The peripheral subunit-binding domain of the dihydrolipoyl acetyltransferase component of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*: preparation and characterization of its binding to the dihydrolipoyl dehydrogenase component

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The peripheral subunit-binding domain of the dihydrolipoyl acetyltransferase polypeptide chain of the pyruvate dehydrogenase multienzyme complex of *Bacillus stearothermophilus* was released by limited proteolysis from a di-domain (lipoyl domain plus binding domain) encoded by a subgene over-expressed in *Escherichia coli*. The domain was characterized by N-terminal sequence analysis, electrospray m.s. and c.d. spectroscopy. It was found to be identical in all respects to a chemically synthesized

peptide of the same sequence. The association of the di-domain and binding domain (both natural and synthetic) with dihydrolipoyl dehydrogenase was analysed in detail and a tight binding was demonstrated. As judged by several different techniques, it was found that only one peripheral subunit-binding domain is bound to one dimer of dihydrolipoyl dehydrogenase, implying that the association is highly anti-cooperative.

INTRODUCTION

The 2-oxo acid dehydrogenase multienzyme complexes are well-characterized assemblies of three enzymes that together catalyse oxidative decarboxylation reactions in the citric acid cycle and branched-chain amino acid metabolism [for recent reviews, see Patel and Roche (1990); Perham (1991); Mattevi et al. (1992a)]. In the pyruvate dehydrogenase (PDH) complex the three enzymes are: pyruvate decarboxylase [pyruvate dehydrogenase (lipoamide); E1p; EC 1.2.4.1], dihydrolipoyl acetyltransferase (E2p; EC 2.3.1.12) and dihydrolipoyl dehydrogenase (E3; EC 1.8.1.4). The structural core of the complex consists of multiple copies of the E2p polypeptide chain arranged with either octahedral (24-mer) or icosahedral (60-mer) symmetry, depending on the source of the enzyme (Reed, 1974; Perham, 1991). The E2p chain itself exhibits a highly segmented structure (Perham and Packman, 1989; Guest et al., 1989; Reed and Hackert, 1990; Perham, 1991), comprising (from the N-terminus) one, two or three lipoyl domains, a domain responsible for binding the peripheral E3 and, in icosahedral complexes, E1p subunits, and a catalytic (acetyltransferase) domain, which binds E1p in octahedral complexes and whose aggregation dictates the symmetry of the structural core. The lipoyl domains interdigitate between the E1p and E3 subunits and are linked by extended but flexible segments of polypeptide chain that allow them to move, facilitating delivery of the substrate to the successive active sites (Texter et al., 1988; Miles et al., 1988; Radford et al., 1989a,b; Wagenknecht et al., 1991; Green et al., 1992; and references cited therein). These structural features, broadly speaking, are common to all 2-oxo acid dehydrogenase complexes, although there are some significant differences with regard to the location of the E3-binding domain in protein X, an additional subunit in the E2

core of eukaryotic PDH complexes (Neagle and Lindsay, 1991; Lawson et al., 1991), and at the N-terminus of the E1 component of the mammalian 2-oxoglutarate dehydrogenase complex (Rice et al., 1992).

The structures of the lipoyl domain (Dardel et al., 1993) and peripheral subunit-binding domain (Kalia et al., 1993) from the E2p chain of the *Bacillus stearothermophilus* PDH complex, and of the peripheral subunit-binding domain from the corresponding dihydrolipoyl succinyltransferase (E2o) chain of the *Escherichia coli* 2-oxoglutarate dehydrogenase complex (Robien et al., 1992), have been determined by means of n.m.r. spectroscopy. Similarly, the structure of the acetyltransferase (octahedral core-forming) domain of the *Azotobacter vinelandii* E2p chain has been established by means of X-ray crystallography (Mattevi et al., 1992b). Together with what we know of the structure of the flexible interdomain segments of polypeptide chain (Radford et al., 1989a; Green et al., 1992), this lends support to the hope that it will be possible to piece together the structure of the intact E2 chain from the structures of its domain-and-linker segments.

Sub-genes encoding the lipoyl domains from the *B. stearothermophilus* (Dardel et al., 1990), *E. coli* (Ali and Guest, 1990) and human (Quinn et al., 1993) PDH complexes have been over-expressed in *E. coli*, making possible the production of large amounts of these proteins for detailed structural analysis. Recently we succeeded in similarly over-expressing a sub-gene encoding a di-domain (residues 1–170 of the E2p chain) comprising the *B. stearothermophilus* lipoyl domain attached by means of its linker to the peripheral subunit-binding domain (Hipps and Perham, 1992). We describe here the preparation and characterization of the peripheral subunit-binding domain generated by limited proteolysis of the di-domain. A 43-residue peptide corresponding to the binding domain (residues

Abbreviations used: PDH, pyruvate dehydrogenase; TFA, trifluoroacetic acid; DQF-COSY, double-quantum-filtered correlation spectroscopy.

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Val¹²⁸–Ala¹⁷⁰ of the E2p chain) was also chemically synthesized and compared with the product of limited proteolysis. The interactions of the intact di-domain and the two peripheral subunit-binding domains (natural and synthetic) with *B. stearotherophilus* E3 (a dimer) were investigated in detail.

MATERIALS AND METHODS

Materials

Bacteriological media were from Difco. *E. coli* strains and plasmids were all as described in Hipps and Perham (1992). Trypsin, sequencing grade, was from Boehringer–Mannheim. Trifluoroacetic acid (TFA) was from Pierce and Warriner (Chester, U.K.). Methyl[³H]acetimidate was synthesized by the method of Armstrong et al. (1980). Coated fused-silica capillaries were from SGE (UK) Ltd. (Milton Keynes, Bucks., U.K.). Aquapore RP300 h.p.l.c. columns were from Applied Biosystems. Other reagents were of analytical grade or the purest available.

Preparation of the natural and synthetic binding domains

The di-domain (residues 1–170 of the *B. stearotherophilus* E2p chain) generated from a sub-gene over-expressed in *E. coli* was purified as described by Hipps and Perham (1992). Limited proteolysis of the di-domain with trypsin was monitored using a Beckman P/ACE 2100 capillary electrophoresis system. Peptides and domains (lipoyl and subunit-binding) were separated on an analytical scale by reverse-phase h.p.l.c. (using an Aquapore RP300 column, 2.1 mm × 30 mm, with 0.1% TFA as mobile phase and CH₃CN as organic modifier, flow rate 0.2 ml/min). Detection was at 220 nm. Peptides were identified by means of electrospray m.s. on a VG BioQ mass spectrometer (Dardel et al., 1990; Packman et al., 1991) and N-terminal-sequence analysis on an Applied Biosystems 477A protein sequencer (Packman et al., 1988). On a preparative scale, the binding domain was separated from other digestion products by ion-exchange chromatography on a Pharmacia MonoS 5/5 f.p.l.c. column, using a step gradient [0–300 (5 min), 300–500 (15 min), 500 (5 min) mM NH₄HCO₃, pH 7.8] with a flow rate of 1 ml/min at 230 nm, with the binding domain being eluted at 300 mM NH₄HCO₃.

The synthetic binding domain (residues 128–170 of the *B. stearotherophilus* E2p chain) was synthesized on an Applied Biosystems 430A peptide synthesizer and purified by h.p.l.c. on a Vydac C4 reverse-phase column, as described elsewhere (Robien et al., 1992).

Characterization of the binding domain

Amino acid analysis was carried out on a LKB 4400 analyser after acid hydrolysis. The protein concentration measured in this way was used to determine the molar absorption coefficient of the binding domain for subsequent use in spectrophotometry. A vacuum u.v. c.d. spectrum from 180–260 nm was obtained using a Jovin Yvon CD6 spectrometer, and the Varselec program (Johnson, 1990) was used to predict the secondary structure of the binding domain.

¹H-n.m.r. spectroscopy

Purified di-domain was freeze-dried and redissolved three times in water to remove all traces of NH₄HCO₃ and its pH was

adjusted to pH 5.0 with 1 M HCl. The final sample contained binding domain (1.5 mM) in a volume of 0.5 ml (20% ²H₂O) to which was added NaN₃ (final concentration 0.02%, w/v) and trimethylsilylpropionate (final concentration 20 μM, as an internal standard). Spectra were recorded at 500 MHz and 293 K using a Bruker AM500 WB spectrometer with an 8 kHz spectral width, pulse intervals of 1.0 s and pre-saturation of the ¹H²HO line. A corresponding sample (1.5 mM, pH 5.0) of the synthetic domain was prepared similarly and two-dimensional double-quantum-filtered correlation spectroscopy (DQF-COSY; Piantini et al., 1982) experiments were performed on both samples.

Association with E3

B. stearotherophilus 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). E3 and binding domain (both natural and synthetic) or di-domain were mixed in various molar ratios in 100 mM sodium phosphate buffer, pH 7.8, and then subjected to electrophoresis in a non-denaturing polyacrylamide gel with a Tris/glycine buffer (Laemmli, 1970) but lacking SDS. The gels were stained with Coomassie Brilliant Blue R250 and scanned with a Transidyne 2955 scanning densitometer and Scatchard plots of [free E3] against [free E3]/[complex] were used to determine approximate dissociation constants. Similar samples of E3–di-domain complexes were also examined by means of capillary electrophoresis on a Beckman P/ACE system.

Stoichiometry of association with E3

Further association studies were carried out by means of gel filtration on a Pharmacia Superdex 200 HR 10/30 f.p.l.c. column (10 mm × 30 cm) in 50 mM sodium phosphate buffer, pH 8.0, using E3 and di-domain mixed in various molar ratios. E3–di-domain complex was prepared by gel filtration of a sample of E3 mixed with a 10-fold molar excess of di-domain. The polypeptide chain ratio in the complex was determined by treatment with methyl[³H]acetimidate as described elsewhere (Bates et al., 1975; Hale et al., 1979). The resulting radiolabelled polypeptides were separated by means of SDS/PAGE with a Tricine buffer. (Schägger and von Jagow, 1987) containing 0.02% mercaptoacetic acid. Radioactive bands were excised and the radioactivity in each was measured in an LKB 1215 Rackbeta liquid-scintillation counter. Non-denaturing gel electrophoresis of the E3–di-domain complex, purified by gel filtration, was used to assess the ability of the complex to bind additional subunits.

RESULTS AND DISCUSSION

Purification of the binding domain

Limited proteolysis of the di-domain with trypsin was studied with a view to producing maximal amounts of the binding domain; unlike the lipoyl domain, the binding domain is somewhat susceptible to proteolysis under mild conditions (Packman et al., 1988). A sample of di-domain (16 μg, 800 pmol) was incubated in 50 μl of 300 mM NH₄HCO₃, pH 7.8, at 20 °C with trypsin (0.2%, w/w) in the capillary-electrophoresis sample cup. Portions of the reaction mixture were removed at various times and the components separated by free-solution electrophoresis at 20 kV, and 30 °C in 0.1 M sodium phosphate buffer, pH 2.5, in a fused-silica capillary to check the extent of proteolysis (Packman and Hipps, 1991).

Proteolysis was stopped after 2 h by acidification to pH 3 with

1 % (v/v) TFA and the products were separated by reverse-phase h.p.l.c. The fractions containing protein were further examined by (a) capillary electrophoresis; (b) N-terminal-sequence analysis to identify the fragment; and (c) electrospray m.s. to determine M_r and hence to establish the peptide-chain length. In this way, the products of the digestion were unambiguously identified (Figure 1a). It is evident that trypsin cleaved the -Arg-Arg-sequence immediately preceding the presumed start of the binding domain in a non-uniform manner; the primary cleavage site was confirmed as following the second arginine residue (peak D), as indicated by Packman et al. (1988), but a minor degree of cleavage occurred after the first arginine (peak F) and trypsin was then unable to remove this N-terminal residue before the domain itself was degraded. For a period of over 1 h, the yield of the binding domain was at its maximum (Figure 1b).

A larger-scale digestion (10.8 mg of di-domain in 8.5 ml of 300 mM NH_4HCO_3 , pH 7.8, with 0.1 % trypsin, w/w) was carried out and monitored by capillary electrophoresis. After 2 h the digestion was stopped by cooling to 4 °C and adding phenylmethanesulphonyl fluoride (final concentration 1 mM). The sample was diluted to 100 mM NH_4HCO_3 and applied to a Pharmacia Mono S 5/5 column equilibrated in the same buffer in order to separate the binding domain from the other digestion products (Packman and Hipps, 1991). Most of the protein was unretarded, whereas the binding domain was bound and could be eluted with 300 mM NH_4HCO_3 . Samples (5 μl) of each fraction were screened for purity by capillary electrophoresis, N-terminal sequence analysis and electrospray m.s. and fractions were pooled appropriately. The major peak was the desired binding domain and a minor peak was the same domain with the additional uncleaved arginine residue at its N-terminus. This strategy for proteolysis and purification allowed the production of sufficient binding domain (3.3 mg) for structural analysis by n.m.r. spectroscopy and for detailed comparison with the synthetic peptide.

Characterization of the binding domain

Estimation of the protein by means of amino acid analysis gave a value for the absorption coefficient of the peripheral subunit-binding domain as $A_{280}^{0.1\%}$ 0.31. This low value is to be expected, as there are few aromatic residues present. Electrospray m.s. of the natural and synthetic domains gave measured masses of 4600.17 (S.D. = 1.12) and 4600.22 (S.D. = 0.53) respectively, compared with a predicted M_r of 4600.33. C.d. spectra of the natural and synthetic binding domains were similar (Figure 2) and the secondary structure prediction suggested a predominantly α -helical structure (approx. 55 %) with some β -turn (17.8 %) and little or no β -sheet. These structural comparisons indicate that the domains produced by either limited proteolysis of the genetically engineered di-domain or chemical synthesis are folded identically.

^1H -n.m.r. spectroscopy

One-dimensional ^1H -n.m.r. spectra of the di-domain (Hipps and Perham, 1992), lipoyl domain (Dardel et al., 1990), peripheral subunit-binding domain (this work, results not shown) and *E. coli* linker peptides (Radford et al., 1989a; Green et al., 1992) indicate that the di-domain spectrum is a composite of the major resonances found in the other spectra. This suggests that the component domains indeed fold independently and do not interact detectably (Hipps and Perham, 1992). Two-dimensional DQF-COSY spectra showed identical cross-peaks for the natural

Figure 1 Digestion of di-domain with trypsin

Di-domain was digested at 20 °C with trypsin (0.2%, w/w) and samples were removed at various times for examination by capillary electrophoresis (20 kV, 30 °C, 0.1 M sodium phosphate buffer, pH 2.5). (a) Separation of the products after 2 h of digestion; the products were identified by means of N-terminal-sequence analysis and electrospray m.s. (for details see the text). Position of lipoyl lysine residue, (●). (b) Yield of binding domain during proteolysis. The relative peak heights of the binding domain (peak D) (●) and the di-domain (peak A) (○) are shown as a function of the time of proteolysis.

Figure 2 C.d. spectra of binding domains

Solid line, binding domain released from di-domain by proteolysis with trypsin; broken line, binding domain prepared by chemical synthesis.

Figure 3 'Fingerprint' region of the two-dimensional DQF-COSY ^1H -n.m.r. spectra of binding domains

(a) Binding domain released from di-domain by proteolysis with trypsin. (b) Binding domain prepared by chemical synthesis.

and synthetic binding domains (Figure 3), indicating that the three-dimensional structures of the natural and synthetic domains are indistinguishable by this criterion.

Association with E3

In the E2p chain of the *B. stearotherophilus* PDH complex, as in most 2-oxo acid dehydrogenase complexes, the peripheral subunit-binding domain has a major part to play in binding the E3 subunits to the E2 core (Perham and Packman, 1989; Reed and Hackert, 1990; Perham, 1991; Mattevi et al., 1992a). A comparison of the association of E3 with the di-domain and with the natural and synthetic binding domains was carried out to investigate the stoichiometry of this interaction. Several different

methods were used, including non-denaturing PAGE, capillary electrophoresis, gel filtration and amidation.

E3 (0.1 nmol of dimer) was mixed with various amount of the di-domain or binding domain before being subjected to electrophoresis on a non-denaturing Tris/glycine polyacrylamide gel. The association of E3 dimer with di-domain is shown in Figure 4: the molecules bind to form a 1:1 complex and the dissociation constant can be calculated to be less than 3.4×10^{-10} M. Scanning densitometry was used to determine the concentrations of free E3 and complex, assuming that both stain equally well with Coomassie Blue, but the Scatchard plot (Figure 4) gave an unmeasurably small K_d value. The natural binding domain also bound to E3 (Figure 4) with a dissociation constant that was calculated to be of the order of 1.8×10^{-8} M. The

Figure 4 Association of di-domain and binding domains with E3 revealed by non-denaturing PAGE

E3 and di-domain or binding domain were combined in various proportions before electrophoresis on a non-denaturing polyacrylamide gel. (a) E3 and di-domain: lane 1, 0.1 nmol of E3 dimer; lanes 2-8, 0.1 nmol di-domain plus 0.05, 0.10, 0.12, 0.14, 0.16, 0.18 and 0.20 nmol E3 respectively. (b) E3 and binding domain: lane 1, 0.1 nmol of E3 dimer; lanes 2-6, 0.1 nmol of E3 dimer with 0.050, 0.067, 0.100, 0.150 and 0.200 nmol respectively, of binding domain released from di-domain by proteolysis with trypsin. (c) Scatchard plot of E3-di-domain interaction. Abbreviations: DD, di-domain; BD, binding domain.

synthetic binding domain behaved identically (results not shown). It is unclear why the di-domain should bind E3 more tightly than the binding domain. The lipoyl domain itself is not involved with the interaction with E3 (Hipps and Perham, 1992), but it is possible that amino acid residues in the inter-domain linker that lie on the N-terminal side of the tryptic cleavage site used to release the lipoyl domain from the binding domain are involved in stabilizing the interaction. Nonetheless, it is clear that the binding domain, natural or synthetic, is capable of a strong interaction with the E3 dimer.

The association of E3 with di-domain was also analysed by capillary electrophoresis. Samples containing 5 μM E3 were mixed with increasing amounts of di-domain and then subjected to electrophoresis in a coated capillary equilibrated in 20 mM sodium acetate, pH 6.8, at 30 °C and 20 kV. The results are shown in Figure 5. It can be seen that the peak attributed to the protein complex continues to rise until there is a molar ratio of di-domain to E3 dimer of 1:1. Any excess E3 migrates as a separate peak ahead of the complex.

Gel filtration of E3, di-domain and the complex (results not shown) was carried out in 50 mM sodium phosphate buffer, pH 7.0, using a Pharmacia Superdex 200 HR 10/30 gel-filtration column. The apparent M_r values, calculated from the retention times, were: E3, 110000; di-domain, 31300; and the complex,

Figure 5 Association of di-domain with E3 revealed by capillary electrophoresis

E3 and di-domain were combined in various proportions in the sample cup of a Beckman P/ACE apparatus and the bound and unbound components were separated by free-solution capillary electrophoresis (20 kV, 30 °C, 20 mM sodium acetate buffer, pH 6.8). (a), 2 μl of 5 μM E3 dimer; (b)-(f) 2 μl of 5 μM E3 dimer combined with 2 μl of 0.5, 1, 2, 4 and 8 μM di-domain respectively. Free di-domain is not detected in this system.

186500. The anomalous retention time of the di-domain (true M_r 18573 for the lipoylated di-domain) is almost certainly due to its physical shape in having two defined domains linked by a flexible but extended segment of polypeptide chain (Hipps and Perham, 1992). The apparent M_r of the di-domain-E3 complex is likely to be similarly anomalous. The stoichiometry of the E3-di-domain complex could be estimated by mixing known amounts of E3 with increasing amounts of di-domain in the eluting buffer and observing the changes in the elution profile on gel filtration (Figure 6). As with capillary electrophoresis (Figure 5), the peak attributed to the E3-di-domain complex continues to rise until there is a molar ratio of di-domain to E3 dimer of

Figure 6 Association of di-domain with E3 revealed by gel-filtration chromatography

E3 and di-domain were combined in various proportions and the bound and unbound components were separated by gel filtration on a Superdex 200 HR column in 50 mM sodium phosphate buffer, pH 7.0. (a)–(e) show 0.5 nmol of E3 combined with 0, 0.1, 0.2, 0.5 and 1.0 nmol respectively, of di-domain and (f) shows 0.5 nmol of di-domain.

1:1, after which the excess di-domain migrates as a separate and more retarded peak (Figure 6).

Stoichiometry of binding

Fractions from a Superdex 200 HR gel-filtration column, prepared as described above, that contained only the E3–di-domain complex, were pooled. Addition of further E3 or di-domain to

Figure 7 Non-denaturing PAGE of the E3–di-domain complex

The E3–di-domain complex purified by gel filtration (Figure 6) was combined with additional amounts of E3 or di-domain before being subjected to non-denaturing PAGE. Lane 1, 10 μ g of di-domain; lane 2, 10 μ g of E3; lane 3, 5 μ g of E3–di-domain complex; lane 4, 5 μ g of E3–di-domain complex plus 10 μ g of E3; lane 5, 5 μ g of E3–di-domain complex plus 10 μ g of di-domain. Abbreviation: DD, di-domain.

this purified sample, followed by non-denaturing PAGE, indicated the inability of the E3–di-domain complex to bind additional protein subunits (Figure 7). The E3–di-domain complex was then treated with 0.1 M methyl[3 H]acetimidate for 1 h at pH 10 in the presence of 6 M guanidine hydrochloride and the products were separated by means of SDS/PAGE. Each lane of the gel was cut into slices, and the radioactivity determined in each slice. From the radioactivity in each protein band and the amino acid compositions, the ratio of the two different polypeptide chains in the E3–di-domain complex could then be calculated (Bates et al., 1975). A molar ratio for E3 dimer:di-domain of $1.05 \pm 0.07:1$ was found.

Conclusions

Sufficient quantities of the natural and synthetic peripheral subunit-binding domain of the *B. stearothermophilus* E2p chain have been prepared for detailed characterization. The domain was shown to be folded and biologically active (capable of binding to the E3 component). The natural and synthetic proteins were indistinguishable, offering reassurance that the three-dimensional structures of synthetic peptides representing the E3-binding domains of the *E. coli* 2-oxoglutarate complex (Robien et al., 1992) and the *B. stearothermophilus* PDH complex (Kalia et al., 1993) accurately represent the native structures. This detailed structural information, in conjunction with further biochemical binding studies, should help to characterize the molecular interactions involved.

The analysis of the interaction between the *B. stearothermophilus* E3 and di-domain/binding domain described above shows that tight binding occurs at a molar ratio of 1:1, i.e. the E3 dimer, although a dimer of identical subunits, binds only one peripheral subunit-binding domain. This can be explained, for example, by assuming some form of steric hindrance or by postulating that association with the binding domain induces a conformational change in the E3 dimer that prevents the association of a second binding domain. The true reason remains to be determined. However, it appears that a polypeptide-chain ratio (E3:E2) of 2:1 is the maximum possible; lower ratios (such as 0.5:1 or less) found in the intact *B. stearothermophilus*

(Henderson and Perham, 1980) and *E. coli* (Reed, 1974; Bates et al., 1977) PDH complexes are therefore likely to be due to steric hindrance in the assembled complex, in particular to competition with E1 dimers for access to the limited surface of the E2 core (Reed et al., 1975).

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