Interaction of component enzymes with the peripheral subunit-binding domain of the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus*: stoichiometry and specificity in self-assembly

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The interaction between the pyruvate decarboxylase (E1) component and a di-domain (lipoyl domain plus peripheral subunit-binding domain) from the dihydrolipoyl acetyltransferase (E2) component of the *Bacillus stearothermophilus* pyruvate dehydrogenase multienzyme complex was investigated. Only 1 mol of di-domain (binding domain) was bound to 1 mol of heterotetrameric E1 ($\alpha_2\beta_2$) and the binding was without effect on the kinetic activity of E1. Similarly, the di-domain bound to separate E1 β subunits at a maximal polypeptide chain ratio of 1:2, but no detectable interaction was found with the E1 α subunit. However, addition of the monomeric E1 α subunit to an E1 β -di-domain

complex generated a fully functional E1 ($\alpha_2\beta_2$)-di-domain complex, indicating that the E1 β subunit plays the critical part in binding the E1 component to the di-domain and suggesting that no chaperonin is needed *in vitro* to promote the assembly of the three separate proteins. Mixing the E1 and dihydrolipoyl dehydrogenase (E3) components in the presence of di-domain revealed that E1 and E3 cannot bind simultaneously to the same molecule of di-domain, a new feature of the assembly pathway and an important factor in determining the ultimate structure of the assembled enzyme complex.

INTRODUCTION

The 2-oxo acid dehydrogenase multienzyme complexes are responsible for the oxidative decarboxylation of various 2-oxo acid substrates, 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 an assembly of three different component enzymes, in which the structural core is formed from multiple copies of the dihydrolipoyl acyltransferase (E2) polypeptide chain arranged with octahedral (24-mer) or icosahedral (60-mer) symmetry (Oliver and Reed, 1982; Reed and Hackert, 1990; Perham, 1991). The peripheral components, a 2-oxo acid decarboxylase (E1) and dihydrolipoyl dehydrogenase (E3), are represented in multiple copies tightly, but non-covalently, bound to the surface of the E2 core.

The highly segmented structure of the E2 chain (Perham and Packman, 1989; Guest et al., 1989; Reed and Hackert, 1990; Perham, 1991) holds the key to the structure and mechanism of the enzyme complexes. It contains (from the N-terminus) one, two or three lipoyl domains (according to the source), a peripheral subunit-binding domain and a catalytic domain, with each domain separated from its neighbours by a long and flexible segment of polypeptide chain. Aggregation of the catalytic domain, in which the acyltransferase activity reposes, is responsible for the octahedral or icosahedral symmetry of the E2 core (Reed and Hackert, 1990; Mattevi et al., 1992, 1993a, and references therein). The catalytic domain is also involved in binding the E1 component in the octahedral 2-oxoglutarate dehydrogenase (OGDH) and pyruvate dehydrogenase (PDH) complexes (Perham, 1991; Mattevi et al., 1992). In other complexes, notably the octahedral branched-chain 2-oxo acid de-

hydrogenase (BCDH) (Wynn et al., 1992a) and icosahedral PDH complexes, the E1 component binds principally to the peripheral subunit-binding domain of the E2 chain. This domain is also responsible for binding the E3 component in all 2-oxo acid dehydrogenase complexes irrespective of their symmetry (Mattevi et al., 1992), although in some instances it may be transposed from the E2 chain to protein X, an additional subunit in the E2 core of eukaryotic PDH complexes (Neagle and Lindsay, 1991; Lawson et al., 1991), or to the N-terminus of the E1 component of mammalian OGDH complexes (Rice et al., 1992).

The three-dimensional structures of each of the domains that comprise the E2 component have been solved separately. Those of the lipoyl domains from *Bacillus stearothermophilus* (Dardel et al., 1993) and *Escherichia coli* (Green et al., 1995) PDH complexes and of the peripheral subunit-binding domains from *B. stearothermophilus* PDH (Kalia et al., 1993) and *E. coli* OGDH (Robien et al., 1992) complexes have been determined by means of n.m.r. spectroscopy. The structure of the inner core-forming catalytic domain from *Azotobacter vinelandii* PDH complex (octahedral symmetry) has been solved by means of X-ray crystallography (Mattevi et al., 1993a). The lipoyl domains extend outwards from the inner core, interdigitating between the E1 and E3 components. The flexibility of the inter-domain segments of polypeptide chain (Green et al., 1992; and references cited therein) allows the lipoyl domain to move, facilitating the visits of the lipoyl-lysine residue to the active sites of the three component enzymes. Substrate channelling is ensured by specific molecular recognition between the lipoyl domain and the E1 component of the parent 2-oxo acid dehydrogenase complex (Graham et al., 1989). The three-dimensional structure of the E3 component, a dimer of identical polypeptide chains, from *A.*

Abbreviations used: E2, dihydrolipoyl acyltransferase; E1, 2-oxo acid (pyruvate) decarboxylase; E3, dihydrolipoyl dehydrogenase; OGDH, 2-oxoglutarate dehydrogenase; PDH, pyruvate dehydrogenase; BCDH, branched-chain 2-oxo acid dehydrogenase; ThDP, thiamin diphosphate.

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vinelandii, *Pseudomonas putida* and *Pseudomonas fluorescens* PDH complexes has been determined by means of X-ray crystallography (Mattevi et al., 1993b and references therein). However, no three-dimensional structure of any E1 component is yet available.

E1 catalyses the thiamin diphosphate (ThDP)-dependent oxidative decarboxylation of the 2-oxo acid and the subsequent reductive acylation of the lipoyl-lysine residue of the E2 chain. It can exist in two forms: as a homodimer (α_2), as found in the octahedral OGDH and PDH complexes, or as a heterotetramer ($\alpha_2\beta_2$), as in the octahedral BCDH and icosahedral PDH complexes. In the heterotetrameric form, the α -chain is believed to be involved in the binding of the ThDP whereas the β -chain is involved in the binding of the E1 component to the E2 core (Stepp and Reed, 1985). In keeping with this, a common sequence motif has been detected in all ThDP-dependent enzymes including the E1 (E1 α) chains of all 2-oxo acid dehydrogenase complexes, and its predicted role in ThDP binding (Hawkins et al., 1989) 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 (Dyda et al., 1993), and by the results of site-directed mutagenesis of pyruvate decarboxylase (Diefenbach et al., 1992), all of which are ThDP-dependent enzymes. Likewise, the E1 β subunit of the E1 component from mammalian BCDH complex is capable of binding to its parent E2 component whereas the E1 α subunit is not, suggesting an apparent binding order of E2-E1 β -E1 α (Wynn et al., 1992a). This is in accord with scanning transmission electron micrographs (Hackert et al., 1989) and the results of limited proteolysis studies (Rahmatullah et al., 1989) of the mammalian PDH complex.

Genes expressing the E1 α and E1 β subunits of the PDH complex from *B. stearrowthermophilus* have been over-expressed in *E. coli* and a functional E1 ($\alpha_2\beta_2$) enzyme has been generated *in vitro* from a mixture of pure recombinant E1 α and E1 β subunits, suggesting that no chaperonin is needed *in vitro* to promote the assembly (Lessard and Perham, 1994). This contrasts with the assembly of the E1 ($\alpha_2\beta_2$) enzyme of the mammalian BCDH complex, where chaperonins groEL and groES appear to be involved (Wynn et al., 1992b). A sub-gene encoding a di-domain comprising the *B. stearrowthermophilus* lipoyl domain attached by means of its linker to the peripheral subunit-binding domain has been over-expressed in *E. coli* (Hippes and Perham, 1992). The recombinant di-domain binds tightly to the *B. stearrowthermophilus* E3 component, but the E3 dimer surprisingly is capable of binding only one di-domain (Hippes et al., 1994). In this paper, we describe the interactions of the di-domain with the recombinant E1 enzyme, with the separate recombinant E1 α and E1 β subunits, and with a mixture of the E1 and E3 components. The results offer important new perspectives on the self-assembly of the multienzyme complex and highlight some novel aspects of its structure.

MATERIALS AND METHODS

Materials

Bacteriological media were from Difco Laboratories. *E. coli* host strain TG1 *recO* [K12, $\Delta(lac-proAB)$, *supE*, *thi*, *hsdR5*, *recO::Tn5 Kan^r/F'traD36*, *proA⁺B⁺*, *laq^r*, *lacZAM15*] and plasmids pKBstE1a and pKBstE1b, expressing the recombinant E1 α and E1 β subunits, respectively, of the *B. stearrowthermophilus* PDH complex have been described previously (Lessard and Perham, 1994). *E. coli* host strain BL21(DE3) [*F⁻*, *ompT*, *hsdS_B* (*r_B⁻m_B⁻*), *gal*, *dcm*, (DE3)] was from Novagen. Plasmid pET-11d2D expressing the di-domain (residues 1–170 of the E2 chain of the

B. stearrowthermophilus PDH complex) is the product of unpublished work (Bowden, S. J. and Perham, R. N.). Molecular-mass standards for gel filtration were from Sigma and Boehringer-Mannheim.

Protein purification

The di-domain was purified essentially as described by Hippes and Perham (1992). Pure di-domain was dialysed against 100 mM potassium phosphate, pH 7.0, (buffer A) and stored frozen at -20°C . The recombinant E1 α subunit was purified as described by Lessard and Perham (1994). For the recombinant E1 β subunit and E1 ($\alpha_2\beta_2$) enzyme, a new step was added to the purification protocol (Lessard and Perham, 1994) in order to remove low-molecular-mass material absorbing at 260 nm. After chromatography on a DE52-cellulose column, the recombinant E1 β subunit or E1 enzyme was loaded directly in 2.5 ml samples on to a Pharmacia Superdex 75 or 200 gel-filtration column (1.6 cm $\times$ 60 cm; flow rate 1 ml/min) equilibrated with buffer A at 4°C . Fractions (1 ml) containing the recombinant E1 β subunit or E1 enzyme were pooled and loaded on to a Poros Q/M column (0.46 cm $\times$ 10 cm; Perseptive Biosystem Inc.) as described by Lessard and Perham (1994). *B. stearrowthermophilus* E3 was purified from a sub-gene over-expressed in *E. coli* (Borges et al., 1990; A. Borges, C. F. Hawkins and R. N. Perham, unpublished work), and after dialysis against buffer A was stored at -20°C . Proteins were estimated by the Coomassie Blue dye method (Bradford, 1976), except for pure proteins, which were estimated by amino acid analysis (Packman et al., 1988). Column fractions were analysed by SDS/PAGE (12.5% or 20% acrylamide) using the Pharmacia PhastSystem.

Preparation of E1–di-domain complex

The E1–di-domain complex was prepared by mixing E1 and di-domain in a molar ratio of 1:2 and loaded in batches of 3–4 mg of total protein on to a Pharmacia Resource Q column (0.64 cm $\times$ 3.0 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, (buffer B) at 25°C . The E1–di-domain complex was eluted with a linear gradient (12 ml) of 0–0.2 M NaCl in buffer B at a flow rate of 1 ml/min. Fractions (0.25 ml) containing the E1–di-domain complex were pooled, dialysed overnight against buffer A, and stored frozen at -80°C .

Gel filtration of di-domain and E1

All experiments were performed at 25°C . Protein was detected at 230 nm. The gel-filtration columns were calibrated as in Lessard and Perham (1994) except that, for the Superdex 200 HR column, horse spleen ferritin (480000-*M_r*) was added to the calibration mixture. E1 (0.5 nmol) and di-domain were mixed in various molar ratios (E1:di-domain; 1:0, 5:1, 2:1, 1:1, 1:2 and 0:1) and gel-filtered on a Pharmacia Superdex 200 HR column (1 cm $\times$ 30 cm; flow rate of 0.5 ml/min) equilibrated with buffer A. The interaction of the E1 β subunit (5.0 nmol) with di-domain was studied similarly (molar ratios of E1 β :di-domain; 1:0, 10:1, 5:1, 2:1 and 1:1). The E1 α subunit (0.5 nmol) was mixed with di-domain in various molar ratios (E1 α :di-domain; 1:0, 1:1, 1:2 and 0:1) and gel-filtered on a Pharmacia Superdex 75 HR column (1 cm $\times$ 30 cm; flow rate of 1 ml/min) equilibrated with buffer A. To assess the ability of the E1 α subunit to bind to an E1 β –di-domain complex, an E1 β –di-domain complex was formed by mixing the two proteins in a molar ratio of 2:1 before adding the E1 α subunit (2 nmol) to the complex in various molar ratios (complex:E1 α ; 0:1, 1:4 and 1:2). The mixture was then gel-filtered on a Superdex 200 HR column equilibrated with

buffer A. An equimolar mixture of E1 (1 nmol) and di-domain was gel-filtered identically as a control.

Gel electrophoresis of di-domain and E1

Di-domain (100 pmol) and E1 were mixed in various molar ratios (di-domain:E1; 0:1, 2:1, 1:1, 1:1.2, 1:1.4, 1:1.6, 1:1.8 and 1:2) and subjected to electrophoresis in a non-denaturing polyacrylamide gel (12% acrylamide running gel, 5% acrylamide stacking gel) with a Tris/glycine buffer (Laemmli, 1970) but lacking SDS. Samples of the purified E1–di-domain complex (100 pmol) were also mixed with a 2-fold molar excess of E1 or a 5-fold molar excess of di-domain, respectively, before electrophoresis in order to assess the ability of the complex to bind additional E1 or di-domain. Binding of the separate E1 α and E1 β subunits to the di-domain was assessed by mixing E1 α and E1 β (100 pmol) with a 10-fold molar excess of di-domain before electrophoresis. Similarly, the ability to form an E1–di-domain complex from the separate E1 α and E1 β subunits and di-domain was evaluated by mixing the E1 β subunit (50 pmol) and di-domain (1 nmol) to form the E1 β –di-domain complex, followed by adding the E1 α subunit (50 pmol) before electrophoresis. All gels were stained with Coomassie Brilliant Blue R-250.

Simultaneous binding of E1 and E3 to di-domain

The ability of the di-domain to bind E1 and E3 was studied by means of gel filtration and non-denaturing PAGE. A mixture of E1 and E3, one of which was kept constant (0.5 nmol), was prepared at various molar ratios (0:1, 1:2, 1:1 and 2:1). To each mixture, di-domain (0.5 nmol) was added and they were gel-filtered on a Superdex 200 HR column equilibrated with buffer A as described above. Pure E1 and E3 enzymes (0.5 nmol) were also gel-filtered separately as controls. Similarly, a mixture of E1 and E3, one of which was kept constant (100 pmol), was prepared at various molar ratios (0:1, 1:2, 1:1, 1:2, 1:4 and 1:0). To each mixture, di-domain (100 pmol) was added before submitting the sample to gel electrophoresis as described above.

Enzyme assays

The assay of E1, both intact and during its reconstitution from the individual E1 α and E1 β subunits, was performed as described previously (Lessard and Perham, 1994). The activity of E1 in the presence of di-domain was assayed after E1 and di-domain were mixed in a molar ratio of 1:4 respectively. To assay E1 reconstituted from the individual E1 α and E1 β subunits in the presence of di-domain, samples were prepared by mixing E1 β and di-domain in a molar ratio of 1:8, followed by adding E1 α at various molar ratios, before assaying for E1 activity.

RESULTS AND DISCUSSION

Association of the di-domain with E1

Two different techniques, gel filtration and non-denaturing PAGE, were used to investigate the association of the *B. stearothermophilus* di-domain with E1 and the stoichiometry of binding.

Samples (0.5 nmol) of E1 were mixed with increasing amounts of di-domain and then gel-filtered in 100 mM potassium phosphate buffer, pH 7.0, on a Superdex 200 HR column (Figure 1a). As previously observed (Lessard and Perham, 1994), in the absence of di-domain the recombinant E1 ($\alpha_2\beta_2$) was eluted as a single peak with an apparent M_r of 153000 (calculated as 153262). Addition of di-domain caused the appearance of a new peak of higher apparent M_r (approx. 260000), which was

Figure 1 Association of di-domain with E1 and the E1 β subunit revealed by gel filtration

Intact E1 ($\alpha_2\beta_2$) or E1 β subunit were mixed separately with the di-domain in various molar ratios and gel-filtered on a Superdex 200 HR column in 100 mM potassium phosphate buffer, pH 7.0. (a) Panels A–E, 0.5 nmol of E1 mixed with 0, 0.1, 0.2, 0.5 and 1.0 nmol of di-domain respectively; panel F, 0.5 nmol of di-domain. (b) Panels A–E, 5 nmol of E1 β subunit mixed with 0, 0.5, 1.0, 2.5 and 5.0 nmol of di-domain respectively; panel F, 2.5 nmol of di-domain.

attributed to the E1–di-domain complex (calculated M_r 171635). The anomalous retention time is probably due to the physical shape of the di-domain, which consists of two globular domains linked by a flexible region of polypeptide chain and which itself migrates anomalously in gel filtration (Hipps and Perham, 1992). Any excess of E1 was eluted as a separate peak in its expected position behind the complex. The E1–di-domain complex peak continued to increase in size until the molar ratio of di-domain:E1 reached 1:1, after which the excess di-domain was eluted as a separate peak that emerged later than the smallest marker protein (M_r 87000).

Samples (100 pmol) of di-domain were also mixed with increasing amounts of E1 and subjected to non-denaturing gel

Figure 2 Association of di-domain with E1 revealed by non-denaturing PAGE

Intact E1 was mixed with the di-domain (DD) in various molar ratios and then subjected to non-denaturing PAGE. Lane 1, 100 pmol of E1; lanes 2–8, 100 pmol of di-domain mixed with 50, 100, 120, 140, 160, 180 and 200 pmol of E1 respectively.

Figure 3 Non-denaturing PAGE of E1–di-domain complex

The E1–di-domain complex purified by means of anion-exchange chromatography was mixed with additional amounts of E1 ($\alpha_2\beta_2$) or di-domain (DD) and then subjected to non-denaturing PAGE. Lane 1, 0.5 nmol of di-domain; lane 2, 0.2 nmol of E1; lane 3, 0.1 nmol of E1–di-domain complex; lane 4, 0.1 nmol of E1–di-domain complex plus 0.5 nmol of di-domain; lane 5, 0.1 nmol of E1–di-domain complex plus 0.2 nmol of E1.

electrophoresis (Figure 2). The amount of E1–di-domain complex formed was observed to increase until the molar ratio of E1:di-domain reached 1:1, after which the excess E1 migrated as free E1. Thus di-domain binds tightly to the tetrameric E1 ($\alpha_2\beta_2$) with a limiting molar stoichiometry of 1:1.

A sample of E1–di-domain complex was prepared by means of anion-exchange chromatography as described in the Materials and methods section. Addition of further E1 or di-domain to this purified sample, followed by non-denaturing PAGE, confirmed that it was incapable of binding additional protein subunits (Figure 3).

Kinetic properties of the E1–di-domain complex

In order to test the effect of binding the di-domain on the E1 activity, kinetic parameters (K_m for pyruvate and k_{cat}) were determined for E1 in the presence and absence of di-domain. Initial rate measurements (0–20 s) for the E1–di-domain complex could be fitted to Michaelis–Menten and Hanes plots (data not shown), which yielded a K_m for pyruvate of $0.86 \pm 0.12 \mu\text{M}$ and a k_{cat} of $0.49 \pm 0.03 \text{ s}^{-1}$. These values are similar to those obtained for free E1 (K_m and k_{cat} of $0.83 \pm 0.06 \mu\text{M}$ and $0.47 \pm 0.03 \text{ s}^{-1}$ respectively) [see also Lessard and Perham (1994)]. The integrity of the E1–di-domain complex under E1 assay conditions was confirmed by gel-filtering a sample on a Superdex 200 HR column in assay buffer (results not shown). These results strongly suggest that there is no occlusion of, or conformational change near, the active site of E1 when the enzyme is bound to the di-domain.

Figure 4 Association of di-domain with the separate E1 α and E1 β subunits revealed by non-denaturing PAGE

The separate E1 α and E1 β subunits were mixed with di-domain (DD) and subjected to non-denaturing PAGE. Lane 1, 1 nmol of di-domain; lane 2, 0.1 nmol of E1 α ; lane 3, 1 nmol of di-domain plus 0.1 nmol of E1 α ; lane 4, 0.2 nmol of E1 β ; lane 5, 1 nmol of di-domain plus 0.1 nmol of E1 β ; lane 6, equimolar mixture (0.1 nmol total) of E1 α and E1 β ; lane 7, 1 nmol of di-domain and 0.05 nmol of E1 β plus 0.05 nmol of E1 α ; lane 8, 0.05 nmol of E1 ($\alpha_2\beta_2$); lane 9, 1 nmol of di-domain plus 0.05 nmol of E1 ($\alpha_2\beta_2$).

Association of the di-domain with the E1 α and E1 β subunits

Gel filtration and non-denaturing PAGE were also used to investigate which subunit of the E1 component is primarily responsible for the interaction with the peripheral subunit-binding domain.

Samples containing 5 nmol (2 mg/ml) of E1 β subunit were mixed with increasing amounts of di-domain and then gel-filtered in 100 mM potassium phosphate buffer, pH 7.0, on a Superdex 200 HR column (Figure 1b). As observed previously (Lessard and Perham, 1994), in the absence of di-domain, the E1 β subunit was eluted mainly in a peak with an apparent M_r of 145000. This appears to be a tetrameric form based on the subunit M_r of 35311 calculated from the DNA. It is likely that the broad peak representing the remainder of the E1 β subunit spread over an apparent M_r of < 87000–140000 is generated by an equilibrium between β , β_2 and β_4 forms occurring during the passage of the protein through the column (Lessard and Perham, 1994). Addition of di-domain to the recombinant E1 β subunit resulted in the appearance of a new peak of higher M_r (apparent M_r 330000) attributed to an E1 β –di-domain complex (Figure 1b). The new peak appears to represent an aggregate of four E1 β subunits and two molecules of di-domain (see below); the anomalous retention time observed for such a complex is probably attributable to the physical shape of the di-domain, as explained above for the E1–di-domain complex. The E1 β –di-domain peak continued to increase in size until a polypeptide chain ratio of 2:1 was reached, after which the excess di-domain was eluted as a separate peak (Figure 1b). The tight association between the E1 β subunit and di-domain could also be demonstrated by means of non-denaturing PAGE (Figure 4, lanes 4 and 5).

Samples of the E1 α subunit (0.5 nmol) were mixed with 0, 0.5 and 1 nmol of di-domain and then gel-filtered in 100 mM potassium phosphate buffer, pH 7.0, on a Superdex 75 HR column (Figure 5a). In the absence of di-domain, the E1 α subunit was eluted as a single peak with an apparent M_r of 42100, corresponding to a monomeric form (Lessard and Perham, 1994). Similarly, the di-domain was eluted as a single peak with an apparent M_r of 39800. The anomalous retention time of the di-domain (calculated M_r 18373) is almost certainly due to its physical shape, as explained above. The apparent M_r of the di-domain differs a little from the value (31300) obtained by Hipps et al. (1994) using a Superdex 200 HR gel-filtration column. The similar elution volumes of the E1 α subunit and di-domain meant that no separation could be achieved, but the

Figure 5 Association of the E1 α subunit with di-domain and with the E1 β -di-domain complex revealed by gel filtration

The E1 α subunit was mixed in various molar ratios with di-domain or the E1 β -di-domain complex and then gel-filtered on Superdex 75 HR and Superdex 200 HR columns respectively, in 100 mM potassium phosphate buffer, pH 7.0. (a) Panels A–C, 0.5 nmol of E1 α plus 0, 0.5 and 1.0 nmol of di-domain respectively; panel D, 0.5 nmol of di-domain. (b) Panels A–C, 2 nmol of E1 α plus 0, 0.5 and 1.0 nmol of E1 β -di-domain complex respectively; panel D, 1 nmol of E1 plus 1 nmol of di-domain.

addition of di-domain to E1 α before gel filtration did not generate a new peak of higher apparent M_r ; both proteins were eluted together at their original elution volumes. This strongly suggests that there is no significant interaction between the E1 α subunit and di-domain. The lack of association was confirmed by the results of non-denaturing PAGE of the proteins (Figure 4, lanes 2 and 3).

Association of the E1 α subunit with the E1 β -di-domain complex

E1 α and E1 β subunits are capable of self-assembly when mixed together *in vitro* to produce a functional E1 ($\alpha_2\beta_2$) enzyme (Lessard and Perham, 1994). The ability of the E1 α subunit to bind to an E1 β -di-domain complex was investigated by using three different methods: gel filtration, non-denaturing PAGE, and by measuring the E1 activity of the reconstituted E1-di-domain complex. In all these experiments, the E1 β subunit and di-domain were pre-mixed to form the E1 β -di-domain complex and this was followed by the addition of the E1 α subunit.

Samples containing 2 nmol of the E1 α subunit were mixed with an increasing amount of E1 β -di-domain complex and then gel-filtered in 100 mM potassium phosphate buffer, pH 7.0, on a Superdex 200 HR column (Figure 5b). Addition of the E1 β -di-

domain complex caused a diminution of the E1 α peak and the appearance of a new peak of higher apparent M_r , consistent with an association between the E1 α subunit and the complex to produce an E1-di-domain complex. The new peak showed a similar retention time to the complex formed from an intact E1 ($\alpha_2\beta_2$) enzyme and di-domain. The association of the E1 α subunit with the E1 β -di-domain complex was also shown by submitting the mixture to non-denaturing PAGE, revealing a band with a similar electrophoretic mobility to that of the E1-di-domain complex formed from intact E1 ($\alpha_2\beta_2$) and di-domain (Figure 4, lanes 2, 5, 7 and 9). These results indicate that the E1 α subunit is capable of assembling with an E1 β -di-domain complex to produce an E1 ($\alpha_2\beta_2$)-di-domain complex.

The integrity of the E1-di-domain complex formed from the separate proteins was confirmed by measuring the recovery of the E1 catalytic activity after mixing the E1 α subunit and E1 β -di-domain complex in various molar ratios and comparing it with the activity of intact E1 associated with di-domain (cf. Lessard and Perham, 1994). This indicated (results not shown) that mixing the E1 α subunit and E1 β -di-domain complex generated an intact and fully active E1-di-domain complex. The results of all these experiments demonstrate that the E1 β subunit is responsible for binding E1 to the di-domain and that E1 α , which itself does not exhibit a strong affinity for the di-domain, is capable of binding to an E1 β -di-domain complex to produce a functional E1-di-domain complex.

Competitive association of E1 and E3 with the di-domain

B. stearothermophilus E3 binds to the di-domain (peripheral subunit-binding domain) with an unexpected stoichiometry of one dimeric E3 molecule bound to one di-domain (Hipps et al., 1994). As shown above, E1 ($\alpha_2\beta_2$) also binds to the di-domain with this unexpected stoichiometry. An obvious question to ask is whether a single molecule of di-domain is capable of binding E1 and E3 simultaneously. This question was addressed by means of non-denaturing PAGE and gel filtration.

Samples (100 pmol) of E1 were mixed with increasing amounts of E3 and to each mixture was added di-domain (100 pmol) before submitting the products to non-denaturing PAGE (Figure 6a). As the amount of added E3 rose, the amount of E1-di-domain complex fell, the amount of E3-di-domain complex rose, and free E1 appeared. No E1-di-domain-E3 complex was observed. Similar results were obtained when samples of E3 (100 pmol) were mixed with increasing amounts of E1 before the addition of 100 pmol of di-domain (Figure 6b). As the amount of added E1 rose, the amount of E3-di-domain complex fell, the amount of E1-di-domain complex rose and free E3 appeared. Further, for a mixture of E1 and E3 at a molar ratio of 4:1, after PAGE almost all the added di-domain was found to be associated with E1, leaving nearly all the E3 unbound (Figure 6b, lane 6). In contrast, for a mixture of E3 and E1 at a molar ratio of 4:1, PAGE indicated that the added di-domain was approximately equally distributed between E1-di-domain and E3-di-domain complexes (Figure 6a, lane 6). Thus, it appears that the di-domain has a higher affinity for E1 than for E3, but more quantitative studies will be needed to estimate the relevant binding constants.

Samples (0.5 nmol) of E1 were also mixed with increasing amounts of E3, after which di-domain (0.5 nmol) was added and the products were gel-filtered in 100 mM potassium phosphate buffer, pH 7.0, on a Superdex 200 HR column (results not shown). Although the E1-di-domain and E3-di-domain complexes were not resolved, and E1 and E3 similarly were not resolved, it was clear that increasing the amount of E3 was

Figure 6 Association of di-domain with E1 and E3 revealed by non-denaturing PAGE

E1 and E3 were mixed in various molar ratios followed by the addition of di-domain (DD) before being subjected to non-denaturing PAGE. **(a)** Lane 1, 100 pmol of E1; lane 2, 100 pmol of E1 plus 100 pmol of di-domain; lanes 3–6, 100 pmol of E1 mixed with 50, 100, 200 and 400 pmol of E3 respectively, followed by the addition of 100 pmol of di-domain; lane 7, 100 pmol of E3 plus 100 pmol of di-domain; lane 8, 100 pmol of E3. **(b)** Lane 1, 100 pmol of E3; lane 2, 100 pmol of E3 plus 100 pmol of di-domain; lanes 3–6, 100 pmol of E3 mixed with 50, 100, 200 and 400 pmol of E1 respectively, followed by the addition of 100 pmol of di-domain; lane 7, 100 pmol of E1 plus 100 pmol of di-domain; lane 8, 100 pmol of E1.

accompanied by the appearance of a new peak of lower apparent M_r (120 000–150 000) than the E1–di-domain complex. It is likely that the new peak comprises a mixture of free E1 and E3, whereas the peak of higher M_r (200 000–250 000) consists of both E1–di-domain and E3–di-domain complexes. Most importantly, no peak of apparent M_r higher than that of the E1–di-domain complex expected of a complex between E1 and E3 and the di-domain was observed. Similar results were obtained when the experiment was performed with E1 as the increasingly added component (results not shown).

Conclusions

We have shown that only one di-domain (peripheral subunit-binding domain linked to the lipoyl domain) from the E2 chain of the *B. stearrowthermophilus* PDH complex binds tightly to one tetrameric E1 ($\alpha_2\beta_2$) molecule of the same complex. This unexpected stoichiometry was also found previously for the association of the di-domain with the homodimeric E3 molecule from *B. stearrowthermophilus* PDH complex (Hipps et al., 1994). It is possible that this is due to some form of steric hindrance or conformational change in the E3 dimer or E1 heterotetramer that renders the association of a second binding domain impossible when the first is in place.

A simpler explanation, in that it applies equally well to both E1 and E3, may lie in the dimeric nature of these proteins. X-ray crystallographic analysis of the E3 component from the PDH complexes of *A. vinelandii*, *Ps. putida* and *Ps. fluorescens* (Mattevi et al., 1993b; and references cited therein) shows them to be homodimers with a 2-fold axis of symmetry. The 1:1 stoichiometry of interaction between E3 and the peripheral subunit-binding domain could thus be explained by the binding site on the E3 dimer being located at one end of the C2 axis of symmetry, a unique site at the interface of the two E3 chains. Similar reasoning can be advanced for the 1:1 stoichiometry of the interaction with E1, as the E1 ($\alpha_2\beta_2$) molecule (viewed as a dimer of dimers) could also present a 2-fold axis of symmetry at

which a unique site for binding the peripheral subunit-binding domain is located. This explanation is made more attractive by the observation of a structural motif located across the 2-fold axis of the E3 molecule that closely resembles the three-dimensional structure of the peripheral subunit-binding domain (Brocklehurst et al., 1994). The structure of E3 bound to a peripheral subunit-binding domain is clearly needed to settle this question.

It has been shown elsewhere that the E1 β subunit, but not the E1 α , is capable of binding to the E2 component from the mammalian BCDH complex, suggesting a binding order of E2–E1 β –E1 α (Wynn et al., 1992a), in keeping with the earlier proposal of Stepp and Reed (1985). Our own experiments with the *B. stearrowthermophilus* E1 α and E1 β subunits confirm this binding order, and the successful assembly of an active E1–di-domain complex *in vitro* indicates that this process can take place without the need for molecular chaperones (cf. Lessard and Perham, 1994), unlike that for the mammalian BCDH subunits (Wynn et al., 1992a). The association of the *B. stearrowthermophilus* E1 β subunit and di-domain at a molar ratio of 2:1 again points to the possibility of a unique E1-binding site being located on a 2-fold axis of the E1 molecule, at an E1 β –E1 β subunit interface.

It is now clear that one *B. stearrowthermophilus* peripheral subunit-binding domain (i.e. one E2 chain) is capable of binding either one E3 dimer or one E1 ($\alpha_2\beta_2$) heterotetramer, but not both simultaneously. Moreover, the affinity for E1 appears to be higher than that for E3, although precise values for the dissociation constants remain to be determined. These facts carry important implications for the assembly of the complex and the nature of its quaternary structure. For example, it is difficult to see how the E1 and E3 molecules can be located with strict precision and symmetry over the surface of the E2 core, a subject that has received widespread study by electron microscopy (Oliver and Reed, 1982; Wagenknecht et al., 1983, 1986, 1990, 1991). There may of course be significant differences, yet to be investigated in the detail presented here, in the binding of E1 to the E2 chains of octahedral pyruvate and 2-oxoglutarate dehydrogenase complexes, where the inner core-domain of the E2 chain plays an important part (Mattevi et al., 1992; and references cited therein). Moreover, in the icosahedral PDH complexes from eukaryotes (Neagle and Lindsay, 1991; Lawson et al., 1991) and the octahedral OGDH complex from mammals (Rice et al., 1992), E3 appears to be bound to a novel protein X component of the E2 core or to an E3-binding domain at the N-terminus of the E1 polypeptide chain respectively. All these observations increasingly point to the importance of the internal network of acyl transfer reactions that provide for active-site coupling in 2-oxo acid dehydrogenase complexes which lack equivalence in the numbers of E1, E2 and E3 active sites and strict symmetry in their assembly (reviewed by Perham, 1991).

This work was supported by the Science and Engineering Research Council and The Wellcome Trust. I.A.D.L. was a recipient of a studentship from the Natural Sciences and Engineering Research Council of Canada. We are grateful to Mrs. Z. J. Jacoby for amino acid analysis, Mr. M. D. Allen for help with the purification of E3, Dr. S. J. Bowden for providing the pET-11d2D plasmid and for help with the purification of the di-domain, and Mr. S. M. Brocklehurst for helpful discussion.

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