td>Biotin</td>
<td>11.9 ± 0.3</td>
<td>20.6 ± 1.8</td>
<td>0.58</td>
</tr>
<tr>
<td>Oxalacetate decarboxylation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCCP</td>
<td>9.71 ± 0.21</td>
<td>0.048 ± 0.002</td>
<td>200</td>
</tr>
<tr>
<td>Biotin</td>
<td>4.03 ± 0.07</td>
<td>9.62 ± 0.58</td>
<td>0.42</td>
</tr>
</tbody>
</table>

The kinetic parameters and their standard errors were determined by non-linear regression analysis of the saturation curves as described in the text.

Protein engineering of pyruvate carboxylase

Table III. Oxalacetate decarboxylation activity (min\(^{-1}\)) of PC-(BC + CT) and chimeric PC in the presence or absence of 0.1 mM acetyl-CoA at pH 8.0 and 30°C

<table>
<thead>
<tr>
<th>Acetyl-CoA</th>
<th>PC-(BC + CT)*</th>
<th>Chimeric PC</th>
<th>Native PCba</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>+Oxamate</td>
<td>6.07 ± 0.71 (2.92 ± 0.30)</td>
<td>8.95 ± 0.51</td>
</tr>
<tr>
<td></td>
<td>–Oxamate</td>
<td>0.19 ± 0.01 (0.14 ± 0.01)</td>
<td>1.58 ± 0.19</td>
</tr>
<tr>
<td>Absent</td>
<td>+Oxamate</td>
<td>4.76 ± 0.51 (2.78 ± 0.15)</td>
<td>8.75 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>–Oxamate</td>
<td>0.18 ± 0.01 (0.12 ± 0.01)</td>
<td>1.32 ± 0.15</td>
</tr>
</tbody>
</table>

aActivity was measured in the presence of BCCP or free biotin (data with free biotin are shown in parentheses).

bData were taken from Sueda et al. (2004).

greater than that with biotin. Similarly, the effect of BCCP on the oxalacetate decarboxylation reaction was studied, where the maximum velocity with BCCP was 2.4 times greater than that with biotin and the \( K_m \) for BCCP was 200 times lower than that for biotin. Hence oxalacetate decarboxylation is 480 times catalytically more efficient with BCCP than with biotin as substrate. It is worth noting that the results obtained here are reminiscent of the BC and CT activity of \( E. coli \) ACC with respect to BCCP and free biotin as substrates (Fall et al., 1971; Blanchard et al., 1999).

Discussion

Pyruvate is an end-product of glycolysis and metabolized further to various compounds. For example, it is converted to oxalacetate by carboxylation, acetyl-CoA by decarboxylation, oxidation and lactate by hydrogenation. Some of the products of these reactions are transformed further: oxalacetate is converted into aspartate by transamination and oxalacetate and acetyl-CoA into citrate by condensation. It is not surprising, therefore, to see that PC is regulated by some of these metabolites to partition pyruvate into available pathways. Nevertheless, the mechanism of this allosteric regulation of PC by these agents has remained largely obscure and to explore the mode of action of acetyl-CoA a protein engineering approach was taken in the present study.

As described above, \( Bacillus \) PC was divided between the CT and BCCP domains and a chimeric PC was constructed by replacing the BC domain of \( Bacillus \) PC with the BC subunit of \( A. flexus \) PC. In the former, the selection of the boundary of the CT and BCCP domains was rather arbitrary, and in order to save the activity of CT the N-terminal portion of the BCCP domain may have been sacrificed. Moreover, the PC-(BCCP) protein thus designed (105 residues) was nicked during expression at nearly one-third of the sequence from the N-terminus, leaving a 76-residue truncated protein. Nonetheless, it possessed most of the characteristics expected for the ‘native’ BCCP, as discussed below.

In \( Bacillus \) PC, the BCCP domain is fused with other domains and it is unable to evaluate its intrinsic affinity for them. The \( K_m \) of 39 \( \mu \)M of PC-(BC + CT) for the 76-residue BCCP obtained experimentally was comparable to that of BC of \( E. coli \) ACC for BCCP, (the 87-residue protein formed by truncation in the middle of native BCCP, \( K_m \) 160 \( \mu \)M) (Fall et al., 1971; Blanchard et al., 1999), suggesting that these two enzymes possess affinity for BCCP nearly of the same magnitude. In addition, the substrate activity of truncated PC-(BCCP) in pyruvate carboxylation was much higher than that of free biotin in terms of both \( V_{\text{max}} \) and \( K_m \), 2- and 600-fold, respectively (Table IV). It is therefore obvious that the affinity of biotin is enhanced markedly by the addition of the 76-residue polypeptide chain. In addition to this enhanced affinity, the BCCP made the carboxylation of pyruvate susceptible to allosteric regulation by acetyl-CoA. Although the magnitude of activation of three times is modest compared with that of 280 times with native PC (Table I), the BCCP seemingly interacts with PC-(BC + CT) in a way in which free biotin can never do.

In the present study, it was found that acetyl-CoA affects both pyruvate carboxylation and ATPase reactions of PC-(BC + CT) in the presence of BCCP, but it is without effect in the same reactions with free biotin. Likewise, acetyl-CoA affected the ATPase reaction of PC-(BC) only in the presence of BCCP. By contrast, the oxalacetate decarboxylation reaction of PC-(BC + CT) was not dependent on acetyl-CoA even in the presence of BCCP. From these observations, it is obvious that acetyl-CoA affects the BC reaction of PC, but not the CT reaction. Judging from the difference in the effect of acetyl-CoA on PC activation between BCCP and free biotin, it is inferred that the peptide chain of the former interacts somehow with the acetyl-CoA binding site of BC to promote the catalytic reaction of BC. However, the effect of acetyl-CoA on the activation of the engineered protein with BCCP is considerably smaller than that of native PC. Hence another role, as proposed in our previous paper (Sueda et al., 2004), that the induction of the conformational change between the BC and CT domains of native PC should also be considered.

In the chimeric PC, both the carboxylation of pyruvate and ATPase activity were independent of acetyl-CoA, presumably because the BC domain deriving from \( A. flexus \) PC lacks the acetyl-CoA binding site or alternatively interfacing of the first and second partial reactions (Equations 1 and 2) does not occur properly. The third possibility would be that the conformation of the chimera is moderately proper already from the beginning for the reaction to occur, as inferred from its high basal activity (Table I).
From the Hill coefficients and dissociation constants, it seems certain that acetyl-CoA binds to the BC domain of PC, although the affinity for acetyl-CoA is partially impaired with PC-(BC) and PC-(BC + CT) from that of native PC, because of the truncation of the CT and/or BCCP domains. To identify the exact acetyl-CoA binding motif, information on the three-dimensional structure of PC-(BC) and native PC is essential and such an undertaking is under way in this laboratory.

References