Cross-linking and ^1H n.m.r. spectroscopy of the pyruvate dehydrogenase complex of *Escherichia coli*

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The pyruvate dehydrogenase complex of *Escherichia coli* was treated with *o*-phenylene bismaleimide in the presence of the substrate pyruvate, producing almost complete cross-linking of the lipoate acetyltransferase polypeptide chains as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This took place without effect on the catalytic activities of the other two component enzymes and with little evidence of cross-links being formed with other types of protein subunit. Limited proteolysis with trypsin indicated that the cross-links were largely confined to the lipoyl domains of the lipoate acetyltransferase component of the same enzyme particle. This intramolecular cross-linking had no effect on the very sharp resonances observed in the ^1H n.m.r. spectrum of the enzyme complex, which derive from regions of highly mobile polypeptide chain in the lipoyl domains. Comparison of the spin–spin relaxation times, T_2 , with the measured linewidths supported the idea that the highly mobile region is best characterized as a random coil. Intensity measurements in spin-echo spectra showed that it comprises a significant proportion (probably not less than one-third) of a lipoyl domain and is thus much more than a small hinge region, but there was insufficient intensity in the resonances to account for the whole lipoyl domain. On the other hand, no evidence was found in the ^1H n.m.r. spectrum for a substantial structured region around the lipoyl-lysine residues that was free to move on the end of this highly flexible connection. If such a structured region were bound to other parts of the enzyme complex for a major part of its time, its resonances might be broadened sufficiently to evade detection by ^1H n.m.r. spectroscopy.

The 2-oxo acid dehydrogenase multienzyme complexes catalyse the following overall reaction:

(R typically = CH_3 — or CH_2 — CH_2 — CO_2H)

and consist of multiple copies of three enzymes that function successively in the mechanism [reviewed by Reed (1974)]. For the pyruvate dehydrogenase complex, these enzymes are pyruvate decarboxylase (E1, EC 1.2.4.1), lipoate acetyltransferase (E2, EC 2.3.1.12) and lipoamide dehydrogenase (E3, EC 1.6.4.3). For the 2-oxoglutarate dehydrogenase complex, the corresponding enzymes are 2-oxoglutarate decarboxylase (E1, EC 1.2.4.2), lipoate succinyltransferase (E2, EC 2.3.1.61) and lipoamide dehydrogenase (E3, EC 1.6.4.3). 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. The substrate is carried in thioester linkage by lipoic acid residues attached covalently to lysine side chains of the subunits of the E2 core.

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) have recently been shown by ^1H n.m.r. spectroscopy to contain extensive regions of polypeptide chain that enjoy remarkable conformational mobility with respect to the rest of the enzyme structure. Correlation of the ^1H n.m.r. spectra with the results of limited proteolysis of trypsin demonstrated that, for the pyruvate dehydrogenase complex of *E. coli*, these regions are part of the lipoate acetyltransferase (E2) core component and are associated with the lipoyl-lysine residues (Perham *et*

et al., 1981). The close similarity of the ^1H n.m.r. spectra obtained for the pyruvate and 2-oxoglutarate dehydrogenase complexes from this and other organisms strongly suggests that high conformational mobility of the lipoic acid-containing regions of the polypeptide chain is a general feature of these enzymes (Perham & Roberts, 1981; Wawrzynczak *et al.*, 1981; Duckworth *et al.*, 1982).

The results are in accord with, and have added a new dimension to, earlier work that indicated that the lipoyl-lysine residues of these complexes are located in regions of the E2 core that are accessible to proteinases and which might have a measure of flexibility (Hale & Perham, 1979*a,b*; Bleile *et al.*, 1979, 1981; Kresze & Ronft, 1980; Kresze *et al.*, 1981; Perham & Wilkie, 1980). Polypeptide-chain mobility provides the molecular basis of a novel system of active-site coupling in these complexes and permits a given lipoyl group to serve more than one E1 or E3 active site, overcoming the otherwise considerable problem posed by the unequal numbers of E1, E2 and E3 active sites in the native enzymes (Perham *et al.*, 1981; Perham & Roberts, 1981; Berman *et al.*, 1981; Stepp *et al.*, 1981; Duckworth *et al.*, 1982).

The lipoic acid-containing regions of the E2 polypeptide chains that are released by limited proteolysis with trypsin have been purified from the E2 core of the pyruvate dehydrogenase complex of *E. coli* (Bleile *et al.* 1979) and ox heart (Bleile *et al.*, 1981). They are highly acidic, migrate anomalously on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, and have a swollen or extended structure as judged by the high values (about 2.0) found for the frictional ratio, f/f_0 . In consequence it has been proposed that they form elongated domains that are attached to the rest of the E2 core by means of flexible hinges (Bleile *et al.*, 1979, 1981). On the other hand, the appearance of the ^1H n.m.r. spectra of the complexes suggested that the lipoic acid-containing regions contain extensive segments of polypeptide chain with the conformational mobility of random coils, though the existence of secondary structure for other segments was envisaged (Perham *et al.*, 1981; Perham & Roberts, 1981; Wawrzynczak *et al.*, 1981; Duckworth *et al.*, 1982).

Each E2 chain in the pyruvate dehydrogenase complex of *E. coli* contains two lipoic acid residues that can be reductively acetylated in the presence of the substrate pyruvate (Danson & Perham, 1976; Bates *et al.*, 1977; Collins & Reed, 1977; Speckhard *et al.*, 1977). The polypeptide chains in the E2 core can then be cross-linked intramolecularly through the lipoyl moieties. This is achieved by treating the reductively acetylated enzyme with bismaleimides (Collins & Reed, 1977) which react with the free thiol group on the *S*-acetyldihydrolipoic acid residues. In the present paper we show that, after

such cross-linking, the pyruvate dehydrogenase complex of *E. coli* retains essentially all of the polypeptide-chain mobility associated with the lipoyl domains of the E2 core. This result, together with an assessment of the size of the random-coil segment, imposes important restrictions on structural models for these regions.

Materials and methods

Enzymes and reagents

Pyruvate dehydrogenase complex was purified from a constitutive mutant of *E. coli* by the method of Reed & Mukherjee (1969), as described by Danson *et al.* (1979). The polypeptide-chain ratios of the complex, determined by means of the radioamidation method (Bates *et al.*, 1975; Hale *et al.*, 1979) using methyl [^3H]acetimidate (Armstrong *et al.*, 1980), were found to be 1.5:1.0:0.8 (E1/E2/E3). Protein concentrations were estimated by amino acid analysis. Trypsin was purchased from Worthington and *o*-phenylene bismaleimide from Aldrich. Other reagents were as specified by Danson *et al.* (1979).

Enzyme assays

Pyruvate dehydrogenase-complex activity and E3 activity were measured in the direction of NAD^+ reduction as described by Danson & Perham (1976). The activity of the E1 component was measured at 30°C and 600nm by the reduction of 2,6-dichloroindophenol in the presence of pyruvate, as suggested by Khailova *et al.* (1977). The assay medium consisted of 70 μM 2,6-dichloroindophenol in 50 mM-potassium phosphate buffer, pH 7.0, containing 1 mM- MgCl_2 , 0.2 mM-thiamin pyrophosphate and 2 mM-sodium pyruvate.

Cross-linking of lipoyl domains with *o*-phenylene bismaleimide

Two incubation mixtures were set up in acid-washed vials, each fitted with a rubber bung containing a small hole through which reagents were added or samples removed. The vials contained 9.3 ml of 20 mM-sodium phosphate buffer, pH 7.0, containing 1.5 mM- NAD^+ , 0.2 mM-thiamin pyrophosphate and 1 mM- MgCl_2 . The stirred solution was bubbled with N_2 for 30 min at room temperature (23°C) and a gentle flow of N_2 was then maintained above the solution throughout the experiment.

Pyruvate dehydrogenase complex (0.65 ml, 48 mg/ml) was added to each vial to give a final protein concentration of 3 mg/ml and the solution was maintained under the flow of N_2 for a further 50 min. Samples (2–10 μl) were removed for analysis by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and for assay of E1, E3 and overall pyruvate dehydrogenase complex activities. At this

point, 0.1 ml of degassed 0.1 M-sodium pyruvate was added to one vial (final concn. 1 mM) and 0.1 ml of degassed water was added to the other. After 30 s, each vial received 50 μ l of 2 mM-*o*-phenylene bismaleimide 10 μ M). The overall pyruvate dehydrogenase-complex activity of the enzyme solution containing pyruvate rapidly fell to about 60% of its starting value. A further addition of 50 μ l of 2 mM-*o*-phenylene bismaleimide was made to each vial, causing a similar loss in catalytic activity, although a third addition of *o*-phenylene bismaleimide had little further effect. A final decrease in catalytic activity (to a level of less than 2%) was achieved by adding 20 μ l of degassed 0.1 M-sodium pyruvate to the relevant vial. The cross-linking reaction was then quenched by adding 2-mercaptoethanol to a final concentration of 1 mM. The E1 and E3 activities of the pyruvate dehydrogenase complex were monitored throughout the experiment and found to be unaffected by the treatment.

Sodium dodecyl sulphate / polyacrylamide-gel electrophoresis

Analysis of proteins in 5%-polyacrylamide gel containing 0.1% sodium dodecyl sulphate was performed as described by Hale & Perham (1979a) using the phosphate-buffered system.

¹H n.m.r. spectroscopy

After being cross-linked as described above, the pyruvate dehydrogenase complex was centrifuged at 165 000 *g* ($r_{av.}$ = 5.91 cm) for 2.6 h at 4°C to concentrate the enzyme. The clear yellow pellet was resuspended in 4 ml of 20 mM-potassium phosphate buffer, containing 2.7 mM-EDTA, pH 7. The solution was clarified in a bench centrifuge and the enzyme complex was recovered by centrifuging again at 165 000 *g*. These two steps ensured removal of unbound protein components (typical $s_{20,w}$ = 20 S) which are normally present in preparations of *E. coli* pyruvate dehydrogenase complex (Danson *et al.*, 1979). The protein pellets were resuspended with 100 μ l of ²H₂O containing 20 mM-sodium phosphate, 2 mM-EDTA, apparent p²H = 7.6, and dialysed against 4 $\times$ 20 ml of the same buffer at 4°C for a minimum of 8 h with each buffer change.

¹H n.m.r. spectra were obtained at 270 MHz with a Bruker WH270 spectrometer, with quadrature detection, a 6 kHz spectral width and a pulse interval of 0.34–5.00 s, as described previously (Perham *et al.*, 1981; Perham & Roberts, 1981). The sample temperature was 25($\pm$ 1)°C. Chemical shifts are expressed relative to internal (1 mM) dioxan (3.71 p.p.m. downfield from 2,2-dimethylsilapentane-5-sulphonate). Spin-spin relaxation times, T_2 , were estimated using the 'spin-echo' method (Hahn, 1950; Carr & Purcell, 1954) with the pulse sequence:

$$(90^\circ - \tau - 180^\circ - \tau - \text{acquire} - T)_n$$

where τ is a variable delay (1–100 ms) and T is a fixed delay of 2.00 s ($>5T_1$). The T_2 values were in the range 3–15 ms, so that no modulation of the decay by spin-spin coupling was observed. T_2 values were calculated from the slope of a plot of the logarithm of resonance intensity as a function of the delay τ .

Other methods

Amino acid analysis of proteins was performed on an LKB 4400 amino acid analyser, using samples that had been hydrolysed for 24 h at 105°C in 6 M-HCl. Analytical ultracentrifugation was performed as described previously (Danson *et al.*, 1979).

Results

Cross-linking of pyruvate dehydrogenase complex with o-phenylene bismaleimide

Initial attempts to cross-link the acetyldihydro-lipoamide groups in the E2 core of the pyruvate dehydrogenase complex employed the conditions described by Collins & Reed (1977), but resulted in poor yields of cross-linked E2, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. We thought it likely that the relatively high concentration (0.2 mM) of *o*-phenylene bismaleimide might be at fault, and better results were indeed obtained when the *o*-phenylene bismaleimide was introduced in small serial additions. The use of very low concentrations of *o*-phenylene bismaleimide presumably favours intramolecular cross-linking by limiting the concentration of *o*-phenylene bismaleimide in free solution.

The results of a typical experiment in which serial additions of low concentrations of *o*-phenylene bismaleimide were made are shown in Fig. 1. The E1 and E3 activities of the enzyme complex were unaffected. The third addition of *o*-phenylene bismaleimide produced little further inhibition of the complex. The final loss in activity brought about by adding more pyruvate (0.2 mM) at this point suggests that the initial pyruvate (1 mM) had all been turned over by the enzyme during the incubation, despite the absence of CoASH. Gradual depletion of pyruvate could explain why equal additions of *o*-phenylene bismaleimide cause progressively less inhibition of the complex activity unless fresh substrate is added.

The extent of cross-linking achieved by this procedure was impressive, with almost total disappearance of monomeric E2 chains from the sodium dodecyl sulphate/polyacrylamide gels and no significant bands appearing with apparent M_r less than pentameric E2 (Fig. 2a). Heavier loadings of protein on the gels showed the presence of some minor bands covering a wide range of M_r values, corresponding to various combinations of inter- and

Fig. 1. Reaction of pyruvate dehydrogenase complex with *o*-phenylene bismaleimide

Two samples of pyruvate dehydrogenase complex (3 mg/ml) were incubated at 23°C under N₂ with 20 mM-sodium phosphate buffer, pH 7.0, containing 1.5 mM-NAD⁺, 0.2 mM-thiamin pyrophosphate and 1 mM-MgCl₂. Sodium pyruvate (final concn. 1 mM) was added to one sample of enzyme. After 30 s, *o*-phenylene bismaleimide (*o*-PBM) (final concn. 10 μM) was added to each sample of enzyme (zero time) and the whole-complex activity of the enzyme incubated in the absence (●) and presence (○) of pyruvate was monitored. Further additions of *o*-phenylene bismaleimide (final concn. 10 μM) were made to each incubation at the times shown by the arrows. At 170 min, extra pyruvate (final concn. 0.2 mM) was added to one sample of enzyme as indicated. The modification reaction was arrested at 230 min by adding 2-mercaptoethanol (final concn. 1 mM) to each sample.

intra-chain cross-linked products. Densitometric scanning of the gels revealed a 95–99% disappearance of monomeric E2 chains and an apparent 10–15% loss of E3 chains. Since no E3 dimer bands were visible, loss of E3 could be due to cross-links formed to E2 with no obvious concomitant loss in E3 catalytic activity. In the absence of pyruvate, *o*-phenylene bismaleimide caused no significant cross-linking of E2 or E3 chains. Identical results were obtained when the cross-linking experiment was carried out in buffer in which ²H₂O fully replaced ¹H₂O, as described below.

To test whether the cross-links in E2 introduced by treatment with *o*-phenylene bismaleimide were restricted to the lipoyl domains of the E2 core, cross-linked and unmodified samples of pyruvate dehydrogenase complex (4 mg/ml) were treated with trypsin (2%, w/w) at 0°C for 30 min in 20 mM-sodium phosphate buffer, pH 7.0. The products were

examined by sodium dodecyl sulphate/polyacrylamide gel-electrophoresis. Identical gel patterns were obtained (Fig. 3), which closely resembled those described previously for the native complex (Hale & Perham, 1979a; Bleile *et al.*, 1979). In particular, the proteolytic fragment of E2 with an apparent *M_r* of 36 000 was found in good yield for both complexes. Since this fragment of the E2 chain constitutes the inner core of the enzyme that has lost its lipoyl domains, it is clear that the cross-links in the *o*-phenylene bismaleimide-treated complex must have been largely restricted to the lipoic acid-containing regions of the E2 polypeptide chains.

A striking feature of *o*-phenylene bismaleimide as a modifying agent for the complex was its potency as an inhibitor compared with *N*-ethylmaleimide. Under identical conditions, 30 μM-*o*-phenylene bismaleimide inhibited pyruvate dehydrogenase complex activity twice as rapidly as 0.5 mM-*N*-ethylmaleimide, with half-times of inactivation of 2 and 4 min respectively.

¹H n.m.r. spectroscopy of cross-linked pyruvate dehydrogenase complex

Protein samples used for ¹H n.m.r. spectroscopy were checked by analytical ultracentrifugation in 20 mM-potassium phosphate in ²H₂O, containing 2 mM-EDTA, apparent p²H 7.6. The cross-linked and control sample each showed a major peak with an *s*_{20,w} value of approx. 57 S at 4.7 mg/ml. A minor peak, commonly found in preparations of the complex and possibly a dimer (Danson *et al.*, 1979), was also present. No low-*M_r* aggregates were observed and no interparticle cross-linking of the enzyme complex, induced by *o*-phenylene bismaleimide in the presence or absence of pyruvate, was detected.

The 270 MHz ¹H n.m.r. spectra of the native and cross-linked pyruvate dehydrogenase complexes are shown in Fig. 4. The two spectra are essentially identical, both containing, in addition to the very broad component from the bulk of the complex, the relatively sharp lines between –0.5 and –3.0 p.p.m., which have been shown to arise from the lipoyl domains of the E2 chains (Perham *et al.*, 1981). The deconvoluted spectra shown as inserts in Fig. 4 demonstrate that the chemical shifts of the sharp lines are the same in the spectra of the native and cross-linked complexes. The intensities (measured relative to the EDTA resonances in spectra obtained with a pulse delay of 5.0 s) and linewidths of these sharp lines are also indistinguishable in the two spectra.

When the cross-linked enzyme complex was stored for 2 weeks at 4°C, the E2 band could be detected once more after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, signifying a slow breakdown of the cross-links (Fig. 2b). To

Fig. 2. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of cross-linked pyruvate dehydrogenase complex* (a) Samples ($5\mu\text{g}$) of untreated complex (A) and complex modified with *o*-phenylene bismaleimide in the presence (B) and absence (C) of pyruvate were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Extensive cross-linking of E2 chains by *o*-phenylene bismaleimide in the presence of pyruvate was observed. The polymerized E2, which failed to enter the gel, is at least pentameric in size. Smaller amounts of E2 polymers of lower M_r are observed at high loadings ($15\mu\text{g}$) of protein (D). (b) Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of samples ($15\mu\text{g}$) of cross-linked (A) and unmodified (B) complex after storage for 2 weeks at 4°C . Reappearance of monomeric E2 chains and decrease in the amount of highly polymerized material in (A) is evidence for slow breakdown of the cross-links.

ensure that rupture of the cross-links was not taking place before the protein was examined by n.m.r. spectroscopy, a further sample of cross-linked pyruvate dehydrogenase complex was prepared as before except that all buffers were made up in $^2\text{H}_2\text{O}$. The modified enzyme was collected by ultracentrifugation at $165\,000\text{g}$ ($r_{\text{av.}} = 5.91\text{ cm}$) for 6 h at 4°C , resuspended overnight in 20 mM-potassium phosphate in $^2\text{H}_2\text{O}$ containing 2 mM-EDTA, apparent p^2H 7.6, and analysed at once by ^1H n.m.r. spectroscopy. The spectrum of this cross-linked enzyme complex was essentially unchanged from that shown in Fig. 4, except for the presence of several additional very sharp resonances. These were also present in the buffer from which the cross-linked complex was prepared by ultracentrifugation and were readily identified as being due to trace amounts of reagents from the cross-linking reaction being carried through in the enzyme pellet. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the cross-linked complex after n.m.r. spectroscopy revealed no breakdown of the cross-links. We conclude that cross-linking of the lipoyl domains through their lipoic acid residues has no detectable effect on the sharp resonances in the ^1H n.m.r. spectrum of the pyruvate dehydrogenase complex.

The widths of the relatively sharp resonances from the lipoyl domain are in the range 35–70 Hz. To establish whether there is any contribution to these linewidths from chemical-shift heterogeneity, we estimated the spin–spin relaxation times, T_2 , of the corresponding protons in the native complex by the spin-echo method. The resonances at -2.43 p.p.m. (alanine $\beta\text{-CH}_3$ and threonine $\delta\text{-CH}_3$) and -0.76 p.p.m. (lysine $\epsilon\text{-CH}_2$ and perhaps arginine $\delta\text{-CH}_2$) are both characterized by a T_2 value of $13 (\pm 2)\text{ ms}$, which corresponds to a linewidth of $25 (\pm 5)\text{ Hz}$. The measured linewidth of these resonances is $35 (\pm 5)\text{ Hz}$ (after correction for the line broadening applied to improve the signal-to-noise ratio), which includes a contribution of $\leq 7\text{ Hz}$ (alanine and threonine) to $\leq 15\text{ Hz}$ (lysine) from scalar coupling. The methylene resonances between -1.3 and -2.2 p.p.m. were characterized by T_2 values of 4–6 ms. Precise measurements of the linewidths of these overlapping resonances were not possible, but the values of 50–75 Hz calculated from the T_2 values are clearly consistent with the spectra. It can be concluded that the linewidths are dominated by spin–spin relaxation with no substantial contribution from chemical-shift heterogeneity.

Fig. 3. Limited proteolysis of modified pyruvate dehydrogenase complex

Samples of unmodified and cross-linked pyruvate dehydrogenase complex were treated with trypsin for 30 min at 0°C in 20 mM-sodium phosphate buffer, pH 7.0, as described in the text. The products were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. A, untreated complex and B, cross-linked complex, each before limited proteolysis; C, untreated complex and D, cross-linked complex after proteolysis.

The spin-echo experiments also gave the opportunity for a more accurate determination of the intensities of the sharp lines in the spectrum. These intensity measurements cannot be made accurately in normal spectra (such as those in Fig. 4), owing to the obvious difficulty in drawing a baseline to take proper account of the large broad component of the spectrum (Perham *et al.*, 1981). This broad component will, however be characterized by a very short T_2 (<0.1 ms) and will therefore be absent from spectra obtained by using the spin-echo pulse sequence with $\tau \geq 1$ ms. With $\tau = 1$ ms (the shortest value feasible on our instrument), the sharper lines, with longer T_2 values, will still have $\geq 80\%$ of their intensity, and this can be measured without interference from the broad component.

These measurements indicate that the relatively sharp lines in the spectrum of the complex have intensity corresponding to a total of some 500 ($\pm 20\%$) protons per protomer. We define a protomer as a 1/24 part of the enzyme complex, since the E2 core appears to possess octahedral symmetry (Reed, 1974; Danson *et al.*, 1979). This value is consistent with, though somewhat larger than, our previous estimate (Perham *et al.*, 1981) based on approximate integration of the normal spectrum.

Fig. 4. ^1H n.m.r. spectroscopy of cross-linked pyruvate dehydrogenase complex

^1H n.m.r. spectra (270 MHz) of (a) native pyruvate dehydrogenase complex and (b) complex cross-linked with *o*-phenylene bismaleimide are shown. The insets show the -0.5 to -5 p.p.m. regions of the deconvoluted spectra. Resonances labelled E arise from 2 mM-EDTA in the buffer, and X is an instrumental artefact.

The absence of sharp resonances in the region of the spectrum (downfield of the water peak) characteristic of aromatic protons, which has already been noted (Perham *et al.*, 1981), shows that the mobile region of the protein contains very few, if any, aromatic residues. Since aliphatic amino acid residues contain an average of 4.5 protons/residue with resonances to high field of dioxan, we conclude that approx. 110 residues/protomer have sufficient backbone mobility in the complex to give rise to these sharp lines.

Discussion

By means of serial additions of very low concentrations of *o*-phenylene bismaleimide to the pyruvate dehydrogenase complex in the presence of pyruvate (Fig. 1), almost complete cross-linking of the E2 chains could be achieved, as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 2). This took place with no effect on the catalytic activities of the E1 or E3 components

and with little evidence of cross-links being formed between E2 and E1 or E3 polypeptide chains. No intermolecular cross-linking of enzyme particles was detected in the ultracentrifuge. Limited proteolysis of the native and cross-linked complexes with trypsin at pH 7 and 0°C gave the same pattern of fragments (Fig. 3), generating in particular the E2 fragment of apparent M_r 36 000. This fragment remains aggregated as the inner core of the *E. coli* pyruvate dehydrogenase complex when the lipoic acid-containing regions of E2 chains are excised (Hale & Perham, 1979*a,b*; Bleile *et al.*, 1979). There is thus good evidence that the cross-links formed by treating the complex with *o*-phenylene bismaleimide in the presence of pyruvate are mostly to be found between lipoyl domains in the same enzyme particle. Another interesting conclusion from the experiments was that *o*-phenylene bismaleimide is a more potent inhibitor of the enzyme complex under these conditions than is *N*-ethylmaleimide. Reaction of an *S*-acetyldihydrolipoic acid residue with *o*-phenylene bismaleimide might be favoured by a hydrophobic binding site on the enzyme.

The intramolecular cross-linking of lipoyl domains in the E2 core had no effect on the ¹H n.m.r. spectrum of the enzyme complex (Fig. 4). This implied in turn that the remarkable polypeptide-chain mobility associated with these regions is largely unimpaired, despite the physical links forged between them. The simplest conclusion to be drawn from this result is that the mobility of this large segment of a lipoyl domain is independent of the restraint to movement imposed at the lipoyl-lysine residues. Thus, it is unlikely that the lipoyl domains are more or less rigid structures hinged to the inner part of the E2 core by a small region of flexible polypeptide chain, as they appear in an earlier model based on other evidence (Bleile *et al.*, 1979, 1981).

On the other hand, the lack of effect of cross-linking on the ¹H n.m.r. spectrum is readily understood if a substantial part of the lipoyl domain has the characteristics of a random coil, since the segmental motion of this part of the chain could continue unaffected by cross-linking. In a random coil, all the alanine methyl protons, for example, would be expected to have the same chemical shift (see Jardetzky & Roberts, 1981), and in fact what appear to be single resonances at -2.34 and -2.77 p.p.m. (close to the expected positions for alanine β -CH₃ and/or threonine γ -CH₃ and for valine γ -CH₃, leucine δ -CH₃ and/or isoleucine γ,δ -CH₃ respectively) are consistently observed in the spectra of 2-oxo acid dehydrogenase complexes from several sources (Perham *et al.*, 1981; Wawrzynczak *et al.*, 1981; Perham & Roberts, 1981; Duckworth *et al.*, 1982). The width of these resonances (35–50 Hz) left open the possibility that significant chemical-shift heterogeneity, reflecting significant tertiary structure,

was concealed within the linewidth. However, our present estimates of T_2 by the spin-echo method, though of limited precision, show clearly that the linewidth is determined by relaxation, with little or no contribution from chemical-shift heterogeneity. The chemical shifts observed for the sharp lines in the spectrum are thus entirely consistent with a random-coil structure for the highly mobile region of the E2 chain.

The best estimate of the extent of this highly mobile region, from the spin-echo spectra, is some 90–130 residues. This is clearly more than just a local hinge between two folded domains. Equally, however, it is too small to represent the whole of the lipoyl domain. The lipoic acid-containing fragment of the E2 chain, which is released by trypsin and contains all the highly mobile region of the polypeptide chain (Perham *et al.*, 1981), has M_r 31 600 by sedimentation equilibrium (Bleile *et al.*, 1979). If we take this fragment as the complete lipoyl domain, then the highly mobile region represents perhaps one-third of it. The existence of a part of the lipoyl domain other than the mobile segment seen by n.m.r. is also indicated by the observation that fragments of the E2 polypeptide chain containing the lipoyl-lysine residues can be excised from the 2-oxoglutarate dehydrogenase complex of *E. coli* (Perham & Roberts, 1981) and from the pyruvate dehydrogenase complex of *Bacillus stearothermophilus* (Duckworth *et al.*, 1982) without substantial changes to the ¹H n.m.r. spectra.

The possibility exists, therefore, that the lipoyl domain contains a region of ordered structure in the vicinity of the lipoyl-lysine residues. On this hypothesis, the E2 polypeptide chain would consist of three domains: one of M_r 36 000 (Hale & Perham 1979*a,b*; Bleile *et al.*, 1979, 1981), which forms the structural core of the complex and contains the lipoate acetyltransferase active site; a second structured domain that contains the lipoyl-lysine residues; and a large highly flexible random-coil domain that links the two.

If a substantial structured region around the lipoyl-lysine residues were free to move on the end of this highly flexible connection, one would expect to see its resonances as relatively sharp signals in the n.m.r. spectrum of the enzyme complex. They would appear at positions distinct from those characteristic of the random coil, reflecting the ordered structure of the region. Such signals are seen, for example, in the ¹H n.m.r. spectrum of the *lac* repressor, in which the structured *N*-terminal 'headpiece' domain is able to move rapidly with respect to the rest of the molecule (Wade-Jardetzky *et al.*, 1979). In the case of the pyruvate dehydrogenase complex, there is no evidence for any such resonances in the spectrum; as noted above, the sharp resonances have the characteristics of a random coil.

However, if the structured part of the lipoyl domain were to spend most of its time in contact with the rest of the complex, its motional freedom would be substantially decreased and its resonances broadened so as to become undetectable. Lipoic acid, in the form of lipoyl-lysine residues, is a substrate for the three component enzymes of the pyruvate dehydrogenase complex and therefore has affinity for the three different types of active site with which it interacts. This affinity could provide the basis for the putative broadening of n.m.r. signals from a structured part of the lipoyl domain. In support of this possibility, we note that free lipoic acid is a substrate for the E3 component, but not the E1, component in the pyruvate dehydrogenase complex, whereas it has been reported that the lipoic acid residue in the purified lipoyl domain will serve as a substrate for the pyruvate decarboxylase (E1) of the ox heart complex (Bleile *et al.*, 1981). Thus there may be additional structural features of the lipoyl domain beyond the immediate vicinity of the lipoyl-lysine residues that mediate reaction of the lipoyl group with the E1 active site, already envisaged as some form of protein-protein interaction (Perham *et al.*, 1981).

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