Domain structure and ^1H -n.m.r. spectroscopy of the pyruvate dehydrogenase complex of *Bacillus stearothermophilus*

Leonard C. PACKMAN,* Richard N. PERHAM* and Gordon C. K. ROBERTS†

*Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge CB2 1QW, U.K., and †Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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The pyruvate dehydrogenase complex of *Bacillus stearothermophilus* was treated with *Staphylococcus aureus* V8 proteinase, causing cleavage of the dihydrolipoamide acetyltransferase polypeptide chain (apparent M_r 57 000), inhibition of the enzymic activity and disassembly of the complex. Fragments of the dihydrolipoamide acetyltransferase chains with apparent M_r 28 000, which contained the acetyltransferase activity, remained assembled as a particle ascribed the role of an inner core of the complex. The lipoic acid residue of each dihydrolipoamide acetyltransferase chain was found as part of a small but stable domain that, unlike free lipoamide, was able still to function as a substrate for reductive acetylation by pyruvate in the presence of intact enzyme complex or isolated pyruvate dehydrogenase (lipoamide) component. The lipoyl domain was acidic and had an apparent M_r of 6500 (by sedimentation equilibrium), 7800 (by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) and 10 000 and 20 400 (by gel filtration in the presence and in the absence respectively of 6M-guanidinium chloride). ^1H -n.m.r. spectroscopy of the dihydrolipoamide acetyltransferase inner core demonstrated that it did not contain the segments of highly mobile polypeptide chain found in the pyruvate dehydrogenase complex. ^1H -n.m.r. spectroscopy of the lipoyl domain demonstrated that it had a stable and defined tertiary structure. From these and other experiments, a model of the dihydrolipoamide acetyltransferase chain is proposed in which the small, folded, lipoyl domain comprises the *N*-terminal region, and the large, folded, core-forming domain that contains the acetyltransferase active site comprises the *C*-terminal region. These two regions are separated by a third segment of the chain, which includes a substantial region of polypeptide chain that enjoys high conformational mobility and facilitates movement of the lipoyl domain between the various active sites in the enzyme complex.

The 2-oxo acid dehydrogenase complexes (M_r 5×10^6 – 10×10^6) consist of multiple copies of three enzymes that function successively to catalyse the following overall reaction:

For the pyruvate dehydrogenase complex, these enzymes are pyruvate dehydrogenase (lipoamide) (E1, EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12) and dihydrolipoamide dehydrogenase (E3, EC 1.6.4.3). Corresponding

enzymes comprise the 2-oxoglutarate dehydrogenase and branched-chain 2-oxo acid dehydrogenase complexes. In all cases, the E2 component forms a structural core of high M_r , to which the E1 and E3 components are bound tightly but non-covalently (for a review see Reed, 1974).

During catalysis the substrate is carried in thioester linkage by lipoyl-lysine 'swinging arms' that are part of the E2 component and serve to transfer the substrate between the physically separate active sites (Reed, 1974; Ambrose & Perham, 1976; Grande *et al.*, 1976). These lipoyl-lysine residues in turn are located in regions of the E2 polypeptide chains that physically protrude from

Abbreviation used: SDS, sodium dodecyl sulphate.

the complexes and are readily removed by limited proteolysis (Hale & Perham, 1979a; Bleile *et al.*, 1979, 1981; Kresze & Ronft, 1980; Perham & Wilkie, 1980; Perham & Roberts, 1981; Kresze *et al.*, 1981). It has been shown by ^1H -n.m.r. spectroscopy that the 2-oxo acid dehydrogenase complexes from *Escherichia coli* (Perham *et al.*, 1981; Perham & Roberts, 1981), ox heart (Wawrzynczak *et al.*, 1981) and *Bacillus stearothermophilus* (Duckworth *et al.*, 1982) contain extensive regions of polypeptide chain that have substantial conformational mobility with respect to the bulk of the structures. The mobile regions are part of the E2 polypeptide chains and appear to tether folded domains in which the lipoyl-lysine residues are located to inner core-forming domains of the E2 chains that contain the dihydrolipoamide acetyltransferase active sites and also provide, at least in part, binding sites for the E1 and E3 components (Perham *et al.*, 1981; Packman *et al.*, 1982; Duckworth *et al.*, 1982). Such polypeptide-chain mobility is part of the system of active-site coupling in these complexes and enables a given lipoyl group to serve more than one E1 or E3 active site (Perham *et al.*, 1981; Berman *et al.*, 1981; Stepp *et al.*, 1981; Roberts *et al.*, 1983), overcoming the puzzle of unequal numbers of E1, E2 and E3 active sites in the enzymes and the physical heterogeneity of the complexes (Bates *et al.*, 1975; Schmitt & Cohen, 1980; Gilbert & Gilbert, 1980; Wagenknecht *et al.*, 1983).

In the present paper, we describe the isolation of a stable proteolytic fragment (probable M_r approx. 6500) of the E2 polypeptide chain from *B. stearothermophilus* pyruvate dehydrogenase complex, which contains the lipoyl group and has the ^1H -n.m.r. spectrum of a highly ordered structure. This lipoyl domain, unlike free lipoic acid, is a substrate for reductive acetylation by E1. The structural and catalytic properties of the E2 chain inferred from these experiments appear to be of general applicability to the 2-oxo acid dehydrogenase complexes.

Materials and methods

Enzymes and reagents

Pyruvate dehydrogenase complex from *B. stearothermophilus* was prepared by the method of Henderson & Perham (1980) and stored at -20°C in 50 mM-sodium phosphate buffer, pH 7.0, containing 2.7 mM-EDTA and 0.02% NaN_3 . The concentrations of proteins of known amino acid composition were determined by amino acid analysis. Other proteins were assayed spectrophotometrically assuming an absorption coefficient, $A_{280}^{1\%}$, of 1.0cm^{-1} , and correcting for light-scattering by linear extrapolation from

320 nm and 360 nm. *Staphylococcus aureus* V8 proteinase was purchased from Miles Laboratories, Slough, Berks., U.K.

Sodium $[2\text{-}^{14}\text{C}]$ pyruvate and *N*-ethyl $[2,3\text{-}^{14}\text{C}]$ -maleimide were from Amersham International, Amersham, Bucks., U.K. Guanidinium chloride and urea were from BDH Chemicals, Poole, Dorset, U.K., and were recrystallized from 96% (v/v) ethanol before use. All other reagents were as specified by Danson *et al.* (1979).

Enzyme assays

Pyruvate dehydrogenase-complex activity and E1 and E3 partial activities were measured as described by Packman *et al.* (1982). The dihydrolipoamide acetyltransferase activity of E2 was assayed by the method of Reed & Willms (1966), except that the formation of *S*-acetyldihydrolipoamide was monitored spectrophotometrically at 240 nm instead of colorimetrically after reaction with hydroxylamine.

Preparation of the lipoyl domain from *B. stearothermophilus* pyruvate dehydrogenase complex

B. stearothermophilus pyruvate dehydrogenase complex (0.9 ml; 37 mg/ml) in 50 mM-sodium phosphate buffer, pH 7.0, containing 2.7 mM-EDTA and 0.02% NaN_3 , was diluted to 3.3 ml with distilled water and kept at 30°C . Samples were removed for assay of complex enzymic activity and for analysis by SDS/polyacrylamide-gel electrophoresis. *S. aureus* V8 proteinase (0.33 mg in 0.1 ml of 20 mM-sodium phosphate buffer, pH 7.0) was added and the digestion allowed to proceed at 30°C . Samples (5 μl) were removed after various times.

Within 2 min, the light-scattering by the solution decreased markedly, and during the interval 6–10 min a heavy precipitate formed. At 100 min a second addition of proteinase (0.3 mg) was made, and after a total digestion time of 180 min phenylmethanesulphonyl fluoride (0.16 M in ethanol) was added to a final concentration of 1 mM. [Recent experiments suggest that this step may be ineffective against this serine proteinase from *S. aureus* (L. C. Packman, unpublished work), but no proteinase could be detected in the fractions from the subsequent gel-filtration column (see Fig. 2) that showed absorbance at 280 nm.] The precipitate was collected in a bench centrifuge and washed twice with 0.25 ml of 5 mM-sodium phosphate buffer, pH 7.0, containing 0.7 mM-EDTA and 0.005% NaN_3 . The supernatant solutions were combined, and the precipitate, comprising the inner core of the E2 component of the complex, was resuspended in 3.5 ml of 5 mM-sodium phosphate buffer, pH 7.0, containing 0.7 mM-EDTA and 0.005% NaN_3 .

The supernatant solution was made 0.3M in NaCl and 0.2mM in 2-mercaptoethanol by adding 5M and 2M stock solutions respectively. The resulting solution was applied to a column (150cm $\times$ 2 cm) of Sephadex G-50 (superfine grade) equilibrated and developed at 23°C with 20mM-sodium phosphate buffer, pH 7.0, containing 0.1M-NaCl, 2.7mM-EDTA and 0.02% NaN₃, to resolve the lipoyl domain from other soluble protein.

Detection of lipoyl domain by reductive acetylation

Samples (50 μ l) of each fraction from the Sephadex G-50 column were mixed at 23°C with 50 μ l of 20mM-sodium phosphate buffer, pH 7.0, containing 3mM-NAD⁺, 4mM-MgCl₂, 0.8mM-thiamin pyrophosphate and 10 μ g of *B. stearothermophilus* pyruvate dehydrogenase complex. After 5 min, 10 μ l of 1.7mM-sodium [2-¹⁴C]pyruvate in water was added and the mixture was left for 25 min, at which time 10 μ l of bovine serum albumin (10mg/ml in water) as carrier protein was introduced. The proteins were denatured by immediate addition of 2ml of ice-cold trichloroacetic acid (10%, w/v) and, after 5 min at 0°C, the precipitate was filtered off on Whatman GF/C glass-fibre discs. The precipitate was washed with 20ml of 10% trichloroacetic acid, dried under vacuum and counted for radioactivity in an LKB Rackbeta 1215 scintillation counter (Stanley *et al.*, 1981).

Kinetics of reductive acetylation of lipoyl domain

To study the kinetics of reductive acetylation, pure lipoyl domain (1–10 μ M) was incubated at 23°C in a final volume of 0.3 ml of 20mM-sodium phosphate buffer, pH 7.0, containing 0.1mM-thiamin pyrophosphate, 0.6mM-MgCl₂, 1.5mM-NAD⁺, *B. stearothermophilus* pyruvate dehydrogenase complex (20 μ g/ml) and bovine serum albumin (0.3mg/ml). After approx. 5 min, 4 μ l of 15mM-sodium [2-¹⁴C]pyruvate was added and samples (45 μ l) were then withdrawn at various times between 0.5 and 4.5 min. The samples were mixed immediately with 1 ml of ice-cold trichloroacetic acid (10%, w/v) and kept at 0°C for 5 min. The precipitated protein was filtered off and counted for radioactivity as described above. Control incubations were treated identically except that lipoyl domain was omitted.

The initial rate of reductive acetylation was measured from a plot of [¹⁴C]acetyl group incorporation versus time. Apparent *K_m* and *V_{max}* values were derived from a direct linear plot.

Polyacrylamide-gel electrophoresis

The discontinuous buffer system of Laemmli (1970) was used for polyacrylamide-gel electrophoresis in the presence and in the absence of 0.1% SDS. Urea/polyacrylamide gels were run in the

same buffer system, omitting SDS but incorporating 8M-urea in the stacking and resolving gels and 9M-urea in the sample application buffer. All gel systems included 2mM-2-mercaptoacetic acid in the cathodic well buffer. Gel compositions are described according to the Hjerten (1962) terminology. All stacking gels were 5%T, 2.6%C. Gels were stained with Coomassie Brilliant Blue.

Analytical ultracentrifugation

A sample of the E2 inner core was dialysed against 25mM-sodium potassium phosphate buffer, pH 7.0, containing 0.1M-NaCl (*I*0.15) and subjected to ultracentrifugation in a Beckman model E centrifuge at 42040 rev./min at 17°C. Sedimentation-equilibrium analysis of the lipoyl domain was performed at 30000 rev./min at 18°C in 25mM-sodium potassium buffer, pH 7.0, containing 0.15M-NaCl (*I*0.2).

¹H-n.m.r. spectroscopy of lipoyl domain and E2 inner core

The lipoyl domain was dialysed against 50mM-NH₄HCO₃ with the use of Spectrapor 3 dialysis membrane (Spectrum Medical Industries, Los Angeles, CA, U.S.A.), and concentrated by freeze-drying. The process of freeze-drying did not affect its ability to become reductively acetylated when assayed with pyruvate dehydrogenase complex and sodium [2-¹⁴C]pyruvate. The lipoyl domain was redissolved in 20mM-potassium phosphate buffer in ²H₂O containing 0.02% NaN₃, apparent pD 7.6. EDTA was added, as required, from a concentrated solution in ²H₂O. Small amounts of undissolved material, if present, were removed in a bench centrifuge.

The precipitate that formed during the release of the lipoyl domains with *S. aureus* V8 proteinase (see above) comprised the inner core (Perham & Wilkie, 1980) of the E2 component. It was dissolved in approx. 1 ml of 20mM-potassium phosphate buffer in ²H₂O containing 0.2M-NaCl and 0.02% NaN₃, apparent pD 7.6. The solution was dialysed at 4°C against several changes of the same buffer except that the NaCl concentration was lowered to 0.1M.

¹H-n.m.r. spectra were obtained at 270 MHz by using a Bruker WH 270 spectrometer and at 500 MHz by using a Bruker AM 500 spectrometer. Spectra of the intact enzyme complex and of the E2 inner core were obtained at 270 MHz, with quadrature detection, a 6 kHz spectral width and pulse intervals of 0.34–5.00 s; the sample temperature was 25 ($\pm$ 1)°C. Before Fourier transformation, the free-induction decay was multiplied by an exponential function leading to a line-broadening of 5 Hz. Spectra of the lipoyl domain were obtained at both 270 MHz and 500 MHz, with spectral

widths of 4.2 kHz and 8 kHz respectively, with pulse intervals of 0.51–1.02 s. Spectra were obtained at sample temperatures between 25 and 45°C ($\pm 1^\circ\text{C}$); a line-broadening of 1 Hz was employed. Chemical shifts were measured relative to internal dioxan, and are quoted relative to 2,2-dimethylsilapentane-5-sulphonate (taking the dioxan chemical shift as 3.71 p.p.m. downfield from 2,2-dimethylsilapentane-5-sulphonate).

Other methods

Protein chemical techniques, such as amino acid analysis on an LKB 4400 amino acid analyser, were performed as described elsewhere (Perham, 1978).

Results

Digestion of pyruvate dehydrogenase complex with S. aureus V8 proteinase

When *B. stearothermophilus* pyruvate dehydrogenase complex was digested with *S. aureus* V8 proteinase at 30°C, there was a decrease in light-scattering during the first 2 min, suggesting disassembly of the complex, and this was followed by the formation of a precipitate. The overall catalytic activity of the complex fell to 1% of its starting

value in 15 min and to 0.5% after 180 min, whereas the E3 activity was unaffected. The E1 activity fell rapidly, but within 20 min stabilized at 20% of its initial value. The dihydrolipoamide acetyltransferase activity of the E2 core was also retained. The products of the proteolysis were analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 1). The E2 subunit was cleaved rapidly, generating a stable fragment of apparent M_r 28000, which resembles that of the E2 inner core described by Perham & Wilkie (1980) and which was found to comprise the precipitate that formed during the digestion (Fig. 1). The E3 and E1 β chains were resistant to proteolysis, but the E1 α chain was slowly degraded. This degradation did not correlate with the loss of E1 activity.

When the experiment was repeated with *B. stearothermophilus* complex in which the lipoic acid residues were labelled with *N*-ethyl[2,3- ^{14}C]maleimide (Perham & Wilkie, 1980), a similar pattern of digestion products was found. Autoradiography of the SDS/polyacrylamide gel (Fig. 1) showed that the second addition of proteinase after 100 min was required to yield a lipoyl domain that was homogeneous by this criterion.

Gel filtration of the digest, after removal of the

Fig. 1. Digestion of *B. stearothermophilus* pyruvate dehydrogenase complex with *S. aureus* V8 proteinase. Pyruvate dehydrogenase complex (10 mg/ml) was digested at 30°C and pH 7 with *S. aureus* V8 proteinase (1%, w/w) as described in the text. A second addition of proteinase was made at 100 min. (a) Samples (5 μl) were removed at intervals and analysed by SDS/polyacrylamide-gel (20%T, 0.6%C) electrophoresis. The compositions of the supernatant (S) and precipitate (P) fractions isolated at the end of the digestion are also shown. (b) In a parallel experiment, the lipoic acid residues of the pyruvate dehydrogenase complex (1 mg) were radiolabelled by treatment with *N*-ethyl[2,3- ^{14}C]maleimide (Perham & Wilkie, 1980). The radioactive protein was digested with *S. aureus* V8 proteinase and the products were analysed by SDS/polyacrylamide-gel electrophoresis, as above. The autoradiograph of the gel is shown, the position of radioactive bands a, b and c in the Coomassie Blue-stained gel being indicated by horizontal arrows.

precipitate of E2 inner core in a bench centrifuge, enabled the lipoyl domain to be separated from the remaining soluble protein (Fig. 2). The lipoyl domain could be detected by its ability to become reductively acetylated by intact pyruvate dehydrogenase complex in the presence of sodium [2-¹⁴C]pyruvate. Some material with apparent M_r (on SDS/polyacrylamide-gel electrophoresis) similar to that of the lipoyl domain was excluded from the column and may represent, at least in part, the smaller cleavage product of the E1 α chain that has remained bound to E1 α and/or E1 β subunits.

When tested individually, the resolved products of the proteolytic digest, i.e. the lipoyl domain of E2, the inner core and the mixture of E1 and E3 components, showed no pyruvate dehydrogenase complex activity in the NAD⁺-reduction assay (in the buffer for which the precipitate of E2 inner core was found to dissolve quite freely). Mixtures of any two products were also inactive, but the presence of all three products, in relative amounts similar to those present in the intact complex, led to a low pyruvate dehydrogenase-complex activity equivalent to 0.5% of that found for the intact enzyme at the same overall protein concentration.

Since all of the disassembled components retained substantial activities (20–100%) in their part reactions, this small amount of catalytic activity in the overall reaction may represent the activity of these components when they lack the benefit of association into the normal multimeric structure.

Characterization of the lipoyl domain

The lipoyl domain migrated rapidly as a single band during electrophoresis in polyacrylamide gels in the presence and in the absence of 0.1% SDS and in the presence of 8M-urea (Fig. 2). The apparent M_r of the domain, as judged from the SDS/polyacrylamide-gel electrophoresis, was about 7800. A similar value was obtained with a continuous phosphate-buffered system of SDS/polyacrylamide-gel electrophoresis (Hale & Perham, 1979b). On the other hand, gel filtration of the domain at 4°C on a Sephadex G-75 column (132cm × 0.6cm) equilibrated with 20mM-sodium phosphate buffer, pH 7.0, containing 2.7mM-EDTA and 0.02% NaN₃ or with 50mM-NH₄HCO₃ gave an apparent M_r of 20400. This same value was also found for the domain that had been reductively acetylated, and it was unaffected by the inclusion of 2mM-

Fig. 2. Gel filtration of the *S. aureus* V8 proteinase digest of *B. stearothermophilus* pyruvate dehydrogenase complex. Pyruvate dehydrogenase complex was digested with *S. aureus* V8 proteinase as indicated in Fig. 1 legend and the precipitate was removed by centrifugation. The soluble fraction was applied to a Sephadex G-50 (superfine grade) column (150cm × 2cm) equilibrated at 23°C with 20mM-sodium phosphate buffer, pH 7.0, containing 0.1M-NaCl, 2.7mM-EDTA and 0.02% NaN₃. The flow rate was 12ml/h. Fractions of volume approx. 6ml were collected and monitored for absorbance at 280nm (●) and 230nm (○). Samples (50μl) of each fraction were tested for their ability to become reductively acetylated in the presence of intact enzyme complex and sodium [2-¹⁴C]pyruvate (Δ), as described in the text. Selected fractions were then examined by polyacrylamide-gel electrophoresis: (A) and (B) electrophoresis of samples of fractions 16 and 24 respectively in an SDS/polyacrylamide gel (20%T, 0.6%C); (C) and (D) electrophoresis of a sample of fraction 24 (C) in a polyacrylamide gel (10%T, 2.6%C) containing 8M-urea and (D) in a polyacrylamide gel (24%T, 2.6%C) under non-denaturing conditions. Apparent M_r values are those of a set of marker proteins and mobilities (R_m) are quoted relative to the Bromophenol Blue dye front.

dithiothreitol in the buffer. Gel filtration of the lipoyl domain at 23°C in 6M-guanidinium chloride, pH 5.0, containing 2mM-dithiothreitol on a Sephacryl S300 column (132cm × 0.6cm) was consistent with an M_r of approx. 10000. Sedimentation-equilibrium analysis (see the Materials and methods section) gave a plot of $\ln c$ versus r^2 that was somewhat curved, indicative of heterogeneity. This curvature was accurately modelled by assuming that the preparation contained 92% by weight of a domain with apparent M_r 6500 (calculated from the slope of the linear portion of a plot of $\ln c$ versus r^2) and 8% by weight of another species with apparent M_r of 26000 (calculated from the slope of the secondary plot of $\ln c$ versus r^2). The latter might be an oligomeric form of the domain.

By protein chemical criteria, the lipoyl domain appeared to have a robust secondary and tertiary structure. For example, when treated with 1% (w/w) trypsin, chymotrypsin, elastase, subtilisin or *S. aureus* V8 proteinase at 37°C for 2h, no proteolysis was detectable, as judged by SDS/polyacrylamide-gel electrophoresis. The inclusion of 2–4M-urea in the digestion had no effect. Some degradation was achieved by using thermolysin at 55°C, indicating perhaps that the structure is more open or flexible as the physiological growth temperature of *B. stearothermophilus* is reached.

The lipoyl domain acted as a substrate for reductive acetylation with sodium [2- 14 C]pyruvate in the presence of *B. stearothermophilus* pyruvate dehydrogenase complex (Fig. 2). This was equally true of the domain before and after freeze-drying, emphasizing its robust nature. Reductive acetylation was also achieved by partly proteolysed E1 component obtained from the digestion of *B. stearothermophilus* complex with *S. aureus* V8 proteinase. The radioactivity incorporated into the domain was released by treatment with performic acid and the incorporation of acetyl groups was matched by the generation of an equal number of thiol groups that could react with *N*-ethyl[2,3- 14 C]maleimide. All these results are characteristic of reductive acetylation of a lipoyl group. From kinetic measurements at 23°C, the K_m value of the pyruvate dehydrogenase complex for the isolated lipoyl domain was 4.3 μ M (assumed M_r 6500) and the V_{max} value for reductive acetylation of the domain under the same conditions was 0.4 nkat/mg of complex.

Characterization of the E2 inner core

The E2 inner core was readily purified as the precipitate that formed during the digestion of the pyruvate dehydrogenase complex with *S. aureus* V8 proteinase (see above and Fig. 1). Although it was insoluble at neutral pH in buffers of low ionic strength, it readily dissolved when the ionic

strength was raised above 0.1. Sedimentation of the inner core in 25mM-sodium potassium phosphate buffer, pH 7.0, containing 0.1M-NaCl (10.15) showed a predominant peak with $s_{20,w}$ 27.4 ± 0.6 S at a protein concentration of 5.6 mg/ml. In some preparations a minor peak was also observed having $s_{20,w}$ 40S, consistent with it being a dimeric form of the particle (Danson *et al.*, 1979).

As mentioned above, the inner core was found to retain the dihydrolipoamide acetyltransferase activity of the intact core.

^1H -n.m.r. spectroscopy

The 270MHz n.m.r. spectra of the native complex and of the E2 inner core are shown in Fig. 3. As noted earlier (Duckworth *et al.*, 1982), the spectrum of the native complex contains a number of resonances in the aliphatic region with linewidths of 50–100Hz, which must arise from a region or regions of polypeptide chain having substantial mobility. By contrast, the spectrum of the inner core produced by *S. aureus* V8 proteinase digestion shown at approximately equivalent gain contains virtually no sharp resonances. The peak at 1.36p.p.m. varied somewhat in intensity from one preparation to another and may arise, at least in part, from a non-protein contaminant. In any

Fig. 3. 270MHz ^1H -n.m.r. spectra of (a) the intact *B. stearothermophilus* pyruvate dehydrogenase complex and (b) the E2 inner core obtained by *S. aureus* V8 proteinase digestion

The vertical gain of the two spectra has been adjusted according to the protein concentration used so that they are directly comparable. Resonances marked d and e arise respectively from dioxan and EDTA. The resonance marked x arises from a non-protein component, possibly silicone anti-foam, which is present in variable trace amounts in different preparations of the complex.

Fig. 4. 500 MHz ^1H -n.m.r. spectrum of the lipoyl domain isolated from *B. stearothermophilus* pyruvate dehydrogenase complex

The region of the spectrum immediately around the large water resonance has been omitted. The aromatic region is presented at $8\times$ and the inset high-field region at $16\times$ the vertical gain of the aliphatic region of the spectrum. The sharp resonance at approx. 0 p.p.m., marked X, arises from a non-protein contaminant (see the legend to Fig. 3).

case, it is clear that the proteolytic treatment has removed essentially all the mobile segment(s) from the E2 subunit.

The 500 MHz ^1H -n.m.r. spectrum of the isolated lipoyl domain, shown in Fig. 4, is characteristic of a protein with small M_r that has substantial tertiary structure. The clearest indication of the existence of a folded structure in this domain is the appearance of a number of methyl resonances to high field of 0.7 p.p.m., and in particular of four methyl resonances between 0.1 and -0.5 p.p.m. These signals arise from methyl groups held close to the face of aromatic rings by the folded structure of the protein. Similarly, the non-equivalence of the phenylalanine residues is apparent in the aromatic region of the spectrum. In the absence of a complete analysis of the spectrum, we cannot rule out the possibility that, as isolated, the lipoyl domain also contains a random-coil component, but the existence of a stable well-defined tertiary structure in a substantial part of the domain is clearly demonstrated. The stability of this structure is indicated by the observation that the ^1H -n.m.r. spectrum, and specifically the structural 'markers' such as the high-field methyl resonances, are unaffected by temperature over the range 25–45°C.

The aromatic proton resonances of the lipoyl domain are not visible in the spectrum of the intact complex (Fig. 3a). This shows that the lipoyl domain is not free to move rapidly on the end of the random-coil polypeptide chain that links it to the

structural core of the E2 subunit. It must spend a major part of the time bound to one of the other components of the complex; this is clearly consistent with the low K_m value observed for the isolated lipoyl domain acting as a substrate for the pyruvate dehydrogenase complex.

Discussion

Treatment of the *B. stearothermophilus* pyruvate dehydrogenase complex with *S. aureus* V8 proteinase led principally to cleavage of the E2 chains (apparent M_r 57000) and disassembly of the complex (Figs. 1 and 2). The inner E2 core formed a precipitate at low ionic strength but was freely soluble at neutral pH at ionic strengths above 0.1. Under these conditions it sedimented with $s_{20,w}$ 27S at 5.6 mg/ml and, since it was composed of polypeptide chains with an apparent M_r of 28000 as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 1), it can be concluded that it was present as a macromolecular aggregate of large M_r . It also retained the lipoate acetyltransferase activity of the intact E2 core, but lacked the highly mobile segment of polypeptide chain seen in the native complex. It closely resembles the inner E2 core ($s_{20,w}$ 33S at an estimated protein concentration of 1.2 mg/ml) generated from the *B. stearothermophilus* complex by tryptic digestion, which displays the icosahedral (60-fold repeat) symmetry of the parent particle and is composed of poly-

peptide chains with an apparent M_r of 28000 (Perham & Wilkie, 1980). There is a marked concentration-dependence of sedimentation coefficient for these enzyme complexes and their E2 cores (Danson *et al.*, 1979; Henderson, 1979). A similar inner E2 core can be generated, again with accompanying disassembly of peripheral subunits, by limited proteolysis of the ox heart pyruvate dehydrogenase complex (Kresze & Ronft, 1980; Bleile *et al.*, 1981), which also has icosahedral symmetry (Reed, 1974). Thus it would appear to be a general feature of this class of pyruvate dehydrogenase complexes.

The 2-oxo acid dehydrogenase complexes of octahedral symmetry behave somewhat differently. Limited proteolysis of the E2 component excises the lipoic acid-containing domains from the pyruvate dehydrogenase (Hale & Perham, 1979a; Bleile *et al.*, 1979) and 2-oxoglutarate dehydrogenase (Perham & Roberts, 1981) complexes, but the inner E2 cores retain their binding sites for E1 and E3 components. However, treatment of the *B. stearothermophilus* pyruvate dehydrogenase complex with chymotrypsin releases lipoic acid-containing segments of the E2 chains without causing disassembly, the residual E2 core being composed of polypeptide chains with apparent M_r of 45000 that retain the mobile segment (Duckworth *et al.*, 1982). This implies that the binding sites for E1 and E3 components receive an essential contribution from that part of the E2 chain which is removed by trypsin but not by chymotrypsin, and that the mobile segment is also contained in this part of the chain. However, the basic design of the E2 component of 2-oxo acid dehydrogenase complexes is obviously common to both symmetry classes.

The lipoyl domain released from the *B. stearothermophilus* complex by treatment with *S. aureus* V8 proteinase is still able to undergo reductive acetylation by sodium [2- 14 C]pyruvate in the presence of intact complex or isolated E1 component; indeed, this formed the assay by which it could be detected during purification (Fig. 2). With the intact complex the apparent K_m for this reductive acetylation was $4.3\ \mu\text{M}$, a value some 5-fold lower than that reported for a lipoyl fragment (apparent M_r 28000) of ox heart pyruvate dehydrogenase complex acting as a substrate for the isolated E1 component (Bleile *et al.*, 1981). In both instances this can be construed as evidence of a high affinity of the lipoyl domain for the E1 active site, in keeping with the inferences from ^1H -n.m.r. spectroscopy of the complexes (Perham *et al.*, 1981; Packman *et al.*, 1982; Roberts *et al.*, 1983). Since free lipoic acid or lipoamide is not a substrate for reductive acetylation by the E1 component, it would appear that presentation of the lipoamide to

the E1 active site by the lipoyl domain is a prerequisite for catalysis. Such presentation may be desirable for the subsequent reactions with the E2 and E3 active sites, but it is certainly not essential, since free lipoamide will serve as a substrate for them.

The rapid migration of the lipoyl domain during electrophoresis on polyacrylamide gels of high %T both under non-denaturing conditions and in the presence of 8M-urea (Fig. 2) suggests that the domain is highly acidic in its folded and unfolded states. The acidic nature and anomalous electrophoretic mobilities of similar but larger lipoic acid-containing fragments of the E2 polypeptide chains from the pyruvate dehydrogenase complexes of *E. coli* (Bleile *et al.*, 1979) and ox heart (Bleile *et al.*, 1981) have previously been noted and the hydrodynamic properties interpreted in terms of swollen or extended conformations. The apparent M_r of 20400 estimated for the *B. stearothermophilus* lipoyl domain by means of gel filtration at pH 7.0 compared with value of 6500 and 10000 estimated by means of sedimentation-equilibrium centrifugation at pH 7.0 and of gel filtration in the presence of 6M-guanidinium chloride at pH 5.0 respectively indicates that the structure of the lipoyl domain itself contributes to these anomalous properties.

Whatever the tertiary structure of the lipoyl domain may be, its robust nature is indicated by its resistance to further proteolysis by several enzymes tested and by its ability to survive freeze-drying in a functional form. This was duly reflected in its ^1H -n.m.r. spectrum (Fig. 4), which is that of a well-defined folded structure and which remains the same up to at least 45°C .

Previous studies of the *B. stearothermophilus* pyruvate dehydrogenase complex by limited proteolysis and ^1H -n.m.r. spectroscopy have led to the view that each E2 polypeptide chain comprises a relatively small but structured domain housing the lipoic acid residue, which is separated from a large core-forming domain by a highly flexible region or regions of some 50 amino acid residues that are the source of the sharp n.m.r. signals (Duckworth *et al.*, 1982; Roberts *et al.*, 1983). The results of the present investigation are wholly in agreement with this model and provide direct evidence that the lipoyl domain does indeed have a defined tertiary structure. Recent experiments (L. C. Packman, R. N. Perham & J. Walker, unpublished work) have shown that the structured lipoyl domain forms the *N*-terminal region of the E2 polypeptide chain, which therefore establishes the order of the three segments that we have described.

The structure of the pyruvate dehydrogenase complex of *E. coli* is similar, but differs significant-

ly in one respect. Each E2 polypeptide chain is thought to contain three lipoyl-lysine residues (Hale & Perham, 1979b), and it has been proposed that the amino acid sequence around each lipoyl-lysine residue is identical, i.e. that the sequence is repeated in the polypeptide chain (Hale & Perham, 1980). DNA-sequence analysis of the gene for the *E. coli* E2 chain has confirmed that this amino acid sequence does repeat and is in fact part of three successive highly homologous regions each of about 100 amino acid residues that form the *N*-terminal half of the polypeptide chain (Stephens *et al.*, 1983). It is clear then that the structure of the E2 chains of the pyruvate dehydrogenase complexes of *E. coli* and *B. stearothermophilus* are formally similar except that the *E. coli* chain has two additional lipoyl domains as an *N*-terminal extension. Since all other lipoate acetyl- and succinyl-transferases appear to contain only one lipoic acid residue per E2 chain (Perham & Roberts, 1981; Stanley *et al.*, 1981, and references cited therein), it is likely that they conform to the structural model that we propose for the E2 chain of the *B. stearothermophilus* enzyme.

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