

Expression in *Escherichia coli* of a sub-gene encoding the lipoyl and peripheral subunit-binding domains of the dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*

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A sub-gene encoding the *N*-terminal 170 residues of the dihydrolipoamide acetyltransferase chain of the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus* was over-expressed in *Escherichia coli*. The expressed polypeptide consists of the lipoyl domain, inter-domain linker and peripheral subunit-binding domain; these were found to have folded into their native functional conformations as judged by reductive acetylation of the lipoyl domain, limited proteolysis of the linker region and ability to bind the dihydrolipoamide dehydrogenase dimer. The di-domain was largely (80%) unlipoylated; a small proportion (4%) was correctly modified with lipoleic acid and the remainder (16%) was aberrantly modified with octanoic acid. A polyclonal antiserum was raised that recognized both the di-domain and the individual component domains. The 400 MHz ¹H-n.m.r. spectrum of the di-domain showed resonances corresponding to those seen in spectra of the lipoyl domain, plus others characteristic of amino acid residues in the flexible linker region. Further, as yet unidentified, resonances are likely to be derived from the peripheral subunit-binding domain. The existence and independent folding of the peripheral subunit-binding domain is thus confirmed and its purification in large-scale amounts for detailed structural analysis is now possible.

INTRODUCTION

The 2-oxo acid dehydrogenase multienzyme complexes are well-characterized enzymes that catalyse oxidative decarboxylation reactions in the citric acid cycle and branched-chain amino acid metabolism [for recent reviews see Perham (1991) and Patel & Roche (1990)]. They are composed of multiple copies of three component enzymes and are classical examples of a 'swinging-arm' mechanism whereby the substrate is channelled through the catalytic reaction by its attachment in thioester linkage to a lipoyl group on the dihydrolipoamide acyltransferase (E2) component. Apart from harbouring the lipoyl group and the acyltransferase active site, the E2 component also provides the inner structural core (octahedral or icosahedral) of the complexes, to which the other two enzymes are bound tightly but non-covalently. Limited proteolysis of numerous 2-oxo acid dehydrogenase complexes and DNA sequence analysis of cloned genes that encode them have revealed that the E2 components possess a multi-domain structure (Perham *et al.*, 1987; Perham & Packman, 1989; Guest *et al.*, 1989; Reed & Hackert, 1990; Perham, 1991) consisting of (from the *N*-terminus) one to three lipoyl domains, a domain responsible for binding the peripheral dihydrolipoamide dehydrogenase (E3) and, in icosahedral complexes, 2-oxo acid decarboxylase (E1) subunits, and a catalytic (acyltransferase) domain that aggregates to form the complex structural core. The various domains are connected by flexible segments of polypeptide chain that allow the lipoyl domains to move, facilitating the delivery of the substrate to the successive active sites (Texter *et al.*, 1988; Miles *et al.*, 1988; Radford *et al.*, 1989*a,b*, and references cited therein).

The lipoyl domain serves two important functions: by virtue of its attachment to this domain, the lipoyl group is turned into an active substrate for reductive acylation by the E1 component, and specificity is conferred on it so that it is reductively acylated only by the E1 component of its parent 2-oxo acid dehydrogenase complex (Graham *et al.*, 1989). The folded structure of the lipoyl domain appears to be crucial for both functions since a synthetic lipoylated decapeptide is ineffective as a substrate, implying the need for a specific molecular recognition event between the lipoyl domain and its cognate E1 (Graham *et al.*, 1989). To throw light on this unusual aspect of the enzyme mechanism, we have recently expressed in *Escherichia coli* a sub-gene encoding the single lipoyl domain (85 amino acid residues) of the E2 chain (EC 2.3.1.12) of the pyruvate dehydrogenase (PDH) complex of *Bacillus stearothermophilus* (Dardel *et al.*, 1990) and embarked on a full determination of its structure by n.m.r. spectroscopy (Dardel *et al.*, 1991).

In the present paper, we take this project one stage further by demonstrating that we can express in *E. coli* a specially created sub-gene that encodes the lipoyl domain and the E3 (EC 1.8.1.4)-binding domain of the *B. stearothermophilus* E2 chain, still linked together by the flexible inter-domain segment of polypeptide chain (Packman *et al.*, 1988; Borges *et al.*, 1990). Both domains were correctly folded, as judged by several biochemical and biophysical criteria, and proved capable of generating a sub-complex with the E3 component. This demonstrates the independent folding of the two domains and opens the way to a structural analysis of the peripheral subunit-binding domain and its mode of interaction with the E3 subunits of the enzyme complex.

Abbreviation used: PDH complex, pyruvate dehydrogenase complex.

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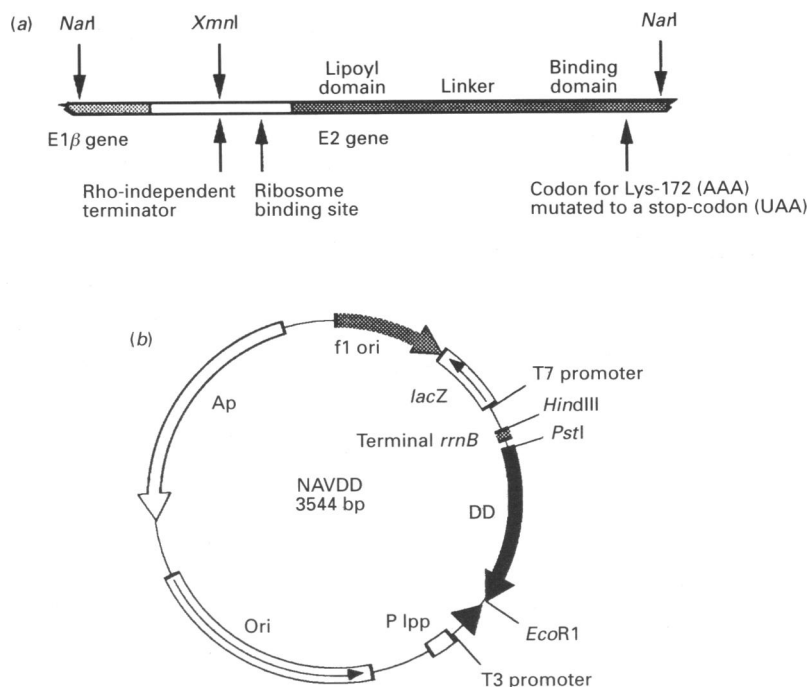


Fig. 1. Construction of NAVDD encoding a di-domain of the E2 chain of *B. stearothermophilus* PDH complex

(a) An *NarI*-fragment of pBst42 encoding the C-terminal region of the E1 β chain and N-terminal region of the E2 chain was ligated into *AccI*-cut M13mp8 and a stop-codon was inserted after the binding domain. (b) The sub-gene was excised by using *XmnI* (to destroy the terminator) and *BamHI*, which cuts the M13 polylinker. It was ligated into *HincII*-*BamHI*-cut pUC19, from which it was subsequently excised with *EcoRI*-*PstI* and ligated into pBSTNAV cut with the same enzymes to create NAVDD.

MATERIALS AND METHODS

Materials

Bacteriological media were from Difco. *E. coli* strain TG1 *recO* (Hawkins *et al.*, 1990) was used as the host strain for the mutagenesis and sub-cloning. The proteinase-deficient strain CH1764 used as the expression host was a gift from C. F. Higgins and S. R. Pearce (I.C.R.F., Oxford, U.K.) and is a derivative of AD5827 made *lon*⁻ and *htpR*⁻ by co-transduction with Tn10 to produce the genotype F *ilv his sup*^o *strA proC*:: Tn10 *galOP IS1 bio* (λ *Bam N*⁺ *c1857 H1*[*cro*-RAJ-*bio*] *wvrB*) *pro*⁺ *tet*^S *proC*-Tn10- Δ *lon*, *tet*^R, *htpR*-Tn10. Restriction enzymes were used as recommended by their suppliers (Amersham, Pharmacia, Boehringer Mannheim and Gibco-Bethesda Research Laboratories). DNA Sequence was determined by dideoxy chain-termination sequencing (Sanger *et al.*, 1980) with the use of the Sequenase kit (U.S. Biochemical) and [α -³⁵S]thio]dATP (Amersham). The mutagenic oligonucleotide was synthesized by Mr. M. Weldon (Department of Biochemistry) on a Milligen BioSearch Cyclon Synthesizer and the mutagenesis (Taylor *et al.*, 1985*a,b*) was performed with the Amersham kit. Trypsin (Tos-Phe- CH_2Cl -treated) and chymotrypsin (Tos-Lys- CH_2Cl -treated) were from Sigma Chemical Co. Peptides were characterized by amino acid analysis on a LKB 4400 analyser after acid hydrolysis and by sequence analysis on an Applied Biosystems 477A protein sequencer (Packman *et al.*, 1988). Reagents were of analytical grade or the purest available.

Gene cloning and expression

Plasmid pBst42 (Borges *et al.*, 1990) was digested with *NarI* to produce a 738 bp fragment of DNA comprising the 3'-end of the *B. stearothermophilus* E1 β gene, the intergenic region and first 182 codons of the E2 gene. This fragment was subcloned into *AccI*-cut M13mp8, and the codon for Lys-172 (AAA) was

converted into a stop-codon (UAA) by site-directed mutagenesis by using the mutagenic oligonucleotide 5'-GGCGCATAA-CCGGCACCG-3'. The fragment was then excised by using *BamHI* and *XmnI*, this latter enzyme being used as it bisects, and so destroys, the putative rho-independent terminator (Borges *et al.*, 1990) immediately preceding the sequence motifs that initiate translation of the E2 gene. The fragment was ligated into *HincII*/*BamHI*-cut pUC19, but expression was poor from this vector and it was therefore excised again by using *EcoRI*/*PstI* and ligated into pBSTNAV (Meinzel *et al.*, 1988) cut with the same enzymes (Fig. 1). The resulting construct (NAVDD) was fully sequenced and shown to encode the N-terminal two domains of the *B. stearothermophilus* E2 chain under the control of the T7 promoter. NAVDD was transformed into the *E. coli* proteinase-deficient strain CH1764 already carrying the plasmid pGp1-2 (Tabor & Richardson, 1985), which encodes the T7 polymerase under the control of the lambda P_L/c1857 temperature-sensitive promoter. Cultures were grown at 30 °C in 2TY medium (Maniatis *et al.*, 1982) until they reached an A₆₀₀ value in the range 1.0–2.0; they were then heat-induced by addition of an equal volume of medium at 65 °C, which swiftly raised the temperature to 42 °C, after which they were incubated in an orbital shaker for 5 h at 37 °C. The *E. coli* host cells (CM1764) are temperature-sensitive and cease to grow, so that the di-domain is the major product of subsequent protein synthesis.

Purification of di-domain

A culture (4 litres) of *E. coli* CH1764 pGp1-2 transformed with NAVDD was grown in 2TY medium as described above. The cells were harvested, resuspended in 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂, 1 mM-2-mercaptoethanol and 0.02% NaN₃, and disrupted in a French press. Cell debris were removed by centrifugation (130000 g for 30 min) and the supernatant was fractionally precipitated with (NH₄)₂SO₄. Through-

out purification, the presence of di-domain was assessed by SDS/PAGE analysis with a continuous Tris/Tricine buffer system (Schägger & von Jagow, 1987). The protein precipitated at 30–70% saturation contained the di-domain and was dissolved in buffer A (10 mM-ammonium bicarbonate buffer, pH 7.8) and dialysed overnight against the same buffer. The solution was applied to a DE52 DEAE-cellulose column (100 ml volume, 2 ml/min flow rate) equilibrated with buffer A and the di-domain was eluted with 250 mM-ammonium bicarbonate buffer, pH 7.8. Fractions containing the di-domain were pooled and concentrated by ultrafiltration (Amicon PM10 membrane) followed by concentration to a final volume of 6 ml by rotary evaporation. This was applied in 1 ml batches to a Superose 12 gel-filtration column equilibrated with buffer A. Fractions containing di-domain were pooled and loaded directly onto a Hiload S f.p.l.c. column (Pharmacia) equilibrated with buffer A, from which it was eluted in an apparently homogeneous peak with 500 mM-ammonium bicarbonate buffer, pH 7.8. The purity of the di-domain was confirmed by amino acid and *N*-terminal sequence analysis (Packman *et al.*, 1988).

Preparation of antiserum and immunoblotting

A rabbit polyclonal antibody against the di-domain was raised by Mr. G. Dew (Strangeways Research Laboratory, Cambridge, U.K.). An Old English white rabbit was injected intramuscularly in two sites with 50 µg purified di-domain on days 0 and 28. Bleeds were taken on days 0, 28, 56 and 70 (bleeds 0–4 respectively) and, after clotting, serum was removed by centrifugation (3000 g for 10 min) and stored frozen at –20 °C. Serum from bleed 3 was used at 1/1000 dilution in immunoblots and dot-blots with goat anti-(rabbit IgG) antibody coupled to alkaline phosphatase (Tago, Burlingame, CA, U.S.A.) as the second antibody (Maniatis *et al.*, 1982).

Reductive acetylation assays

These were carried out as described by Packman *et al.* (1984). Di-domain was incubated with purified *B. stearothermophilus* PDH complex in the presence of [2-¹⁴C]pyruvate, precipitated with trichloroacetic acid and the amount of ¹⁴C incorporated was measured in an LKB Rackbeta liquid-scintillation counter. Preincubation (1 h, 30 °C) with an *E. coli* TG1 *recO* cell lysate (150 µg) supplied with lipoic acid (0.4 mg/ml final concentration) and ATP (1 mM final concentration) was also carried out in an attempt to lipoylate, and so to render functional, any unlipoylated di-domain in the preparation before the acetylation assay.

Limited proteolysis

The di-domain, dissolved in 100 mM-ammonium bicarbonate buffer, pH 7.8, was subjected to limited proteolysis with trypsin (5% ; for 2 h at 22 °C) and chymotrypsin (1% ; for 2 h at 22 °C). The products were separated by SDS/PAGE in a Tricine buffer (Schägger & von Jagow, 1987) and stained with Coomassie Brilliant Blue R250 or with silver stain (Merrill *et al.*, 1981).

Interaction with E3

B. stearothermophilus E3 was purified from an over-expressing system in *E. coli* (A. Borges, C. F. Hawkins & R. N. Perham, unpublished work). The di-domain and E3 were chromatographed separately, and after being incubated together in roughly equimolar concentrations (1–2 nmol in 0.5 ml) in 100 mM-ammonium bicarbonate buffer, pH 7.8, on a Superose 12 f.p.l.c. gel-filtration column (Pharmacia) equilibrated with the same buffer and developed at 0.25 ml/min. Fractions from the column were analysed by SDS/PAGE in a Tricine buffer. The complex of E3 with the binding domain was subjected to limited proteolysis with chymotrypsin (1%, w/w, chymotrypsin/di-domain;

for 2 h at 22 °C) to release the lipoyl domain and the remaining complex was analysed by gel filtration and SDS/PAGE as above.

¹H-n.m.r. spectroscopy

Purified di-domain was freeze-dried and dissolved in 50 mM-potassium phosphate buffer, pH 7.0, followed by freeze-drying from ²H₂O three times. The final sample contained di-domain (0.2 mM) in a volume of 0.5 ml to which was added NaN₃ (final concentration 0.02%) and trimethylsilylpropionate (final concentration 20 µM, as an internal standard). Spectra were recorded at 400 MHz with a Bruker AM400 WB spectrometer with a 6 kHz spectral width, pulse intervals of 1.0 s and pre-saturation of the ¹H²O line. Before Fourier transformation, a Gaussian multiplication function was applied, leading to a line broadening of approx. 1 Hz.

RESULTS AND DISCUSSION

Purification of the di-domain

A sub-gene encoding residues 1–171 of the E2 chain of *B. stearothermophilus* PDH complex was created and placed under the control of the T7 promoter in plasmid NAVDD, as described in the Materials and methods section. The plasmid was transformed into *E. coli* CH1764pGp1-2 cells and the sub-gene encoding the *B. stearothermophilus* di-domain was found to be strongly expressed, generating a polypeptide chain with an apparent *M_r* of 23 500 on SDS/PAGE.

The putative di-domain was purified by means of fractional precipitation with (NH₄)₂SO₄, ion-exchange chromatography on DE52 DEAE-cellulose, gel filtration on Superose 12 and further ion-exchange chromatography on a Hiload S f.p.l.c. column, as described in the Materials and methods section. The course of the purification, as judged by SDS/PAGE, is illustrated in Fig. 2. A yield of approx. 12 mg of purified protein was obtained from 12.2 g wet wt. of *E. coli* cells. The identity of the di-domain was confirmed by amino acid analysis and by determining its *N*-

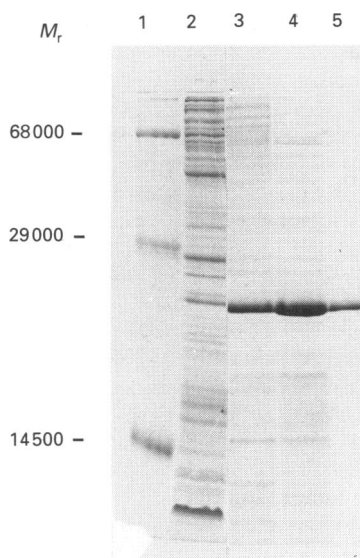


Fig. 2. Purification of di-domain analysed by SDS/PAGE

Samples of di-domain during purification were analysed by SDS/PAGE. Lane 1, *M_r* standards (BSA 68 000, carbonic anhydrase 29 000 and lysozyme 14 500); lane 2, 400 µg of cell lysate; lane 3, 20 µg of product from DE52 DEAE-cellulose chromatography; lane 4, 5 µg of product from Superose 12 column; lane 5, 1.25 µg of product from Hiload S column.

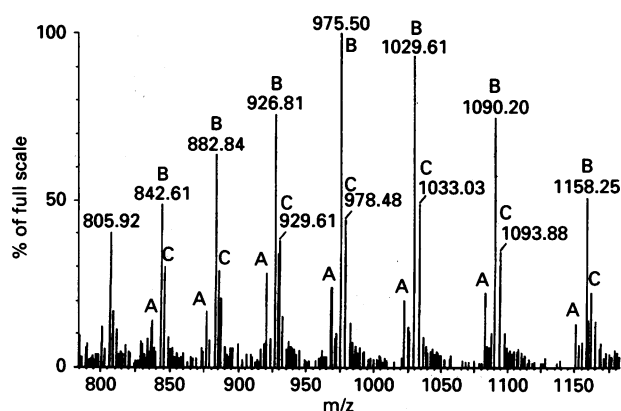


Fig. 3. Electrospray m.s. of the mixture of lipoylated and octanoylated di-domain (15 pmol/ μ l)

Series A, unlipoylated di-domain; series B, octanoylated di-domain; series C, lipoylated di-domain.

terminal sequence for six amino acid residues. The di-domain was found to lack the initiating *N*-terminal methionine residue encoded in the DNA sequence (Borges *et al.*, 1990). The same was true for the single lipoyl domain (residues 2–86) previously expressed in *E. coli* from its sub-gene in a comparable plasmid (Dardel *et al.*, 1990). The purified di-domain thus represents residues 2–171 of the *B. stearothermophilus* E2 chain encoded by the *pdhC* gene.

The purified protein migrated with an apparent M_r of 23 500, in contrast with the predicted M_r of 18 573 for a lipoylated di-domain (Fig. 2). This anomalous migration is due, at least in part, to the linker region separating the lipoyl and peripheral subunit-binding domains, as is demonstrated below by the limited-proteolysis studies. Amino acid analysis gave a value for the absorption coefficient of the di-domain as $A_{280}^{0.1\%} = 0.48$.

Reductive acetylation and m.s. of the di-domain

The biological activity of the di-domain was tested by measuring its ability to become reductively acetylated by the *B. stearothermophilus* PDH complex in the presence of [14 C] pyruvate. This would be expected to depend on the degree of lipoylation of the expressed protein as well as on its having assumed a correctly folded conformation. Only low levels of radioactivity were found to be incorporated: 50 c.p.m./ μ g above background, in comparison with 12 500 c.p.m./ μ g for the single lipoyl domain previously expressed (Dardel *et al.*, 1990). Treatment of the di-domain with an *E. coli* extract supplied with lipoic acid and ATP doubled the radioactivity incorporated in the acetylation assay, suggesting that the *E. coli* lipoylating enzymes can recognize and act on the *B. stearothermophilus* di-domain, though the effect was not large.

Chromatography on a MonoQ f.p.l.c. column (Pharmacia) was used to separate the apparently homogeneous di-domain into two components, which, although behaving identically on SDS/PAGE, migrated slightly differently on non-denaturing PAGE (with an identical Tricine gel electrophoresis system but omitting SDS from the sample and running buffers). A gradient (20 ml) of 100–500 mM-ammonium bicarbonate buffer, pH 7.8, eluted a major peak at \sim 200 mM-ammonium bicarbonate, which was followed by a smaller peak at approx. 300 mM-ammonium bicarbonate. The major peak migrated more slowly when submitted to non-denaturing PAGE, and was not active in the reductive acetylation assay. The minor peak showed an incorporation of 200 c.p.m./ μ g in the same assay, suggesting that the major peak was unlipoylated and the minor peak lipoylated, at least in part.

Electrospray m.s. was used to confirm that the major peak was unlipoylated: its M_r was estimated as $18\,387 \pm 4.0$ (s.d.) (predicted M_r for the unlipoylated di-domain is 18 385). The minor peak showed three components on electrospray m.s.: the smallest (Fig. 3, series A) had a measured M_r of 18 385 and was therefore likely to be contaminating unlipoylated di-domain; the major species (Fig. 3, series B) had a calculated M_r of $18\,515 \pm 1.9$ (s.d.) and a third species (Fig. 3, series C) had an M_r of $18\,573 \pm 5.5$ (s.d.). The predicted M_r for a lipoylated di-domain is 18 573, whereas an M_r of 18 515 corresponds to that predicted for the di-domain carrying an octanoyl group. Aberrant post-translational modification of the single lipoyl domain of *B. stearothermophilus* with octanoic acid, a biosynthetic precursor of lipoic acid, has been noted before (Dardel *et al.*, 1990). Octanoylation of an *E. coli* lipoyl domain expressed in a lipoic acid-deficient strain of *E. coli* has also been reported (Ali *et al.*, 1990) but there is no evidence for octanoylation in the wild-type *E. coli* PDH complex (Packman *et al.*, 1991).

In the present experiments, the proportion of substituted di-domain was estimated to be approx. 20% of the total preparation, with only 4% being lipoylated and the remainder (16%) being octanoylated. This accounts for the low incorporation of [14 C]acetyl groups observed when the di-domain was tested in the reductive acetylation assay. Although in previous experiments the single lipoyl domain was found to be 16% lipoylated and 4% octanoylated (Dardel *et al.*, 1990), that protein was produced by over-expression of the cloned gene under the control of the strong constitutive *lpp* promoter in growing *E. coli* cells (strain TG1 *recO*). The low level of lipoylation of the di-domain in the present experiments is therefore likely to be due, at least in part, to the heat-induction of the T7 polymerase simultaneously switching off host cell (*E. coli* CH1764) growth, leaving insufficient lipoylating enzyme activity to substitute more than a small proportion of the over-expressed di-domain and perhaps leading to a higher level of aberrant octanoylation. Subtle differences in folding of the lipoyl domain that contribute to this cannot of course be ruled out, but there is no evidence for this from any of the criteria (limited proteolysis, n.m.r. spectroscopy) used thus far to characterize the di-domain.

Limited proteolysis of the di-domain

Limited proteolysis of the intact *B. stearothermophilus* E2 chain with chymotrypsin releases the lipoyl domain by cleavage at Phe-85 (Duckworth *et al.*, 1982; Packman *et al.*, 1988). Exposure of the di-domain to chymotrypsin (1%; for 2 h at 22 °C), cleaved it into the expected two halves (Fig. 4a): the lipoyl domain and the binding-domain-plus-linker. These were separated by chromatography on a MonoQ f.p.l.c. column. A sample (20 μ g) of di-domain digested with chymotrypsin was loaded on to the MonoQ column equilibrated with buffer A and peptides were eluted with a gradient of 0.1–1 M-ammonium bicarbonate buffer, pH 7.8, over 5 column volumes. The binding-domain-plus-linker was eluted at 150 mM-ammonium bicarbonate, whereas the lipoyl domain was eluted at 400 mM-ammonium bicarbonate; their identities were confirmed by amino acid analysis and by determining their *N*-terminal sequences for five residues. On SDS/PAGE (Fig. 4a), the two 85-residue peptides migrated with apparent M_r values of 9700 (lipoyl domain; predicted M_r 9264 unlipoylated) and 14 250 (binding-domain-plus-linker; predicted M_r 9133). The presence of the linker peptide still attached to the peripheral subunit-binding domain is likely to be a major cause of its anomalous migration (Graham *et al.*, 1986; Miles *et al.*, 1988). The binding-domain-plus-linker fragment contains three sites (Lys-98, Lys-107 and Arg-127; wild-type E2 numbering) for limited proteolysis with trypsin (Packman *et al.*, 1988). As expected, the binding-domain-plus-

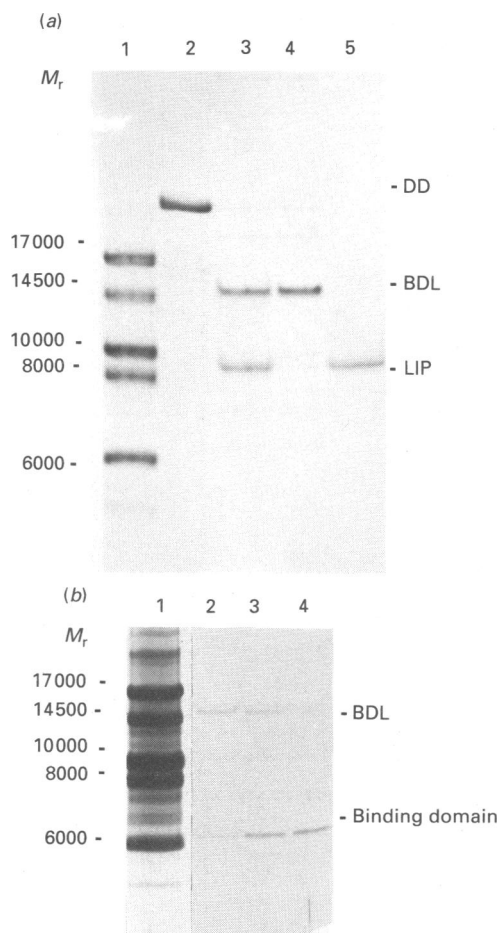


Fig. 4. Limited proteolysis of di-domain

(a) Di-domain (DD) was digested with chymotrypsin (1%; for 2 h at 22 °C) and the products were separated by anion-exchange chromatography on a MonoQ f.p.l.c. column, developed with a gradient of 0.1–1 M-ammonium bicarbonate buffer, pH 7.8, before analysis by SDS/PAGE. Lane 1, low- M_r standards (BDH Chemicals); lane 2, undigested di-domain; lane 3, digest before chromatography; lane 4, fraction 14 from MonoQ column (binding-domain-plus-linker; BDL); lane 5, fraction 36 from MonoQ column (lipoyl domain; LIP). (b) The binding-domain-plus-linker (BDL) was digested with trypsin (5%; at 22 °C) for various times and the products were analysed by SDS/PAGE. The gel was silver-stained. Lane 1, low- M_r standards, as above; lanes 2–4, products of tryptic digestion of binding-domain-plus-linker after 10, 30 and 150 min respectively.

linker could be further cleaved with trypsin to release the binding domain alone, but the linker fragments were undetected. The binding domain migrated on SDS/PAGE with an apparent M_r of 6100 compared with its predicted value of 4600 (Fig. 4b). Immunoblots of the purified peptides showed that the antiserum raised against the di-domain also recognized the individual lipoyl and peripheral subunit-binding domains and binding-domain-plus-linker (results not shown). The relative antigenicities of the separate domains, as determined by dot-blots as well as Western blots, suggested that the major antigenic epitopes in the di-domain reside in the linker region and peripheral subunit-binding domain, the lipoyl domain being relatively weakly antigenic.

Binding of the E3 component

In the E2 chain of the *B. stearothermophilus* PDH complex, as in all 2-oxo acid dehydrogenase complexes of icosahedral sym-

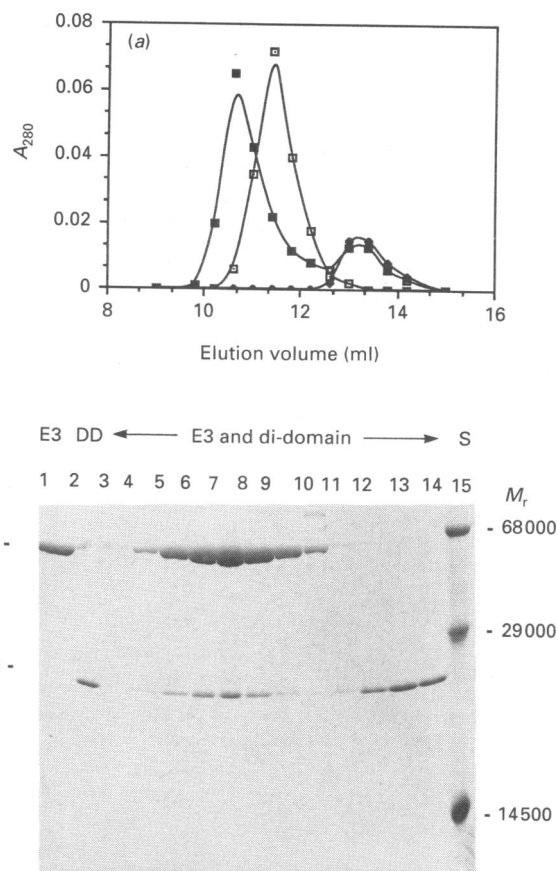


Fig. 5. Formation of complex of E3 and di-domain

(a) Samples were gel-filtered on a Superose 12 column in 100 mM-ammonium bicarbonate buffer, pH 7.8. The absorbance of the effluent was monitored at 280 nm. □, E3 alone (run I); ◆, di-domain (DD) alone (run II); ■, complex of E3 and di-domain (run III). E3 and di-domain were combined in a 1:2 molar ratio and eluted as complex and excess free di-domain. (b) SDS/PAGE analysis of fractions collected from gel filtrations shown in (a). Lane 1, peak at 11.3 ml, run I; lane 2, peak at 13.3 ml, run II; lanes 3–14, fractions eluted at 10.0, 10.2, 10.4, 10.6, 10.8, 11.0, 11.4, 11.8, 12.8, 13.0, 13.2 and 13.6 ml, run III; lane 15, M_r standards as in Fig. 2. There is a slight (0.1 ml) discrepancy between the A_{280} maximum and the fraction on the gel showing the most protein, owing to the dead volume between the spectrophotometer and the fraction collector.

metry studied thus far, the peripheral subunit-binding domain has a major part to play in binding the E3 subunits to the E2 core (Perham & Packman, 1989; Reed & Hackert, 1990). To test whether the *B. stearothermophilus* di-domain expressed in *E. coli* was similarly capable of binding E3 subunits, a sample (40 μg , 2.2 nmol) of the di-domain was mixed with a sample (145 μg , 1.2 nmol) of *B. stearothermophilus* E3 and incubated in 100 mM-ammonium bicarbonate buffer, pH 7.8, at 22 °C for 30 min. The mixture was then applied to a Superose 12 gel-filtration column equilibrated with 100 mM-ammonium bicarbonate buffer, pH 7.8, and eluted with the same buffer (Fig. 5a). Samples of the column effluent were examined by SDS/PAGE (Fig. 5b). It was evident that the elution behaviour of the mixture of E3 and di-domain was different from that of the E3 and di-domain applied to the same column separately, with the major protein peak being eluted earlier, suggestive of a higher M_r (Fig. 5a). The di-domain was found to have split into two parts, one emerging with the E3 component, the other remaining in its original elution position (Figs. 5a and 5b). These results indicate that most if not all of the

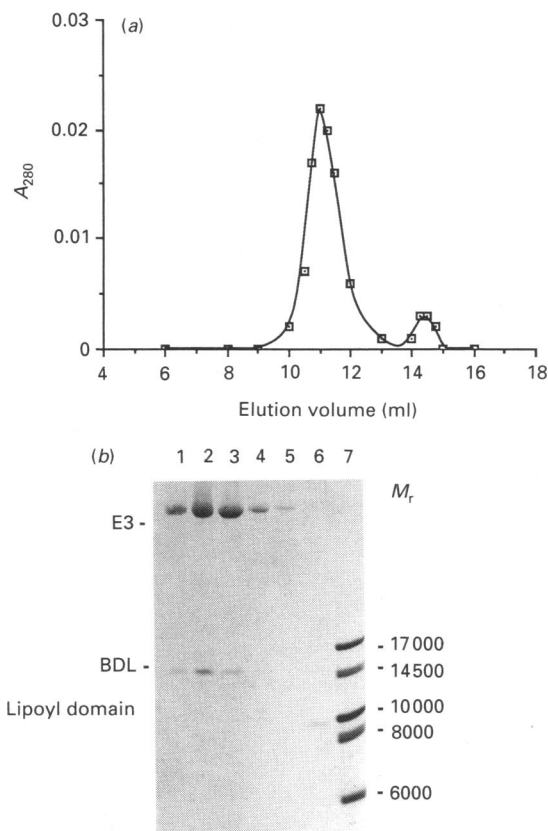


Fig. 6. Chymotryptic digestion of complex of di-domain and E3

(a) Equimolar amounts of E3 and di-domain were allowed to form a complex and then subjected to chymotryptic digestion (1% for 2 h at 22 °C) before being subjected to gel filtration. The putative E3-binding-domain-plus-linker (BDL) complex was found to be eluted at 11.0 ml and free lipoyl domain at 14.3 ml. (b) SDS/PAGE of fractions from the gel-filtration column. Lanes 1–6, fractions eluted at 10.5, 11.0, 11.5, 12.0, 12.5 and 14.0 ml respectively; lane 7, low- M_r standards as in Fig. 4(b).

E3 has formed a complex with the di-domain, with some excess of free di-domain remaining.

The complex of E3 and di-domain was allowed to form again but then subjected to chymotryptic digestion to release the lipoyl domain, as above, before applying the mixture to the gel filtration column. The result is shown in Fig. 6. An E3-binding-domain-plus-linker complex and the free lipoyl domain were clearly resolved by gel filtration and identified by SDS/PAGE. The experiment could be reversed, so that the chymotryptic digestion of the di-domain was carried out before any complex with E3 was allowed to form (results not shown). The results were identical, indicating that association of E3 with the di-domain is independent of the lipoyl domain.

¹H-n.m.r. spectroscopy

The di-domain sample prepared for n.m.r. spectroscopy contained both lipoylated and unlipoylated species, but the former was present only in small amounts (< 5%). Dardel *et al.* (1990) showed that the 400 MHz ¹H-n.m.r. spectra of lipoylated and unlipoylated lipoyl domains are essentially identical, differing only in those resonances that can be assigned to the lipoamide moiety. It was therefore assumed that the mixture of di-domain species would not cause complications in the n.m.r. spectroscopy.

The spectra shown in Fig. 7 are those of the purified lipoyl domain (kindly provided by Dr. F. Dardel) and the di-domain.

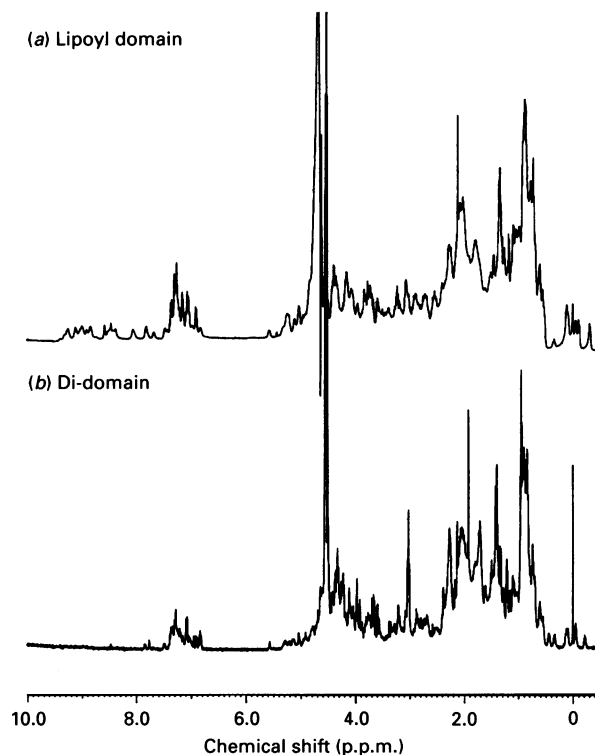


Fig. 7. 400 MHz ¹H-n.m.r. spectra of lipoyl domain and di-domain

The spectra were recorded at 400 MHz at 45 °C. (a) Unlipoylated lipoyl domain, 128 transients; (b) di-domain, 600 transients. The chemical shifts are expressed relative to internal trimethylsilyl-propionate.

There are many conserved resonances seen in both spectra, which are likely to be derived from the lipoyl domain. There are also resonances, e.g. that at 1.39 p.p.m., which are characteristic of the linker regions in the *B. stearothermophilus* (Packman *et al.*, 1984) and *E. coli* (Perham *et al.*, 1981; Texter *et al.*, 1988; Radford *et al.*, 1989a) E2 chains. Others as yet unidentified presumably arise from the binding domain. The high-field methyl resonances were well-conserved over the temperature range 25–55 °C, indicating that the di-domain has a stable tertiary structure, although above this temperature some unfolding was apparent.

Conclusions

The generation of a folded and biologically active di-domain encompassing the lipoyl domain, linker region and peripheral subunit-binding domain of the E2 chain of the *B. stearothermophilus* PDH complex proves that these domains are capable of independent folding. The status of the peripheral subunit-binding domain, hitherto identified only in limited digests of the native E2 chain (Packman & Perham, 1986; Packman *et al.*, 1988), has been confirmed by its ability to form a complex with the *B. stearothermophilus* E3 component, also obtained by over-expression of the cloned structural gene in *E. coli*. The generation of large amounts of the di-domain and thence, by limited proteolysis, of the peripheral subunit-binding domain, opens the way to a full three-dimensional structure analysis of the latter by means of n.m.r. spectroscopy. Such an analysis of the partner lipoyl domain is now well in hand (Dardel *et al.*, 1990, 1991). Structural information about the binding domain, in conjunction with biochemical binding studies such as those outlined above, will help to characterize its interactions with the other components of the PDH complex.

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REFERENCES

- Ali, S. T., Moir, A. J. G., Ashton, P. R., Engel, P. C. & Guest, J. R. (1990) *Mol. Microbiol.* **4**, 943–950
- Borges, A., Hawkins, C. F., Packman, L. C. & Perham, R. N. (1990) *Eur. J. Biochem.* **194**, 95–102
- Dardel, F., Packman, L. C. & Perham, R. N. (1990) *FEBS Lett.* **264**, 206–210
- Dardel, F., Laue, E. D. & Perham, R. N. (1991) *Eur. J. Biochem.* **201**, 203–209
- Duckworth, H. W., Jaenicke, R., Perham, R. N., Wilkie, A. O. M., Finch, J. T. & Roberts, G. C. K. (1982) *Eur. J. Biochem.* **124**, 63–69
- Graham, L. D., Guest, J. R., Lewis, H. M., Miles, J. S., Packman, L. C., Perham, R. N. & Radford, S. E. (1986) *Philos. Trans. R. Soc. London A* **317**, 391–404
- Graham, L. D., Packman, L. C. & Perham, R. N. (1989) *Biochemistry* **28**, 1574–1581
- Guest, J. R., Angier, S. J. & Russell, G. C. (1989) *Ann. N.Y. Acad. Sci.* **573**, 76–99
- Hawkins, C. F., Borges, A. & Perham, R. N. (1990) *Eur. J. Biochem.* **191**, 337–346
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Meinzel, T., Mechulan, Y. & Fayat, G. (1988) *Nucleic Acids Res.* **16**, 8095–8096
- Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) *Science* **211**, 1437–1438
- Miles, J. S., Guest, J. R., Radford, S. E. & Perham, R. N. (1988) *J. Mol. Biol.* **202**, 97–106
- Packman, L. C. & Perham, R. N. (1986) *FEBS Lett.* **206**, 193–198
- Packman, L. C., Perham, R. N. & Roberts, G. C. K. (1984) *Biochem. J.* **217**, 219–227
- Packman, L. C., Borges, A. & Perham, R. N. (1988) *Biochem. J.* **252**, 79–86
- Packman, L. C., Green, B. & Perham, R. N. (1991) *Biochem. J.* **277**, 153–158
- Patel, M. S. & Roche, T. E. (1990) *FASEB J.* **4**, 3224–3233
- Perham, R. N. (1991) *Biochemistry* **30**, 8501–8511
- Perham, R. N. & Packman, L. C. (1989) *Ann. N.Y. Acad. Sci.* **573**, 1–20
- Perham, R. N., Duckworth, H. W. & Roberts, G. C. K. (1981) *Nature (London)* **292**, 474–477
- Perham, R. N., Packman, L. C. & Radford, S. E. (1987) *Biochem. Soc. Symp.* **54**, 67–81
- Radford, S. E., Laue, E. D., Perham, R. N., Martin, S. R. & Appella, E. (1989a) *J. Biol. Chem.* **264**, 767–775
- Radford, S. E., Perham, R. N., Ullrich, S. J. & Appella, E. (1989b) *FEBS Lett.* **250**, 336–340
- Reed, L. J. & Hackert, M. L. (1990) *J. Biol. Chem.* **265**, 8971–8974
- Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. M. & Roe, B. A. (1980) *J. Mol. Biol.* **143**, 161–178
- Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Tabor, S. & Richardson, C. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 1074–1078
- Taylor, J. W., Schmidt, W., Cosstick, R., Okruszek, A. & Eckstein, F. (1985a) *Nucleic Acids Res.* **13**, 8749–8764
- Taylor, J. W., Ott, J. & Eckstein, F. (1985b) *Nucleic Acids Res.* **13**, 8765–8785
- Texter, F. L., Radford, S. E., Laue, E. D., Perham, R. N., Miles, J. S. & Guest, J. R. (1988) *Biochemistry* **27**, 289–301

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