Expression in *Escherichia coli* of Genes Encoding the E1α and E1β Subunits of the Pyruvate Dehydrogenase Complex of *Bacillus stearothermophilus* and Assembly of a Functional E1 Component (α₂β₂) in Vitro*

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The E1α and E1β subunits of the pyruvate decarboxylase (E1) component of the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus* were produced from two genes overexpressed separately in *Escherichia coli*. A functional E1 enzyme was generated from disrupted mixtures of cells containing the separately overexpressed E1α and E1β genes. The purified E1 enzyme exhibited an apparent molecular mass of 150,000 Da, consistent with an αβ₂ structure. The E1α and E1β (30 °C) were found to be 0.9 ± 0.2 μM and 0.47 ± 0.03 s⁻¹, respectively. The purified E1α subunit existed as a monomer (42,000 Da), whereas the E1β subunit existed mainly (95%) in a tetrameric form (145,000 Da). Mixing equimolar amounts of the pure recombinant E1α and E1β subunits in vitro generated a functional E1 enzyme with a molecular mass and an E1 activity similar to those of the E1(αβ₂) enzyme purified from disrupted mixtures of cells containing individually expressed subunits. Mixing individual subunits in vitro with one of the subunits in excess resulted in complete assembly of the lesser subunit into the intact E1(αβ₂) enzyme. Thus, no chaperonin is needed in vitro to promote the assembly of the separate subunits to form the E1 component of the pyruvate dehydrogenase multienzyme complex of *B. stearothermophilus*.

The 2-oxoacid dehydrogenase multienzyme complexes catalyze the irreversible oxidative decarboxylation of pyruvate, 2-oxoglutarate, and branched-chain 2-oxo acids, generating the corresponding acyl-CoA and NADH (for recent reviews, see Patel and Roche (1990), Perham (1991), and Mattevi et al. (1992)). They consist of multiple copies of three component enzymes: a 2-oxoacid decarboxylase (E1), a dihydrolipoyl acyltransferase (E2), and a dihydrolipoyl dehydrogenase (E3). The E2 polypeptide chain is a multidomain structure consisting of (from the NH₂ terminus) one to three lipoyl domains, each of which carries a lipoyl group, a peripheral (E1 and E3) subunit-binding domain, and a catalytic (acyltransferase) domain that aggregates to form the octahedral (24-mer) or icosahedral (60-mer) core of the complex (Oliver and Reed, 1982; Reed and Hackert, 1990; Perham, 1991).

The high flexibility of the interdomain segments of polypeptide chain (Green et al., 1992) permits the lipoyl domains to move, facilitating the shuffling of the substrate, which is in turn attached to the lipoyl group, between the successive active sites (Texter et al., 1988; Miles et al., 1988; Radford et al., 1989a, 1989b). Substrate channeling is ensured by specific molecular recognition between the lipoyl domain and the E1 component of the parent 2-oxoacid dehydrogenase complex (Graham et al., 1989). Although no 2-oxoacid dehydrogenase complex has been crystallized, the three-dimensional structures of each of the domains that compose the E2 component have been solved separately, either by x-ray crystallography (Mattevi et al., 1993a) or NMR spectroscopy (Robien et al., 1992; Dardel et al., 1993; Kalia et al., 1993). The three-dimensional structure of the E3 component, an enzyme common to the different complexes, has been determined by x-ray crystallography (Mattevi et al., 1993b). However, no three-dimensional structure of any E1 component has yet been solved, and only limited structural information is available.

The E1 component catalyzes the first reaction in the complex, the thiamin diphosphate (ThDP)-dependent oxidative decarboxylation of the 2-oxo acid, followed by the reductive acylation of the lipoyl group covalently bound to a lysine residue of the E2 lipoyl domain. The decarboxylation step is the rate-limiting step in the overall reaction catalyzed by the complexes (Danson et al., 1978; Akiyama and Hammes, 1980; Cate et al., 1980). There are two possible forms of the E1 component: the homodimer (α₂) with a subunit molecular mass of ~100 kDa, as found in the octahedral 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes, and the heterotetramer (αβ₂) with subunit molecular masses of ~41 and 36 kDa, respectively, which is found in the octahedral branched-chain 2-oxoacid dehydrogenase and icosahedral pyruvate dehydrogenase complexes. In the heterotetrameric form, the E1α subunit appears to comprise two large domains with molecular masses of 31 and 10 kDa (Koike et al., 1992).

The genes encoding the E1α and E1β chains of the pyruvate dehydrogenase and branched-chain 2-oxoacid dehydrogenase complexes and the unsplit E1 chain of the 2-oxoglutarate dehydrogenase complex may share a common ancestor (Matuda et al., 1991), but the E1 chain from the *Escherichia coli* pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes show a pronounced lack of sequence similarity (Guest et al., 1989). However, a 30-amino acid residue motif, beginning with -GDG- and ending with -NN-, has been detected in all known ThDP-dependent enzymes, irrespective of their lack of sequence homology otherwise, and predicted to be involved in...
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ThDP binding (Hawkins et al., 1989). Thus, it is found in the E1 (E1a) chain of all 2-oxoacid dehydrogenase complexes, the subunit associated with ThDP binding (Stepp and Reed, 1985). Its predicted role has been substantiated by the recent determination of the three-dimensional structures of transketolase (Lindqvist et al., 1992), pyruvate oxidase (Muller and Schulz, 1993), and pyruvate decarboxylase (Iida et al., 1993) and by the results of site-directed mutagenesis of pyruvate decarboxylase (Diefenbach et al., 1992), all ThDP-dependent enzymes.

Genes expressing the E1a (Davie et al., 1992) and E1β (Wynn et al., 1992a) chains of a mammalian branched-chain 2-oxoacid dehydrogenase complex have recently been overexpressed in E. coli. The recombinant E1β subunit, but not the E1α subunit, binds to the E2 component (Wynn et al., 1992a), in accord with predictions made from scanning transmission electron micrographs of the complex (Hackert et al., 1989) and from limited proteolysis studies of the mammalian pyruvate dehydrogenase complex (Rahmatullah et al., 1989). Coexpression of genes encoding the E1α subunit, as a fusion protein with E. coli maltose-binding protein (MBP), and the E1β subunit generates an active E1a(E1bβ) tetramer, which, unlike mixing individually overexpressed subunits in vitro does not (Davie et al., 1992). This suggests that simultaneous expression of both subunits in the same cellular compartment is important for assembly into a functional E1 component in vivo (Davie et al., 1992). Furthermore, chaperonins appear to be involved in promoting the assembly of the active tetrameric E1(E1bβ) enzyme, as indicated by coexpressing the MBP-E1α and E1β genes in an E. coli strain deficient in chaperonins groEL and groES, with and without the concomitant expression of these chaperonins from a second vector (Wynn et al., 1992b).

The pyruvate dehydrogenase complex from the thermophilic Gram-positive organism Bacillus steaerothermophilus is representative of bacterial pyruvate dehydrogenase complexes of i-cosahedral symmetry and correspondingly has an E1 component of the a1b1 type. Both the E1α and E1β genes have been cloned and sequenced (Hawkins et al., 1990). In this paper, we report the overexpression in E. coli of genes encoding the B. stearothermophilus E1α and E1β subunits separately, the purification of the protein subunits, and their assembly into the active E1(a1b1) component in vitro without the need for chaperonins.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bacteriological media were from Difco. The pBS42 vector carrying the E1α and E1β genes and E. coli host strain TG1 reco(K12, lac-proAB), supE, thi, hsdS, recO::Tn5 Kanr/F'traD36, proA+B+, lacIq, lacZAM15) have been described previously (Hawkins et al., 1990). The overexpression vector pKK223-3, restriction endonucleases, Klenow fragment, calf intestinal alkaline phosphatase, Dideoxynucleotide DNA sequencing (Sanger et al., 1986), from Beecham Laboratories. Molecular mass standards for gel filtration were from Sigma, and ampicillin was purchased from Sigma. Ultrapure agarose and cesium chloride were from Life Technologies, and ampicillin was purchased from Sigma. Ultrapure agarose and cesium chloride were from Life Technologies.

**Recombinant DNA Techniques—**Recombinant DNA techniques were carried out as described elsewhere (Sambrock et al., 1989). Small DNA fragments were isolated by the crush-and-soak method of bands excised from 5% polyacrylamide gel electrophoresis (Sambrock et al., 1989). Oxodeoxynucleotide DNA sequencing (Sanger et al., 1977) was carried out using the T7 Sequencing™ kit from Pharmacia and [α-32P]dATP from Amersham International. The techniques used for the double-stranded DNA isolated by using the Magic™ miniprep DNA purification system (Promega). Polymerase chain reactions (PCRs) (Saiki et al., 1985, 1988) were carried out under mineral oil in a reaction buffer (100 µl) containing 100 ng of double-stranded plasmid DNA, 200 mM dNTPs, 2 mM MgCl2, 100 µM of each sense and antisense primers, and 2.5 units of Taq DNA polymerase on a programmable PCR-3 thermal cycler (Techne Corp.). The cycling parameters used were as follows: 2 min at 95 °C (one cycle); 1 min at 94 °C, 2 min at 50 °C, and 3 min at 72 °C (5 cycles); and 10 min at 72 °C (one cycle). The fidelity of the amplified PCR fragment was established by DNA sequencing after subcloning into the vector. The primers used either for PCR or DNA sequencing were synthesized on a Pharmacia Gene Assembler PlusTM.

**Construction of Expression Vector for Mature E1α Subunit—**The pBS42 vector was digested with XmaI and treated with Klenow fragment and then with calf intestinal alkaline phosphatase to produce an intermediate vector, pkBstEla.1 (Fig. 1a). Cleavage with XmaI followed by treatment with Klenow fragment was necessary to regenerate the BstEII restriction site lost by the previous NeoI digestion and mungbean nuclease treatment of the pBS42 vector. To reintroduce the deleted 5′-terminal sequence of the E1α gene, a 725-bp DNA fragment containing the 5′-terminal sequence and a new EcoRI restriction site preceding the initiator region upstream of the E1α gene was amplified by PCR (Scharf et al., 1986) from the pBS42 vector. The sense/antisense primers used were a 19-bp oligonucleotide (5′-AGCCTGAATTCGAATTC-3′ (mis- matched base) and a 17-bp oligonucleotide (5′-AAAACCGATCTTCG-3′), which annealed to the region corresponding to bases 1386–1404 and 2097–2112, respectively, of the DNA insert in the pBS42 vector. The PCR-amplified DNA fragment was digested with BstEII and then with EcoRI. The EcoRI-BstEII DNA fragment (426 bp) isolated from the pBS42 vector gel electrophoresis was subcloned into the pKK223-3 expression vector previously digested with BstEII and EcoRI and treated with calf intestinal alkaline phosphatase (Fig. 1a). The resultant expression vector, pkBstElb1, encodes the entire B. stearothermophilus E1α subunit. The DNA was fully sequenced to ensure its accuracy.

**Construction of Expression Vector for Mature E1β Subunit—**The pBS42 vector was digested with Asp700 and PstI. The 876-bp DNA fragment (3′-terminal sequence of the E1β gene) isolated after 5% polyacrylamide gel electrophoresis was subcloned into the pKK223-3 expression vector previously digested with HindIII, treated with Klenow fragment and then with calf intestinal alkaline phosphatase to produce an intermediate vector, pkBstElb1.1 (Fig. 1b). The 5′-terminal sequence of the gene was reintroduced by PCR amplification of a 185-bp DNA fragment from the pBS42 vector containing a new EcoRI restriction site and the initiator region (Shine-Dalgarno sequence and the downstream sequence between it and the initiation codon) of the E1α gene in front of the E1β gene. The sense/antisense primers used were a 46-bp oligonucleotide (5′-CCCGAATTCGAGGTCACCCAAGT-GCCGGGCAAATGACAATGTCG-3′ (mismatched underlined)) and a 17-bp oligonucleotide (5′-CAAAACGCCGATCTTCG-3′), which annealed to the region corresponding to bases 2543–2566 and 2689–2710, respectively, of the pBS42 vector gel electrophoresis. The PCR-amplified DNA fragment was digested with EcoRI and PstI. The EcoRI-PstI DNA fragment (147 bp) isolated from 5% polyacrylamide gel electrophoresis was subcloned into the pKBstElb1.1 vector previously digested with PstI and EcoRI and treated with calf intestinal alkaline phosphatase (Fig. 1b). The resultant expression vector, pkBstEla1, encodes the entire B. stearothermophilus E1β subunit. The DNA was fully sequenced to ensure its accuracy.

**Overexpression of E1α and E1β Subunits**—E. coli strain TG1 recoO cells transformed with pKBstEla1 or pkBstElb1 were grown as an overnight culture (5 ml) in 2 x YT medium (Sambrock et al., 1989) containing 0.5% glucose and 20 µg/ml ampicillin and 37 °C and used to inoculate 1 liter of the same medium. When the absorbance at 600 nm reached 0.7, the inducer IPTG was added to a final concentration of 1 mM. After 6 h, the cells were harvested by centrifugation; washed with 20 mM potassium phosphate buffer, pH 7.0 (buffer A); and then centrifuged again. The sedimented cells were stored at −20 °C until needed (not more than 4 days).

**Purification of Recombinant E1 Enzyme and Its Separate Subunits, E1α and E1β—**All steps were performed at 4 °C unless otherwise stated. Protein was estimated by the Coomassie dye binding method (Bradford, 1976), except for pure proteins, which were estimated by amino acid analysis (Packman et al., 1986). Column fractions were analyzed with SDS-polyacrylamide gel electrophoreses (PAGE) (12.5% acrylamide) using the Pharmacia PhastSystem™. Cells (1 liter of culture) were resuspended in buffer A (2 ml/g of wet cells) containing 1 mM phenylmethylsulfonyl fluoride. To purify the recombinant E1 enzyme, cells expressing individual E1α and E1β chains were mixed and then disrupted (18,000 pounds/square inch) in a French press (SLM-AMINCO). Cell debris was removed by centrifugation (95,000 x g for 20
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Fig. 1. Scheme for the construction of the expression vectors pKBstElA (a) and pKBstE1b (b). The construction of the expression vectors is described in detail in the text. Genes are shown by differential shading, and the orientations of genes and promoters are indicated with arrows. A, Asp700 site; Amp', ampicillin resistance gene shading, and the orientations of genes and promoter are indicated with arrows. B, BstEII site; CIAP, calf intestinal alkaline phosphatase; C-t E1a (E1B), fragment of the E1a (E1B) gene encoding the COOH-terminal part of the E1a (E1B) chain; EcoRI site (*; introduced); EcoRl (E1B), the E1a (E1B) gene; H, HindIII site; Nt, NcoI site (*; digested and treated with mungbean nuclease); Nl-E1a (E1B), E2), fragment of the E1a (E1B), E2) gene encoding the NH2-terminal part of the E1a (E1B), E2) chain; ori, origin of ColEl plasmid replication; P, PstI site; Psc, tata promoter; Ter*, incomplete sequence of tetracycline resistance gene (sensitive); X, XmaI site.

min), and the supernatant was applied to a Whatman DE52-cellulose anion-exchange column (1.6 × 30 cm; flow rate of 1 ml/min) previously equilibrated with buffer A. The recombinant E1 enzyme eluted with a linear gradient of 0–1 M NaCl in buffer A at 1 ml/min over 600 ml. Fractions (6 ml) containing the recombinant E1 and E1P subunits were pooled, dialyzed against buffer A, and loaded in batches of 3–4 mg of total protein onto a PorosTM 75 gel filtration column (1.6 × 60 cm; flow rate of 1 ml/min) equilibrated with 50 mM potassium phosphate, pH 7.0 (buffer C). Fractions (1 ml) containing the recombinant E1 subunit were pooled and loaded in batches of 1.5–2 mg of total protein onto a PorosTM Q column equilibrated with buffer A. The recombinant E1α subunit eluted with a linear gradient of 0.05–0.2 M NaCl in buffer A at 1 ml/min over 9 ml. Fractions (0.25 ml) containing the recombinant E1α subunit were pooled, dialyzed overnight against buffer C, and stored frozen in liquid nitrogen. For the recombinant E1β subunit, after the DE52 column chromatography, fractions (5 ml) containing subunits were pooled, dialyzed overnight against buffer C, and loaded in batches of 3–4 mg of total protein onto a PorosTM Q column equilibrated with buffer A. The recombinant E1β chain eluted with a linear gradient of 0.3–0.5 M NaCl in buffer A at 1 ml/min over 6 ml. Fractions (0.25 ml) containing the recombinant E1β subunit were pooled, dialyzed overnight against buffer C, and stored frozen at −80 °C. The purity of the proteins (E1, E1α, and E1β) was confirmed by amino acid analysis (Packman et al., 1988) and SDS-PAGE.

Reconstitution of E1 Enzyme from Individually Purified Subunits—Reconstitution of the E1 enzyme from the purified E1α and E1β subunits was analyzed by measuring the E1 activity in mixtures of the individual subunits in various molar ratios (see "Enzyme Assays"). Mixtures (2 mg of total protein) of the pure recombinant E1α and E1β subunits in molar ratios of 1:1, 1:2 and 2:1, respectively, were also loaded onto a PorosTM Q column equilibrated with buffer A at 25 °C, and the different protein species eluted with a linear gradient of 0–0.6 M NaCl in buffer A at 1 ml/min over 12 ml. Fractions (0.5 ml) containing the E1 enzyme were pooled and applied to a calibrated Superdex™ 200HR column for the determination of molecular mass as described above.

Enzyme Assays—The E1 enzyme activity was monitored in 1 ml cuvettes at 30 °C in an HP 8452 diode array spectrophotometer (Hewlett-Packard Co.) using 2,6-dichlorophenolindophenol as substrate (Khailova et al., 1977). The activity was followed by measuring the linear decrease in the absorbance at 600 nm over the first 20 s. A solution containing the recombinant E1 enzyme, 2.1 mM MgCl2, and 0.21 mM ThDP in 104 mM potassium phosphate buffer, pH 7.6, was prepared 10 min before the assay and kept on ice throughout the experiment. Samples (960 μl) of this solution were incubated for 10 min at 30 °C, followed by the addition of 20 μl of 2.5 mM 2,6-dichlorophenolindophenol. The assay was started by adding a sample (20 μl) of pyruvate (0–20 mM). The data were fitted to Michaelis-Menten and Hanes equations (KaleidaGraph, Synergy software, PCS Inc.). For the reconstitution of the E1 enzyme, the individual E1α and E1β subunits were mixed at various molar ratios, and a sample (20 μl) was added to a solution (940 μl) of 106 mM potassium phosphate buffer, pH 7.0, containing 2.1 mM MgCl2 and 0.21 mM ThDP and preincubated for 10 min at 30 °C. Then, 20 μl of 2.5 mM 2,6-dichlorophenolindophenol was added, and the assay was started by adding 20 μl of 20 mM pyruvate.

SDS-Polyacrylamide Gel Electrophoresis—Proteins were separated by SDS-PAGE using a discontinuous Tris/glycine buffer (Laemmli, 1970) at 12% acrylamide and 5% acrylamide resolving gels containing 0.1% SDS. Electrophores buffers were supplemented with 2...
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Fig. 2. Expression of the *B. stearothermophilus* Ela and E1β genes in *E. coli*. *E. coli* TGI recO cells were transformed with the relevant plasmid (pKBstElα or pKBstElβ), grown, and induced with IPTG as described in the text. The cell pastes were mixed and disrupted, and the recombinant E1 enzyme was purified by sequential ion-exchange chromatography on DEAE-cellulose and Poros™ Q/M columns. Samples were analyzed by means of SDS-PAGE with Coomassie Blue staining at each stage of the procedure. Lane 1, molecular mass standards (bovine serum albumin, 66.0 kDa; ovalbumin, 45.0 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa; carbonic anhydrase, 29.0 kDa; and trypsinogen, 24.0 kDa); lane 2, *E. coli* cells transformed with pKK223-3; lane 3, *E. coli* cells transformed with pKBstElα; lane 4, *E. coli* cells transformed with pKBstElβ; lane 5, *B. stearothermophilus* pyruvate dehydrogenase complex (apparent molecular masses: E3, 57.0 kDa; E2, 54.0 kDa; Elα, 42.0 kDa; and E1β, 36.0 kDa (Henderson and Perham, 1980)).

RESULTS AND DISCUSSION

Construction of Expression Vectors pKBstElα and pKBstElβ—We tried initially to coexpress the *B. stearothermophilus* E1α and E1β genes from the same promoter (tac) since there is a Shine-Dalgarno sequence within the 3'-terminal sequence of the E1α gene upstream of the E1β gene. However, all efforts to generate the appropriate plasmid (pKBstEl) were unsuccessful, suggesting that something intrinsic to the genes or the Elα(αββ) protein is toxic to the *E. coli* cells (data not shown). Separate expression of the E1α and E1β genes was therefore attempted, enabling separate studies of the protein subunits and of their assembly in *vivo* to form the E1(αββ) enzyme.

The expression of the Elα and Elβ subunits was determined by total cellular levels of Elα and Elβ proteins that reached ~35 and 50%, respectively, of the total cellular protein (Fig. 2). NH2-terminal sequence analysis revealed that both subunits were processed in *vivo* to remove the NH2-terminal methionine residue and that the NH2-terminal amino acid sequences, GVKTF-(E1α) and AQMTMV-(E1β), matched those encoded by the DNA sequence. All of the E1α subunit was expressed in a soluble form, but 25% of the E1β subunit was found in the cell debris after centrifugation, which may reflect some inclusion bodies formed as a result of the high level of gene expression (Williams et al., 1982). These have been observed for the overexpression of the E1β chain of the mammalian branched-chain 2-oxoacid dehydrogenase complex (Wynn et al., 1992a).

Association of Elα and E1β Subunits to Form Functional E1 Enzyme—*E. coli* TGI recO cells expressing the E1α and E1β genes separately were mixed and then disrupted together. An association of the E1α and E1β subunits was indicated by chromatography of the mixed crude extract on a DE52 column, followed by a Poros™ Q/M column (Fig. 3). Excess E1β subunit did not coelute with the intact E1 enzyme, but emerged later in a position similar to that of the E1β subunit when it was purified alone (Fig. 3, lane 2). The recombinant E1 enzyme was >95% pure as judged by SDS-PAGE (Fig. 3) and amino acid analysis (data not shown). Over 80 mg of pure E1 was produced from a cell mixture comprising 1-liter cultures of each of the two transformed *E. coli* cells.

The pure recombinant E1 enzyme was passed through a calibrated Superdex™ 200HR gel filtration column. It eluted as a single peak with an estimated molecular mass of 150 kDa. This is consistent with an αββ structure based on the molecular mass (153,262 Da) calculated from the amino sequences of the two subunits. Coexpression in the same cell of genes encoding both subunits of the E1 component of the *B. stearothermophilus* pyruvate dehydrogenase complex is evidently not essential for the assembly of a functional E1(αββ) enzyme; mixing the crude extracts of cells separately expressing the E1α and E1β genes is sufficient.

Quaternary Structure of E1α and E1β Subunits—The individual E1α and E1β subunits generated by expression of the E1α and E1β genes from plasmids pKBstElα and pKBstElβ, respectively, were purified as described under "Experimental Procedures." They were >95% pure as judged by SDS-PAGE (Fig. 4) and amino acid analysis (data not shown). The yields of the pure *B. stearothermophilus* E1α and E1β subunits obtained from 1-liter cultures of induced *E. coli* TGI recO cells were 25 and 70 mg, respectively.

To assess their quaternary structure, the E1α and E1β sub-
units were gel-filtered on calibrated Superdex™ 75HR and 200HR columns, respectively. Neither eluted in a dimeric form \((\alpha_2 \beta_2)\) over a range of protein concentrations from 0.5 to 1.5 mg/ml. The Ela subunit eluted as a single peak with an estimated molecular mass of 42 kDa, corresponding to a monomeric form (calculated molecular mass of 41,320 Da). On the other hand, >95% of the ElP subunit eluted in a peak with an estimated molecular mass of 145 kDa. This appears to be a tetrameric form \((E1\beta)^4\) based on the subunit molecular mass of 35,311 Da calculated from the DNA sequence. The remaining 5% was spread over a molecular mass range of <87–140 kDa, which might be explained by an equilibrium between \(E1\beta\), \(E1\beta_2\), and \(E1\beta\) forms occurring during the passage of the protein through the column.

Association of Purified Ela and ElP Subunits into Functional El Component—The reconstitution of the El enzyme from the purified subunits was evaluated by mixing them in different molar ratios and assaying for the catalytic activity of the assembled El enzyme. The observed activities, based on the molar quantity of each Ela or ElP chain, were compared with the value obtained for half of the same molar quantity of the recombinant El(\(\alpha\beta\)) enzyme that had been purified from the mixed crude extract (Fig. 5). When the recombinant ElP subunit was mixed with an excess of the recombinant Ela subunit, the catalytic activity recovered was identical to that of the same molar quantity of recombinant El based on its ElP subunit content. This suggests that all recombinant ElP chains bound to the recombinant Ela chains to create a fully active El enzyme. The same was observed when the recombinant Ela subunit was mixed with an excess of the recombinant ElP subunit, suggesting that all the recombinant Ela chains bound to the recombinant ElP chains to create likewise a fully active El enzyme. However, in an equimolar mixture of Ela and ElP chains, only 92% of the potential El activity was observed, suggesting that 8% of the recombinant Ela and ElP chains present failed to assemble correctly. This was confirmed by anion-exchange chromatography of an equimolar mixture of the chains: the majority of the protein eluted as the assembled El enzyme, whereas the remaining noncomplexed Ela and ElP chains eluted separately (Fig. 610).

### Fig. 5. Activity of the El enzyme reconstituted from the purified Ela and ElP subunits. The purified Ela and ElP subunits were mixed at different molar ratios and assayed for El catalytic activity. The activity recorded for the molar quantity of the El(\(\alpha\beta\)) chain present in the assay was compared with that measured for half of the same molar quantity of the El(\(\alpha\beta\)) enzyme. 

- Adding ElP to a fixed amount (300 pmol) of the Ela chain; 
- Adding Ela to a fixed amount (300 pmol) of the ElP chain.

Fractions containing the reconstituted El enzyme were pooled and gel-filtered on a calibrated Superdex™ 200HR column. The El enzyme eluted as a single peak with an estimated molecular mass of 150 kDa. This value is similar to that obtained for the recombinant El enzyme purified from the mixed crude extract (see above) and is consistent with an \(\alpha\beta\) structure. The same result was observed when ElP was reconstituted from pure recombinant ElP and Ela subunits mixed in a molar ratio of 1:2 or 2:1 and gel-filtered on a Superdex™ 200HR column. Thus, it is clear that no chaperonin is needed in vitro to promote the correct assembly of the \(\alpha\beta\) structure and that excess Ela or ElP chains remain unbound.

### Kinetic Analysis—To achieve the maximal catalytic activity, preincubation of the recombinant El enzyme for 10 min in the presence of the cofactor ThDP and MgCl\(_2\) was necessary. No further increase in activity was detectable after 8 h of preincubation (data not shown), resulting in a 3.5-fold increase in the activity compared with that obtained by starting the assay immediately by the addition of enzyme or cofactor. A lag phase has been observed in the past for different ThDP-dependent enzymes (Horn and Bisswanger, 1983). Initial rate measurements (0–20 s) could be fitted to Michaelis-Menten and Hanes plots (data not shown) and yielded a \(K_m\) for pyruvate and a \(k_{cat}\) of 0.9 ± 0.2 μM and 0.47 ± 0.03 s\(^{-1}\), respectively.

### Conclusion—We have shown that coexpression of genes encoding the Ela and ElP subunits of the El component from the B. stearothermophilus pyruvate dehydrogenase complex is not necessary for enzyme assembly. By mixing and disrupting E. coli cells separately expressing the two genes, we have produced a functional El(\(\alpha\beta\)) enzyme. We also purified each subunit separately and showed that the pure subunits are capable of self-assembly when mixed together to produce a functional El(\(\alpha\beta\)) enzyme. Thus, it would appear that molecular chaperones are not required to promote correct assembly of the El component of the B. stearothermophilus pyruvate dehydrogenase complex, at least in vitro. These results differ from those obtained for the assembly of overexpressed MBP-Ela fusion and ElP subunits of the branched-chain 2-oxoacid dehydrogenase complex of mammalian mitochondria, which suggest that chaperonins groEL and groES are required to promote assembly of this eukaryotic enzyme in E. coli cells deficient in these chaperonins (Wynn et al., 1992b). In the latter work, the reconstitution of the El enzyme was undertaken using the Ela subunit fused to MBP (Davie et al., 1992; Wynn et al., 1992b), and we therefore cannot exclude the possibility of the fused MBP preventing normal assembly of the El enzyme or of it.
interfering in the folding pathway of the attached $E_1\alpha$ subunit. Equally, in the present work, we cannot exclude a role for chaperonins in promoting the folding of the individual $E_1\alpha$ and $E_1\beta$ subunits, which might explain the low recovery of $E_1$ activity when the denatured polypeptide chains of the pyruvate dehydrogenase complex from $B.\text{stearothermophilus}$ are mixed together (Jaenicke and Perham, 1982). With the successful cloning and overexpression of the $E_1\alpha$ and $E_1\beta$ genes, this and other aspects of the structure and function of $E_1$ can now be pursued.

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