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Cloning, Sequencing, and Overexpression of Gene Encoding the *Bacillus stearothermophilus* Lipoate Acetyltransferase

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Lipoate acetyltransferase (E2) comprising sixty subunits is one of the three component enzymes of pyruvate dehydrogenase multienzyme complex from the thermophile, *Bacillus stearothermophilus*. Gene encoding E2 of the *B. stearothermophilus* (NCA 1503) spontaneous mutant was cloned and sequenced. The amino acid sequence of E2 deduced from its nucleotide sequence (1305 bp) was quite similar to that of original strain (GenBank: 40038). The gene was overexpressed in the *Escherichia coli* BL21 (DE3) pLysS to a soluble and active T7–tagged E2 (tE2): E2 plus fifteen extension residues from *N*–terminus. tE2 was purified to homogeneity by ammonium sulfate fractionation, gel filtration, and heat treatment. The sedimentation coefficient of tE2 was estimated 31S, suggesting that tE2 also comprises sixty subunits. tE2 was relatively stable; incubation at 60 °C for 60 min affected its enzyme activity insignificantly, and 90% of original activity was recovered from 6 M guanidine hydrochloride. tE2 was capable of spontaneously non–covalent association with the *B. stearothermophilus* E3: other component of the pyruvate dehydrogenase. Based on these results, tE2 was suggested useful for a model system to examine the stabilizing mechanism of an enzyme complex.

INTRODUCTION

The *Bacillus stearothermophilus* lipoate acetyltransferase [EC 2.3.1.12] (E2) comprises sixty identical subunits and forms a core of pyruvate dehydrogenase complex (PDC) (Perham, 1996; Domingo *et al.*, 1999; Perham, 2000). Each subunit has three structural domains: catalytic domain (CD), E1/E3 binding domain (BD), and lipoyl domain (LD) from *C* to *N* termini. CD is solely responsible for a non–covalent aggregation of sixty subunits besides a catalytic function of E2. Through a BD, either tetrameric pyruvate decarboxylase [EC 1.2.4.1] (E1) or dimeric dihydrolipoamide dehydrogenase [EC 1.8.1.4] (E3) attaches non–covalently to a subunit so that PDC is a multienzyme complex having a molecular size of nearly ten megadalton. E1, E2, and E3 participate differently in the overall reaction catalyzed by PDC: the synthesis of acetyl CoA from pyruvate and CoA. E1 catalyzes the decarboxylative transfer of an acetyl group from pyruvate to thiamine diphosphate. The acetyl group is then transferred to a lipoyl group covalently

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attached to a lysine residue in LD: the center residue of a DKA motif (Dardel *et al.*, 1993). The final acetyl transfer is that in CD from the lipoyl group to CoA, yielding a dihydrolipoamide group and acetyl CoA. The turnover of the overall reaction therefore requires the E3-catalyzed re-oxidation of the dihydrolipoamide group; E3 is a flavoprotein acting with the reduction of NAD^+. We have been concerned with the stabilizing mechanism of a huge assembly composed of many polypeptides and have examined how PDC is disintegrated by several factors such as heat and potassium iodide (Hiromasa *et al.*, 1994; Hiromasa *et al.*, 1997; Aso *et al*, 1998). Since results from studies on stability of a component enzyme isolated from PDC are different from those using PDC, only based on knowledge of properties of free components, it is difficult to give an explanation of changes in PDC; these changes usually accompany the dissociation into and re-association of components (Hiromasa *et al.*, 2000; Jung *et al.*, 2002). Inter- and intra-protein interactions must be responsible for changes in PDC and become fairly complicated with increasing number of components. In this context, we have been in need of construction of a middle-scale model: a protein complex simpler than PDC, but far larger than a common oligomeric protein. The gene encoding E2 is cloned and sequenced (GenBank: 40038), and is expressed in *Escherihia coli* (Borges *et al.*, 1990; Lessard *et al.*, 1998). With reference to these results, we embarked on the preparation of E2 from the *B. stearothermophilus* (NCA 1503) spontaneous mutant, because this strain has been maintained in Kyushu University and because its fresh cells have been available. We examined the sequence of E2 gene from the mutant and constructed its overexpression system in *E. coli*. We furthermore purified resulting T7-tagged E2 and examined its properties; this E2 is abbreviated as tE2 in this paper.

MATERIALS AND METHODS

Chemicals, plasmid, and genome DNA

All chemicals used were of the highest grade commercially available. Sepharose CL-2B and Sephacryl S-400HR were obtained from Pharmacia LKB Biotechnology (Tokyo). The *B. stearothermophilus* diaphorase II (E3) was purchased from Unitika (Kyoto) and used without further purification. KOD' DNA polymerase and DNA ligation kit were from Takara Shuzo. Geneclean kit and Big Dye Terminator Cycle Sequencing kit were obtained from Bio101 (USA) and PE Applied Biosystems (USA), respectively. The cells of the *B. stearothermophilus* (NCA 1503) spontaneous mutant were kindly donated from Dr. M. Kimura (Faculty of Agriculture, Kyushu University). Chromosomal DNA of the cell was principally prepared according to the method of Barker (Barker, 1989). The *E. coli* BL21 (DE3) pLyS strain and the pET11a plasmid were obtained from Stratagene (La Jolla, USA).

Cloning and sequencing of E2 gene

E2 gene was amplified by the step-down polymerase chain reaction (PCR) according to the method of Hecker and Roux (Hecker and Roux, 1996). The reaction mixture comprised 700 ng of the genome DNA, 200 mM dNTPs, 100 pmol of sense primer, 100 pmol antisense primer, and 2.5 units of KOD' DNA polymerase; the sense and antisense primers were 5'-GTGGATCCGTGCTTTTGAATTTAAGCTG-3' and 5'-GTGGATCCCAAC-

TACCATCGTTTTACGCC–3', respectively. The mixture was incubated with thirty–five temperature cycles by the following (I) and (II) methods; each cycle in both the methods included successive three incubations at different temperatures for 30 sec, 15 sec, and 15 sec, respectively. First fifteen cycles were done by the method (I), using the following five temperature series: 95 °C–74 °C–72 °C; 95 °C–70 °C–72 °C; 95 °C–66 °C–72 °C; 95 °C–62 °C–72 °C; 95 °C–58 °C–72 °C. Each series was repeated three times. Residual twenty cycles were done by the method (II); every cycle was repeated, using 95 °C–54 °C–72 °C temperature series. Resulting product was digested by *Bam*HI. The E2 gene was resolved by agarose gel electrophoresis, purified with Geneclean kit, and cloned into a pUC19 vector: pUC/E2. The DNA sequence of E2 gene in pUC/E2 was determined with an ABI PRISM 377 DNA sequencer using the forward and reverse M13/pUC sequencing primers, and a Big Dye Terminator Cycle Sequencing kit.

Overexpression of E2 gene

The pUC/E2 was digested with *Bam*HI and resolved by agarose gel electrophoresis. Resulting E2 gene was purified using Geneclean kit and sub–cloned into a pET11a vector. pET/E2 plasmid thus constructed was overexpressed in *E. coli* BL21 (DE3)pLysS; operation including cultivation in LB–amp medium at 37 °C for 5 h after induction with 1 mM isopropyl–β–D–thiogalactopyranoside (IPTG) was done according to the procedure described in the pET System Vectors and Hosts instruction manual (Stratagene, USA).

Purification of tE2

All procedures except for heat treatment were done at 4 °C. Unless otherwise noted, the buffer used for purification was 20 mM sodium phosphate buffer (pH 7) containing 2 mM EDTA and 0.15 mM phenylmethanesulfonyl fluoride (standard buffer), and centrifugation was done for 30 min at 3,000–5,000 $\times g$. A protein solution was concentrated by the following method; proteins were precipitated by incubation in 50% saturation of $(NH_4)_2SO_4$ for 1 h, re–suspended with a small amount of standard buffer, and centrifuged to remove insoluble matters. The overexpression of E2 gene was done by the method described above. The cells collected by centrifugation were re–suspended in a standard buffer and disrupted ten times by ultrasonic treatment: 30 sec each. Cell debris was removed by centrifugation. Resulting supernatant was brought to 35% saturation of $(NH_4)_2SO_4$. After removal of precipitate by centrifugation, supernatant was concentrated and submitted to the gel filtration on a Sepharose CL–2B column (2.5 $\times$ 110 cm); the column was pre–equlibrated and developed with a standard buffer. Active fractions were combined, concentrated, and filtered again through the same column. Active fractions were combined, concentrated, and incubated at 70 °C for 5 h. After centrifugation, resulting supernatant was dispensed and stored at −30 °C.

Assay of enzyme activity and analytical ultracentrifugation

Enzyme activities of E2 and E3 were measured at 30 °C by the methods reported previously (Hiromasa *et al*, 1993; Hiromasa *et al.*, 1995). Analytical ultracentrifugation was done at 20,000 rpm and 20 °C by sedimentation velocity method, using a Beckman Optima XL–A ultracentrifuge equipped with An–60 Ti rotor and double sector cell (12 mm). Sedimentation pattern was monitored at 280 nm and analyzed with a Beckman

XLAVEL program.

Stability of tE2

Stability of tE2 at various temperatures between 30 °C and 95 °C was examined by the following method: incubation of tE2 solution (standard buffer) for 60 min, cooling on ice for 60 min, and assay of enzyme activity at 30 °C. Stability of tE2 to guanidine hydrochloride (GdnHCl) was examined by the following method: incubation of 0.1 mg/ml tE2 (standard buffer) at 4 °C for 24 h in the presence of various amounts of GdnHCl and assay of enzyme activity at 30 °C. The amount of GdnHCl was confirmed by measuring refrative index (Pace *et al.*, 1989). Effects of the removal of GdnHCl on tE2 were examined by measuring enzyme activity at 30 °C after dialysis against a standard buffer at 4 °C for 48 h.

Preparation of tE2–E3 complex

tE2 and E3 solutions were prepared with a standard buffer. Mixing ratio between tE2 and E3 was confirmed based on total UV absorbancy at 280 nm (A_{280}) of each solution; about 0.3 ml of the mixture containing tE2 (total A_{280}=0.6) and E3 (total A_{280}=1.2) was prepared. The mixture was incubated for 30 min at room temperature and subjected to the gel filtration on a Sephacryl S–400HR (1.5×26 cm) column at room temperature; the column was pre–equilibrated and developed with a standard buffer.

RESULTS AND DISCUSSION

Cloning, sequencing, and overexpression of gene

The E2 gene of the *B. stearothermophilus* (NCA 1503) original strain has an 1.3 kb–open reading frame (GenBank: 40038). With reference to the sequence of the gene, we designed the sense and antisense primers for amplification of the E2 gene from the *B. stearothermophilus* (NCA 1503) spontaneous mutant; both the sequences were further designed so that each primer included a *Bam*HI recognition site. Using these primers, the gene was successfully amplified by step–down PCR. Resulting products were digested by *Bam*HI, and the E2 gene (1.3 kb) was purified by agarosegel electrophoresis. As shown in Fig. 1, the purified gene was ligated into pUC19 vector (2.6 kb) which had been also cut with *Bam*HI, and constructed a pUC/E2 plasmid (3.9 kb). Both the strands of the insert DNA in pUC19 were completely sequenced. As shown in Fig. 2, the gene had an open reading frame composed of 1305 bp encoding 434 amino acids (DDBJ nucleotide sequence accession number: AB044388). The gene was slightly larger than that of the original strain: 427 amino acids deduced (Borges *et al.*, 1990; GenBank: 40038). Comparison of deduced amino acid sequence of the spontaneous mutant with that of original strains is illustrated in Fig. 3. We hereinafter follow the numbering of amino acid residue for the E2 gene from the original strain (GenBank: 40038). There was a single LD (A–1 to F–85) including a DKA motif (41 to 43) in the sequence (Neveling *et al.*, 1998), suggesting occurence of a single lipoyl group. Within the region (A–1 to E–89) including this LD, there was no difference in sequences between the two strains. Only one replacement from V–157 to I–157 was within the region (P–124 to K–171) including BD (V–128 to A–170). Although seven residues within CD (E–192 to A–427) were different from those of original strains, a sequence around a catalytic H–398 was well conserved. Relatively

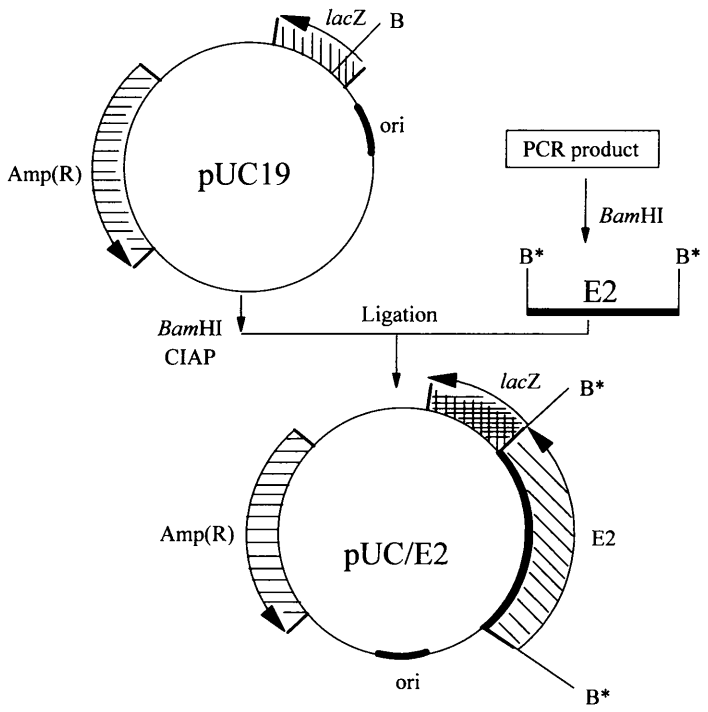

Fig. 1. Construction scheme of pUC/E2. Shadowed portion and arrow of circular arc indicate gene and its orientation, respectively. CIAP, calf intestinal alkaline phosphatase; B, *Bam*HI site; B*, *Bam*HI site introduced; ori, origin of ColE1 plasmid replication; *lac*Z, gene encoding β-galactosidase; Amp(R), ampicillin resistance gene.

large difference in sequences was in a flexible linker region between adjacent domains; gaps were also in the region between BD and CD. Based on these results, it was suggested that drastic mutations are never induced and that highly conserved sequences in the region corresponding to a structural domain are significant.

As shown in Fig. 4, E2 gene was purified from *Bam*HI digestion products of the pUC/E2 plasmid by agarose gel electrophoresis and sub-cloned into a pET11a vector (5.6 kb). Resulting pET/E2 plasmid (6.9 kb) was expressed in the *E. coli* BL21 (DE3) pLysS strain. The addition of IPTG to a culture medium induced the expression of E2 as a soluble and active enzyme efficiently. E2 thus overexpressed was a T7-tagged E2: tE2. As shown in Fig. 5, at a *N*-terminus, amino acid sequence of tE2 was deduced fifteen residues larger than that deduced from E2 gene (Fig. 3). Our several trials to prepare an E2 without the T7 tag or with other tag were unsuccessful and, so far, an efficient expression of the gene is only performed with the pET/E2. We used the pET11a vector to amplify the whole E2 gene. On the other hand, the genes of original strain encoding the *N*- and *C*-terminal regions of E2 are separately amplified by splice overlap extension PCR

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   1 GTG GCT TTT GAA TTT AAG CTG CCG GAC ATT GGC GAA GGC ATC CAC GAA GGT GAA ATT GTC   60
   1 M  A  F  E  F  K  L  P  D  I  G  E  G  I  H  E  G  E  I  V                      20
  61 AAA TGG TTT GTG AAA CCG GGC GAT GAA GTG AAC GAA GAC GAT GTA TTG TGC GAA GTG CAA  120
  21 K  W  F  V  K  P  G  D  E  V  N  E  D  D  V  L  C  E  V  Q                      40
 121 AAC GAC AAA GCG GTC GTC GAG ATC CCA TCT CCT GTC AAG GGG AAA GTG CTT GAG ATT CTC  180
  41 N  D  K  A  V  V  E  I  P  S  P  V  K  G  K  V  L  E  I  L                      60
 181 GTC CCG GAA GGA ACG GTG GCG ACG GTC GGG CAA ACG CTC ATT ACG CTC GAT GCG CCG GGC  240
  61 V  P  E  G  T  V  A  T  V  G  Q  T  L  I  T  L  D  A  P  G                      80
 241 TAT GAA AAC ATG ACG TTT AAA GGA CAA GAA CAC GAA GAA GTG AAA AAA GAG GAA AAA GCG  300
  81 Y  E  N  M  T  F  K  G  Q  E  H  E  E  V  K  K  E  E  K  A                     100
 301 GAA ACG GTG TCG AAA AAG GAA ATG GTT GAG ACC GCT GCT CCG AGC GCA CCG GCG GCC GAA  360
 101 E  T  V  S  K  K  E  M  V  E  T  A  A  P  S  A  P  A  A  E                     120
 361 GCG GAA GCC GAC CCG AAC CGC CGC GTC ATC GCG ATG CCG TCT GTG CGC AAA TAT GCG CGT  420
 121 A  E  A  D  P  N  R  R  V  I  A  M  P  S  V  R  K  Y  A  R                     140
 421 GAA AAA GGC GTC GAT ATC CGC CTC GTC CAA GGC ACG GGG AAA AAC GGC CGC ATT TTG AAA  480
 141 E  K  G  V  D  I  R  L  V  Q  G  T  G  K  N  G  R  I  L  K                     160
 481 GAA GAT ATT GAT GCC TTC CTC GCC GGC GGC GCG AAA GCC GCG GCT GAG CCG ACG CCG CAA  540
 161 E  D  I  D  A  F  L  A  G  G  A  K  A  A  A  E  P  T  P  Q                     180
 541 GCG GCG GAA GAG AAG GCA GCG CCG CAA GCG CCA GCG GCG AAA CCG GTT GTG CCG GAA GGC  600
 181 A  A  E  E  K  A  A  P  Q  A  P  A  A  K  P  V  V  P  E  G                     200
 601 GAA TTC CCG GAA ACG CGC GAG AAA ATG AGC GGC ATC CGT CGG GCG ATC GCC AAG GCC ATG  660
 201 E  F  P  E  T  R  E  K  M  S  G  I  R  R  A  I  A  K  A  M                     220
 661 GTG CAT TCG AAA CAT ACG GCC CCG CAC GTG ACG CTG ATG GAC GAA GCC GAT GTG ACG AAG  720
 221 V  H  S  K  H  T  A  P  H  V  T  L  M  D  E  A  D  V  T  K                     240
 721 CTT GTT GCT CAC CGA AAA AAA TTC AAG GCC ATT GCC GCG GAA AAA GGC ATC AAG CTG ACG  780
 241 L  V  A  H  R  K  K  F  K  A  I  A  A  E  K  G  I  K  L  T                     260
 781 TTC TTG CCG TAT GTC GTC AAA GCG CTA GTT TCC GCG CTG CGT GAA TAT CCG GTG CTG AAT  840
 261 F  L  P  Y  V  V  K  A  L  V  S  A  L  R  E  Y  P  V  L  N                     280
 841 ACG TCG ATC GAC GAT GCG ACG GAG GAA ATT ATT CAT AAG CAT TAC TAC AAC ATC GGC ATC  900
 281 T  S  I  D  D  A  T  E  E  I  I  H  K  H  Y  Y  N  I  G  I                     300
 901 GCC GCT GAT ACG GAT CGA GGG TTG CTC GTG CCG GTC ATT AAA CAT GCC GAC CGG AAG CCG  960
 301 A  A  D  T  D  R  G  L  L  V  P  V  I  K  H  A  D  R  K  P                     320
 961 ATT TTC GCG CTG GCG AAG GAA ATC AAC GAA CTC GCT GAA AAA GCG CGC GAA GGC AAA CTG 1020
 321 I  F  A  L  A  K  E  I  N  E  L  A  E  K  A  R  E  G  K  L                     340
1021 ATG CCA AAC GAA ATG AAA GGC GCA TCG TGC ACG ATC ACG AAC ATC GGC TCA GCC GGC GGG 1080
 341 M  P  N  E  M  K  G  A  S  C  T  I  T  N  I  G  S  A  G  G                     360
1081 CAA TGG TTT ACG CCG GTC ATC AAC CAT CCG GAA GTG GCG ATT CTT GGC ATC GGC CGC ATT 1140
 361 Q  W  F  T  P  V  I  N  H  P  E  V  A  I  L  G  I  G  R  I                     380
1141 GCC GAA AAA CCG ATC GTC CGC GAC GGT GAA ATT GTC GCT GCC CCG ATG CTG GCG CTC TCG 1200
 381 A  E  K  P  I  V  R  D  G  E  I  V  A  A  P  M  L  A  L  S                     400
1201 CTC AGC TTT GAC CAT CGG ATG ATC GAC GGG GCG ACA GCG CAA AAA GCG CTC AAC CAT ATC 1260
 401 L  S  F  D  H  R  M  I  D  G  A  T  A  Q  K  A  L  N  H  I                     420
1261 AAG CAG CTG TTA AGC GAT CCA GAA TTA TTA TTA ATG GAG GCG TAA                      1305
 421 K  Q  L  L  S  D  P  E  L  L  L  M  E  A  *                                    435
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Fig. 2. Nucleotide sequence of E2 gene and its amino acid sequence deduced. Termination codon is indicated by asterisk.

method, and then, an expression plasmid is constructed by connecting the two genes (Lessard *et al.*, 1998).

Purification and characterization of E2

We purified tE2 by a fractionation between 35% and 50% saturation of ammonium sulfate, two consecutive chromatographies using Sepharose CL–2B, and a heat treatment at 70 °C for 5 h. By this procedure, 130 mg of tE2 (162 units/mg) was purified from 1800 ml of a culture medium. Limited proteolysis of PDC by V8 protease yields E2 inner core

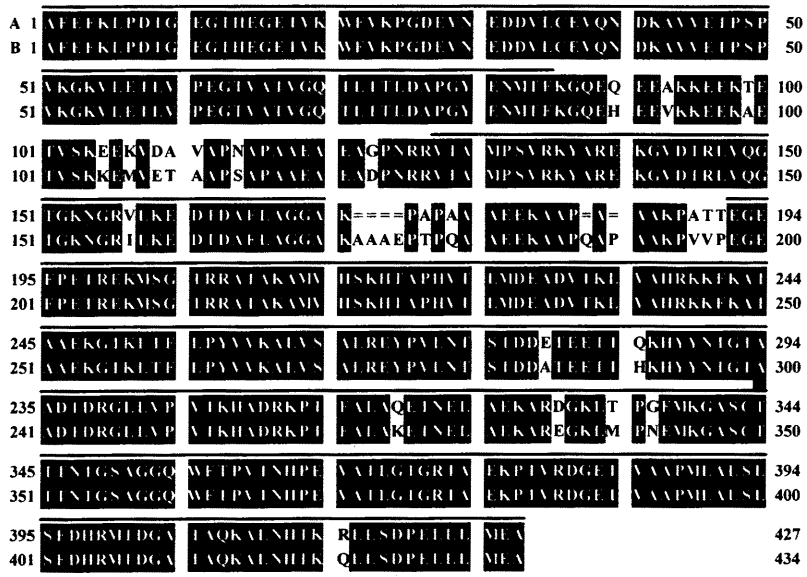

Fig. 3. Comparison of deduced amino acid sequence of E2 from the *B. stearothermophilus* NCA1503 (A) with that from its spontaneous mutant (B). Identical amino acid residue is highlighted on a black background. A gap is indicated by an equal sign. Lines above a vertical pair of residues indicate the three domains: LD (A–1 to F–85), BD (V–128 to A–170), and CD (E–192 to A–427).

(E2ic) that is an assembly of sixty CD polypeptides (Perham and Wilkie, 1980). Previously we examined the GdnHCl–induced denaturation of E2ic and found that a pre–incubation of E2ic at 70 °C and cooling on ice affect its enzyme activity and thermostability insignificantly, but improve the recovery of the activity from GdnHCl–induced denaturation (Hiromasa *et al*, 1998; Aso *et al*., 2001). We therefore adopted the heat treatment in the purification procedures of tE2, although a stabilizing mechanism of E2ic is still under investigation. This treatment might be useful for inactivation of *E. coli* original enzymes including proteins showing affinity for tE2 overexpressed. We confirmed the purity of tE2 by SDS PAGE, comparing with the electrophoretic mobility of an E2 component of PDC separately purified from the *B. stearothermophilus* cells (data not shown). The sedimentation coefficient ($S^{\circ}_{20,w}$) of tE2 was estimated 31S. The sedimentation coefficient of E2ic isolated from PDC is 30S–27S (Packman *et al*, 1984; Hiromasa *et al*, 1998). The molecular size of tE2 is estimated larger than E2ic, but contribution of LD and BD to sedimentation velocity might be less than that of CD, because these domains are connected with flexible region and protruded from the core portion corresponding to E2ic. It was therefore suggested that the value of the sedimentation coefficient of tE2 is similar to that of E2ic and that tE2 also comprises sixty subunits. Since the overexpression of tE2 was never done under any lipoylation conditions such as a use of culture medium supplemented with lipoic acid, tE2 was supposed lipoyl–deficient (Wallis and

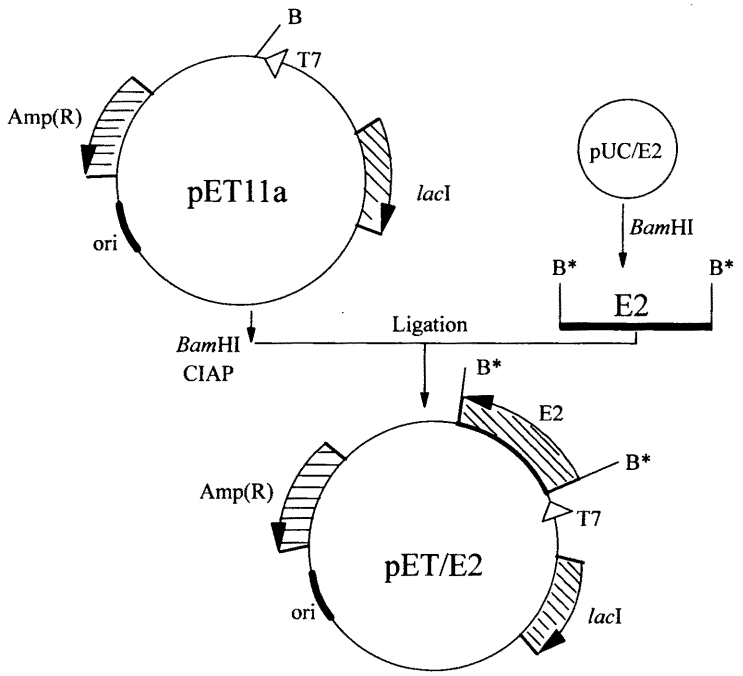

Fig. 4. Construction scheme of pET/E2. Shadowed portion and arrow of circular arc indicate gene and its orientation, respectively. CIAP, calf intestinal alkaline phosphatase; B, *Bam*HI site; B*, *Bam*HI site introduced; ori, origin of ColE1 plasmid replication; *lac*I, gene encoding *lac* repressor; T7; T7 promoter; Amp(R), ampicillin resistance gene.

1	MASMTGGQQM GRGSVAFEFK LPDIGEGIHE GEIVKWFVKP	40
41	GDEVNEDDVL CEVQNDKAVV EIPSPVKGKV LEILVPEGTV	80
81	ATVGQTLITL DAPGYENMTF KGQEHEEVKK EEKAETVSKK	120
121	EMVETAAPSA PAAEAEADPN RRVIAMPSVR KYAREKGVDI	160
161	RLVQGTGKNG RILKEDIDAF LAGGAKAAAE PTPQAAEEKA	200
201	APQAPAAKPV VPEGEFPETR EKMSGIRRAI AKAMVHSKHT	240
241	APHVTLMDEA DVTKLVAHRK KFKAIAAEKG IKLTFLPYVV	280
281	KALVSALREY PVLNTSIDDA TEEIIHKHYY NIGIAADTDR	320
321	GLLVPVIKHA DRKPIFALAK EINELAEKAR EGKLMPNEMK	360
361	GASCTITNIG SAGGQWFTPV INHPEVAILG IGRIAEKPIV	400
401	RDGEIVAAPM LALSLSFDHR MIDGATAQKA LNHIKQLLSD	440
441	PELLLMEA	448

Fig. 5. Deduced amino acid sequence of tE2. A bar indicates *N*-terminal extension sequence.

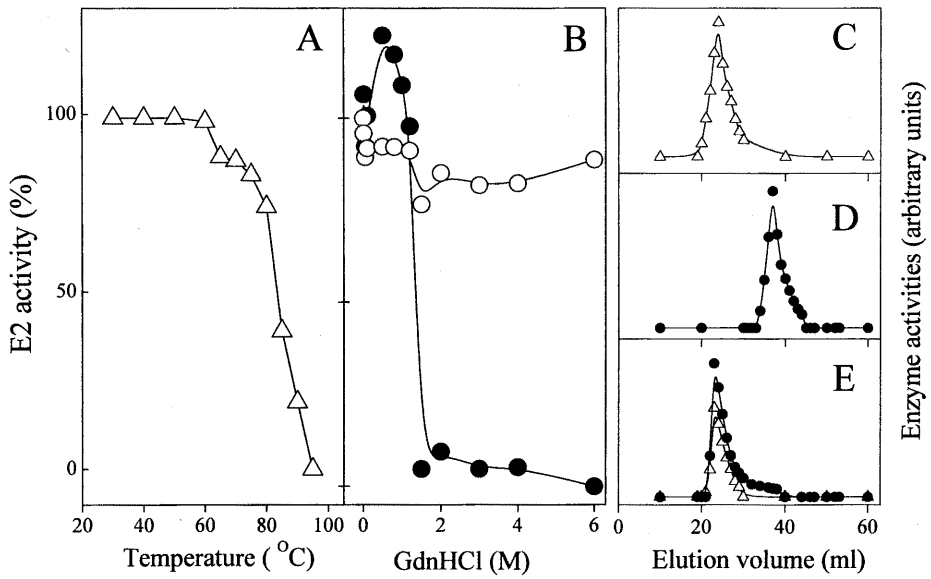

Fig. 6. Temperature– or GdnHCl–induced changes in enzyme activity of tE2 and complex formation between tE2 and E3. Panel A: temperature–induced inactivation of tE2. Residual activities (△) after incubation at various temperatures are plotted as values relative to the activity without incubation. Panel B: GdnHCl–induced changes in activity of tE2; E2 activity after incubation with GdnHCl (●); E2 activity after the incubation and subsequent removal of GdnHCl by dialysis (○). Activities are plotted as values relative to the activity in the absence of GdnHCl. Panel C–E: gel–filtration profiles of tE2 (C; △, E2 activity), E3 (D; ●, E3 activity), and their mixture (E; △, E2 activity; ●, E3 activity).

Perham, 1994; Lessard *et al.*, 1998).

We examined the thermostability of tE2 by incubating at various temperatures, cooling, and measuring residual activity. As shown in Fig. 6A, tE2 lost about 25% of its original activity by incubation at 80 °C for 60 min. Only based on these results, there might be no drastic difference in thermostability between tE2 and E2ic, and tE2 was highly resistant to heat (Aso *et al.*, 2001). We also examined the effects of GdnHCl on tE2. After incubation at 4 °C for 24 h in the presence of GdnHCl, an enzyme activity was measured. Although GdnHCl at concentrations less than 1.0 M had a tendency to improve the activity, as shown in Fig. 6B, tE2 lost almost all its activity in 1.5 M GdnHCl. Gradual removal of GdnHCl by dialysis restored at least 80% of its original activity even after incubation in 6 M GdnHCl. Since the assay of enzyme activity dilutes GdnHCl rapidly, our observations might include changes induced by rapid dilution. It was therefore implied that the rapid dilution and the gradual removal induce irreversible and reversible changes

in tE2, respectively. We also monitored the intensity of light scattering (at 540 nm) of tE2 in the presence of GdnHCl and found that the intensity decreases with increasing amount of GdnHCl and that GdnHCl at concentrations above 1.5 M quenches the scattering (data not shown). Based on these results, tE2 was suggested completely dissociated in 1.5 M. E2ic of original strain is expressed in *E. coli* and purified as an active sixty-mer; this E2ic comprises residues from 173 to 427 (Allen *et al.*, 1997). The E2ic dissociated into monomers in the presence of 6 M GdnHCl is reassociated into fully active species by dialysis against a phosphate buffer with stepwise decrease in GdnHCl content. In our experiments, GdnHCl was never removed by such a carefully designed method. Irreversible loss in activity of tE2, therefore, might occur partially.

Finally we examined whether tE2 is capable of binding non-covalently to the *B. stearothermophilus* E3. tE2, E3, and their mixture were analyzed by gel filtration. The peaks of E2 and E3 activities were detected at elution volumes of 24 ml and 37 ml, respectively (Fig. 6C & 6D); in both cases, an elution profile of activity was overlapped with that of protein (data not shown). When the mixture of tE2 with E3 was prepared in the total A_{280} ratio tE2:E3=1:2 and analyzed, each of E2 and E3 activities was eluted as a single peak at 23 ml (Fig. 6E); proteins were also eluted as a single peak at the same volume (data not shown). This volume was at the exclusion limit of the Sephacryl column and smaller than that of tE2. Upon mixing in the total A_{280} ratio tE2:E3=1:5, E2 activity was also peaked at 23 ml, but E3 activity was split into the two peaks at 23 ml and 37 ml (data not shown). Based on these results, tE2 was strongly suggested to form a complex with multiple copies of E3 molecules.

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