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Recognition of the Lipoyl Domain is the Ultimate Determinant of Substrate Channelling in the Pyruvate Dehydrogenase Multienzyme Complex

D. Dafydd Jones, Katherine M. Stott, Pedro A. Reche and Richard N. Perham*

Cambridge Centre for Molecular Recognition Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge CB2 1GA, UK

Reductive acetylation of the lipoyl domain (E2plip) of the dihydrolipoyl acetyltransferase component of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli* is catalysed specifically by its partner pyruvate decarboxylase (E1p), and no productive interaction occurs with the analogous 2-oxoglutarate decarboxylase (E1o) of the 2-oxoglutarate dehydrogenase complex. Residues in the lipoyl-lysine β-turn region of the unlipoylated E2plip domain ($E2plip^{apo}$) undergo significant changes in both chemical shift and transverse relaxation time (T_2) in the presence of E1p but not E1o. Residue Gly11, in a prominent surface loop between β-strands 1 and 2 in the E2plip domain, was also observed to undergo a significant change in chemical shift. Addition of pyruvate to the mixture of $E2plip^{apo}$ and E1p caused larger changes in chemical shift and the appearance of multiple cross-peaks for certain residues, suggesting that the domain was experiencing more than one type of interaction. Residues in both β-strands 4 and 5, together with those in the prominent surface loop and the following β-strand 2, appeared to be interacting with E1p, as did a small patch of residues centred around Glu31. The values of T_2 across the polypeptide chain backbone were also lower than in the presence of E1p alone, suggesting that $E2plip^{apo}$ binds more tightly after the addition of pyruvate. The lipoylated domain ($E2plip^{holo}$) also exhibited significant changes in chemical shift and decreases in the overall T_2 relaxation times in the presence of E1p, the residues principally affected being restricted to the half of the domain that contains the lipoyl-lysine (Lys41) residue. In addition, small chemical shift changes and a general drop in T_2 times in the presence of E1o were observed, indicating that $E2plip^{holo}$ can interact, weakly but non-productively, with E1o. It is evident that recognition of the protein domain is the ultimate determinant of whether reductive acetylation of the lipoyl group occurs, and that this is ensured by a mosaic of interactions with the Elp.

Keywords: pyruvate dehydrogenase complex; lipoyl domain; lipoylation; protein-protein interaction

**Corresponding author*

Present addresses: D. D. Jones, MRC Centre for Protein Engineering, Hills Road, Cambridge, CB2 2QH, UK; P. A. Reche, DNAX Research Institute, 901 California Ave, Palo Alto, CA 94304-1104, USA.

Abbreviations used: E1o, 2-oxoglutarate decarboxylase; E1p, pyruvate decarboxylase; E2p, dihydrolipoyl acetyltransferase; E2plip, innermost lipoyl domain of *E. coli* E2p; $E2plip^{apo}$, unlipoylated domain; $E2plip^{holo}$, lipoylated domain; HSQC, heteronuclear single quantum correlation; LplA, lipoyl protein ligase A; NMR, nuclear magnetic resonance; PDH, pyruvate dehydrogenase; T_2, transverse relaxation time; OGDH, 2-oxoglutarate; BCDH, branched-chain 2-oxo acid; BSA, bovine serum albumin.

E-mail address of the corresponding author: r.n.perham@bioc.cam.ac.uk

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Introduction

The pyruvate dehydrogenase (PDH) complex catalyses the oxidative decarboxylation of pyruvate, transferring the resultant acetyl group to coenzyme A. It belongs to a family of related 2-oxo acid dehydrogenase complexes, which includes the 2-oxoglutarate (OGDH) and branched-chain 2-oxo acid (BCDH) dehydrogenase complexes. In the PDH complex, the three component enzymes are pyruvate decarboxylase (E1p; EC 1.2.4.1), dihydrolipoyl acetyltransferase (E2p; EC 2.3.1.12) and dihydrolipoyl dehydrogenase (E3; EC 1.8.1.4) (reviewed by Perham, 1991, 2000; de Kok *et al*., 1998). The pyruvate decarboxylase catalyses the initial decarboxylation of the 2-oxo acid, using thiamin diphosphate (ThDP) as a cofactor, and the subsequent reductive acetylation of a lipoyl group bound to the E2p. E2p catalyses the transfer of the acetyl group to CoA, and E3 concludes the process by reoxidizing the dihydrolipoyl group to regenerate the dithiolane ring, with NAD^+ as the final electron acceptor. In the PDH complex from *Escherichia coli* and most Gram-negative bacteria, E2p forms a cubic core consisting of 24 polypeptide chains arranged with octahedral symmetry, whereas in *Bacillus stearothermophilus* and most Gram-positive bacteria, the E2p core is icosahedral and comprises 60 E2p chains (Reed & Hackert 1990; Perham, 1991). E1p and E3 are bound tightly but non-covalently around the core. The OGDH and BCDH complexes follow the same structural pattern.

The lipoyl group is covalently attached in an amide linkage to the N^6-amino group of a specific lysine residue of an independently folded domain that forms the N-terminal part of the E2p chain. In the E2p chain of *E. coli* there are three such lipoyl domains, whereas in that of *B. stearothermophilus* and the E2o chain of *E. coli* there is only one (Reed & Hackert, 1990; Perham 1991). This lipoyl domain plays a vital role in coupling the reactions within the complex in an organized and specific manner (Perham, 1991, 2000; de Kok *et al*., 1998). Acting as a ''swinging arm'' (Reed, 1974), the lipoyl group visits each of the three active sites of the complex, carried by a mobile lipoyl domain that protrudes out from the inner core of the complex.

The nuclear magnetic resonance (NMR) solution structures of the single *E. coli* E2o (Ricaud *et al*., 1996) and the innermost of the three *E. coli* E2p (Jones *et al*., 2000a) lipoyl domains have been solved, as have those of others from various other organisms (Dardel *et al*., 1993; Berg *et al*., 1996, 1997; Howard *et al*., 1998). Their overall backbone structure is virtually identical, comprising a β-barrel formed from two four-stranded β-sheets. The β-sheets have a 2-fold axis of quasi-symmetry, with the lipoyl-lysine residing at the tip of a tight, type I β-turn. Positioning of the target lysine at its exact location within the β-turn is essential for correct post-translational modification (Wallis & Perham, 1994). A prominent surface loop, linking β-strands 1 and 2, is only present in one of the β-sheets and lies close in space to the lipoyl-lysine β-turn. This loop is important for the structure, post-translational modification and catalytic function of the domain (Wallis *et al*., 1996; Jones *et al*., 2000 a,b).

Attachment of the lipoic acid to the lipoyl domain occurs in two ways (Morris *et al*., 1994; Green *et al*., 1995; Jordan & Cronan, 1997a). In the reaction catalysed by lipoyl protein ligase A (LplA), activation of the carboxyl group of lipoic acid by reaction with ATP to form lipoyl-AMP is followed by transfer of the lipoyl group to the target lysine residue and the release of AMP. An alternative ATP-independent reaction is thought to involve lipoic acid attached to the acyl carrier protein of fatty acid synthesis as the activated intermediate (Jordan & Cronan, 1997a,b). Lipoyl domains from a wide range of organisms and 2-oxo acid dehydrogenase complexes, together with the H-protein from the glycine cleavage system, can be lipoylated by the *E. coli* lipoylating machinery (Dardel *et al*., 1991; Quinn *et al*., 1993; Berg *et al*., 1994, 1995; Wallis & Perham, 1994; Macherel *et al*., 1996), but the lipoyl domain of the bovine BCDH complex is an exception (Griffin *et al*., 1990).

With the *E. coli* PDH complex, free lipoate can act as the substrate for E2p and E3 but is a very poor substrate for E1p. However, the E2p lipoyl domain is an excellent substrate (k_{cat}/K_m raised by a factor of 10^4). Moreover, the E2p and E2o lipoyl domains from *E. coli* (Graham *et al*., 1989; Jones *et al*., 2000b) function as substrates only for their cognate E1 s. Similar results have been reported for the E2p and E2o lipoyl domains of *Azotobacter vinelandii* (Berg *et al*., 1998). Thus, the lipoyl domain provides an elegant mechanism for substrate channelling such that reductive acylation is confined to a lipoyl group covalently attached to a specific lysine residue of the intended E2 component (Perham, 1991, 2000; de Kok *et al*., 1998). The question arises as to how the *E. coli* E2p and E2o lipoyl domains display such distinct activities towards their cognate E1, yet possess an identical structural scaffold. The binding of the lipoyl domain to *E. coli* E1p is weak (K_s not less than 1 mM, despite a K_m of ca 20 μM) and transient (Graham & Perham, 1990). Thus co-crystallization of the lipoyl domain with E1 is likely to be impossible. However, NMR has been used to identify regions on the apo-form of the *B. stearothermophilus* E2p lipoyl domain that undergo an interaction with E1p. Alterations in both chemical shift (Wallis *et al*., 1996) and transverse (T_2) relaxation times (Howard *et al*., 2000) have been utilized for this purpose. It is surprisingly that the backbone amide of the lysine to which the lipoic acid is attached does not appear to undergo an interaction with E1p.

The quaternary structures of the *E. coli* (α_2) and *B. stearothermophilus* ($\alpha_2\beta_2$) E1p differ, and therefore so may their interactions with the lipoyl domain. The crystal structure of an $\alpha_2\beta_2$ E1p from *Pseudomo-*

nas putida has recently been solved (Ævarsson *et al*., 1999). The ThDP in the active site is buried at the bottom of a 20 Å deep funnel-shaped hole at the interface between the α and β subunits; in order for the lipoyl group to reach the cofactor site, assuming that no major conformational changes ensue, the lipoyl domain has to come into close contact with the Elp surface and the lipoyl-lysine side-chain must become fully extended. Here, we use NMR to investigate regions of the innermost lipoyl domain of *E. coli* E2p that interact with the *E. coli* α_2 E1p. We analyse the interaction of the holo-domain (E2plip^{holo}) and the apo-domain (E2plip^{apo}) with Elp, and the catalytically unproductive interaction of the E2plip^{apo} and E2plip^{holo} domains with *E. coli* Elo. It has been reported that the α_2 E1p from *A. vinelandii* is more resistant to proteolysis in the presence of pyruvate (Hengeveld *et al*., 1997) and that pyruvate decreases ThDP dissociation from *E. coli* Elp by a factor of 10 (Hennig *et al*., 1997), suggesting a conformational change in Elp in the presence of substrate. We have therefore also measured chemical shift and T_2 changes after the addition of pyruvate to the mixture of *E. coli* E2plip^{apo} and E1p.

Results

Interaction of E2plip^{apo} with E1p

Accompanying a protein-protein interaction, chemical shifts of nuclei are altered as a result of a change in the local electronic environment that differentially shields nuclei from the static magnetic field. Any conformational change that arises from complex formation can further perturb the chemical shift. When the protein-ligand interaction is weak (fast exchange), as with E1p and the lipoyl domain, a weighted ''average'' chemical shift is seen rather than two distinct shifts corresponding to the bound and unbound forms. In addition, the resonances derived from the bound form of the domain would be too broad to detect, given the high molecular mass of E1 (200 kDa per dimer).

Howard *et al*. (2000) have shown that it is also possible to use backbone ^{15}N T_2 experiments to analyse interactions of the *B. stearothermophilus* lipoyl domain with its E1p. Matsuo *et al*. (1999) have used the same method to analyse the interaction between components of the *Methylococcus capsulatus* methane mono-oxygenase system. The rationale behind their approach is based on the observation that the two interacting components are in fast exchange with respect to chemical shift, but slow exchange with respect to T_2. This is clearly the case in the system described here, where titration with the Elp component leads to a progressive change in chemical shifts but all backbone resonances remain observable. Under these conditions the observed T_2 is modified according to the equation (Dwek, 1973; Jardetsky & Roberts, 1981; Lian & Roberts, 1993):

$$^1/T_{2\,\text{interaction}} = {}^1/T_{2\,\text{free}} + {}^1/t_{\text{lip}} \qquad (1)$$

where $T_{2\ free}$ is the T_2 for the lipoyl domain alone, $T_{2\ interaction}$ is the measured T_2 in the presence of the second component, and t_{lip} is the lifetime of the lipoyl domain in the free state. From this, it would be expected that all T_2 values would be reduced to some extent, as governed by t_{lip}. However, certain residues may undergo a reduction in T_2 greater than that experienced by the bulk backbone. This can be attributed to an additional exchange contribution, since the residues which directly contact E1 experience larger chemical shift changes on binding than the rest of the backbone. The applicability of equation (1) to this and similar systems is discussed in full by Howard *et al*. (2000). In order to relate the T_2 values measured for the free E2plip domain to those measured in the presence of E1, the T_2 ratio was calculated for each residue:

$$T_2 \text{ ratio} = T_{2\,E2plip}/T_{2\,E2plip+E1} \qquad (2)$$

The changes in chemical shift observed in the presence of E1p were very small (Figure 1). The largest changes were centred around the lipoyl-lysine β-turn region. With respect to ^1H nuclei, Ala42, Ser43 and, to a lesser extent, Gly11, underwent a significant Δδ. With respect to ^{15}N nuclei, Lys41 (the target for lipoylation) and, to a lesser extent, Asp40, Ser43, Met44, Glu45 and Val46 underwent a significant Δδ. The largest Δδ for the ^1H and ^{15}N nuclei were 0.013 ppm (Ala42) and 0.068 ppm (Lys41), respectively.

In the presence of either E1o or bovine serum albumin (BSA), the resonances associated with E2plip^{apo} did not appear to undergo any significant change in chemical shift (data not shown). This strongly suggests that the chemical shift changes observed in the presence of E1p are significant and not due simply to adventitious changes (in pH, non-specific interactions, etc.) on addition of the second component.

The E2plip^{apo} T_2 values also indicate that the lipoyl-lysine β-turn region interacts with E1p (Figure 1(c)). In general, the T_2 values decreased in the presence of E1p, consistent with a global line broadening that accompanies formation of a transient complex. Over and above this general trend, five residues underwent a significant change due to secondary effects: Lys41, Ala42, Ser43 and, to a lesser extent, Asp40 and Met44. In the presence of either E1o or BSA, T_2 values for E2plip^{apo} did not show any significant change (Figure 1(d) and (e)). There was a slight decrease in the overall T_2 values in the presence of E1o, but this could be attributed to an increase in viscosity.

The effect of 2-oxo acid on the interaction of E2plip^{apo} with E1p

Addition of the substrate, pyruvate, had a noticeable effect on the interaction of E2plip^{apo} with E1p (Figure 2). Not only did many residues

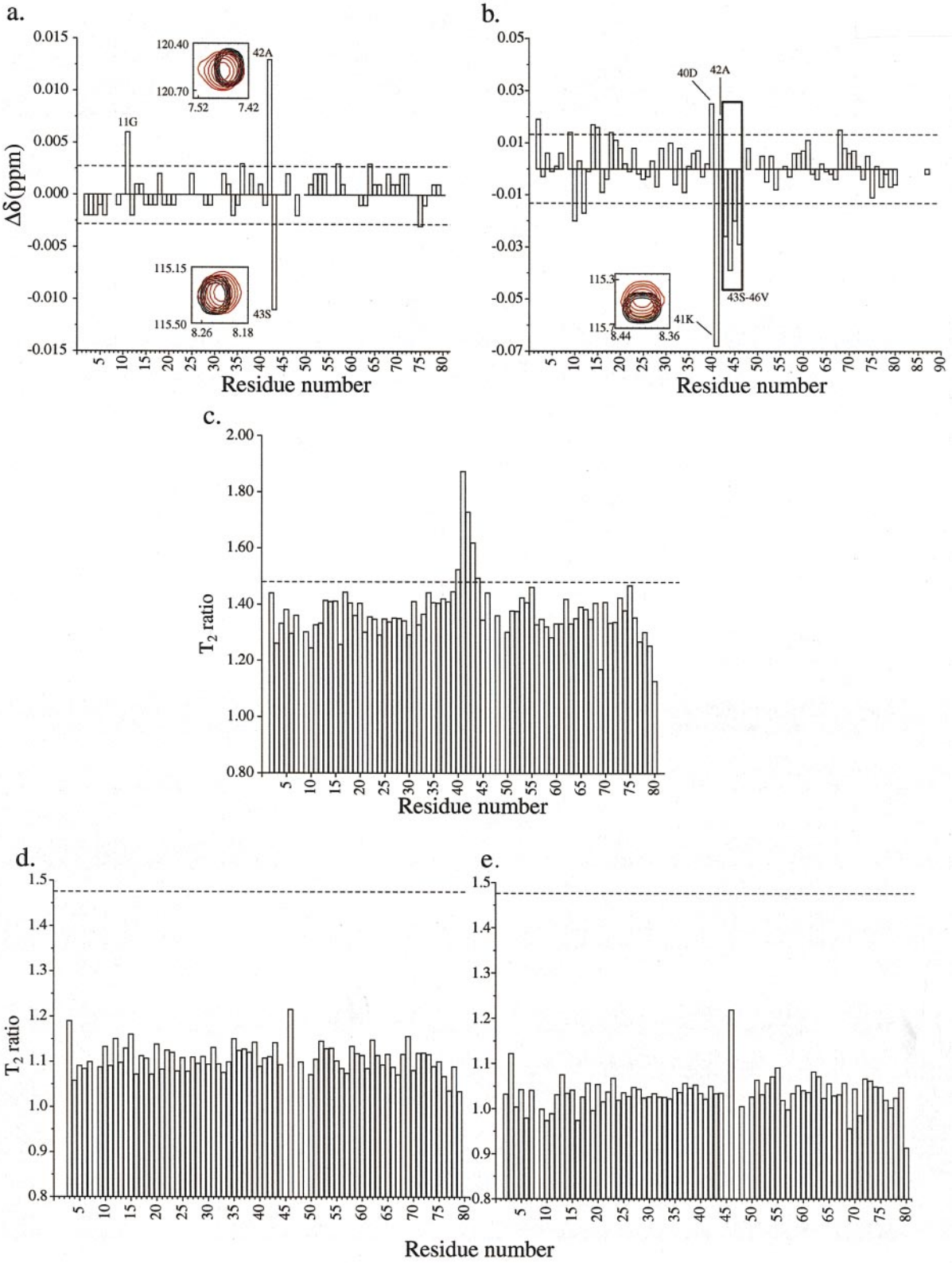

Figure 1. The interaction of E2plip^{apo} with E1p and E1o. (a) change in ^{1}H chemical shift and (b) change in ^{15}N chemical shift for E2plip^{apo} (0.6 mM) in the presence of E1p (0.1 mM). $\Delta\delta$ was calculated by subtracting the E2plip^{apo}:E1p chemical shift from the free E2plip^{apo} chemical shift. Also shown are the resonances for the E2plip^{apo} (black) and E2plip^{apo}:E1p (red) for residues Lys41, Ala42 and Ser43. The effects of (c) E1p, (d) E1o and (e) BSA on the T_2 times of the E2lip^{apo} domain are also indicated. The T_2 ratio was calculated (from equation (2)) as described in Materials and Methods. The broken lines represent the threshold values calculated as described in Materials and Methods.

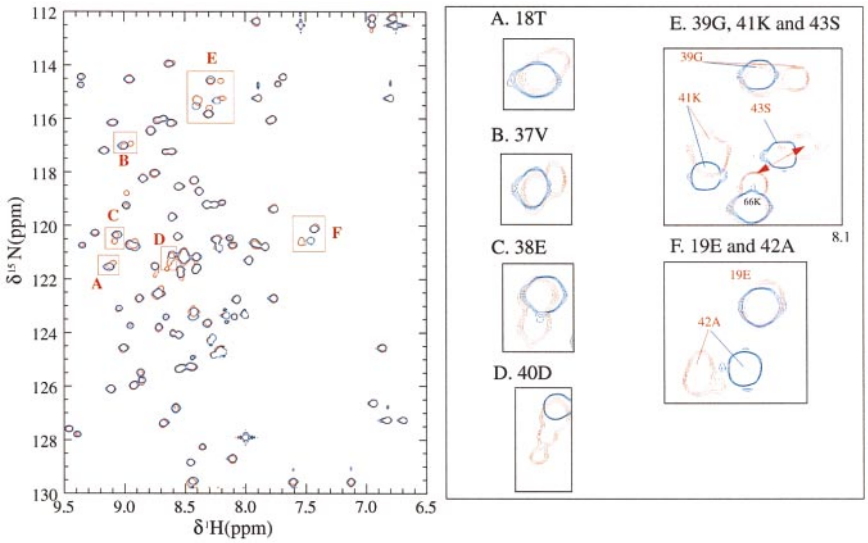

Figure 2. The effect of pyruvate on the interaction of E2plip^{apo} with E1p. The HSQC spectrum in blue represents that of the free E2plip^{apo} domain and the spectrum in red represents that recorded in the presence of E1p and pyruvate. The right-hand panel magnifies several cross-peaks indicated in the left-hand panel. For further details, see the text.

undergo a large change in chemical shift, far greater than seen in the presence of E1p alone, but many residues also displayed multiple resonances. Residues Thr18, Val37, Glu38, Gly39, Asp40, Lys41, Ala42 and Ser43 showed the highest degree of multiplicity (Figure 2). This could be explained by a conformational change in the lipoyl domain taking place on binding to Elp in the presence of substrate, with the conformers in slow exchange. Alternatively, and probably more likely, a conformational change in Elp itself might be responsible. On the addition of more E1p (final concentration 0.2 mM), the intensities of all the peaks diminished, indicating that further line broadening had occurred (data not shown). If the E1p-E2plip^{apo} complex was in slow exchange with E1p, increasing the E1p concentration would reduce the time the domain spends in its free state, resulting in the observed increase in line broadening.

With respect to the ^{1}H nucleus, Thr18, Glu31, Val37, Gly39, Ala42 and Ser43 exhibited a significant $\Delta\delta$ (Figure 3(a)); and with respect to the ^{15}N nucleus, Thr18, Val28, Glu38, Asp40, Lys41, Ser43, Met44, Val46, Ala48 and Lys58 showed a significant $\Delta\delta$ (Figure 3(b)). Most of these residues undergoing significant changes in chemical shift are centred around the lipoyl-lysine β-turn, except for Thr18, Val28, Glu31, Ala48 and Lys58.

The E2plip^{apo} domain was found to exhibit no changes in chemical shift in the presence of pyruvate alone. Likewise, E2plip^{apo} in the presence of pyruvate and Elo showed no significant changes in chemical shift (data not shown). The addition of 2-oxoglutarate, the natural substrate for E1o, caused a slight change in chemical shift for three residues (Gly12, Glu16, Ala29), but the changes were much smaller than those observed in the presence of Elp and pyruvate (Figure 3) and the multiple resonances for specific residues (Figure 2) were not observed. Thus, the presence of 2-oxoglutarate may promote an interaction of E2plip^{apo} with E1o, but any such interaction is very weak and is certainly non-productive catalytically (Graham *et al*., 1989; Jones *et al*., 2000b).

As T_2 values are calculated using resonance intensity, the presence of multiple cross-peaks for particular residues in the presence of Elp and pyruvate will influence their T_2 values. For those that could be measured, some of the T_2 values remained close in value (e.g. for Thr18, Glu38), but those of Val37 (109 ms and 87 ms), Gly39 (112 ms and 55 ms), Asp40 (81 ms and 109 ms) and Ser43 (127 ms and 70 ms) differed depending on which resonance intensity was used to calculate the T_2. Nevertheless, the T_2 values for E2plip^{apo} calculated over the whole backbone were generally lower in the presence of Elp and pyruvate, indicating that the substrate was promoting an interaction (Figure 4(a)). As Figure 4 shows, the residues most affected could not be easily identified as the trend does not appear to be smooth; but Leu34, Asp40, Lys41, Ala42, Ser43 and Met48 appeared to undergo a larger change than the majority. Although Val7 and Ala48 are above the set threshold, in the HSQC spectrum of E2plip^{apo} alone, the cross-peaks overlay, thereby making the calculated T_2 inaccurate, and so these changes were ignored.

In the presence of both E1o and 2-oxoglutarate, the E2plip^{apo} domain exhibited no significant changes in T_2 (Figure 4), as noted previously with E1o alone (Figure 1). This again would suggest that there is no interaction between E2plip^{apo} and E1o.

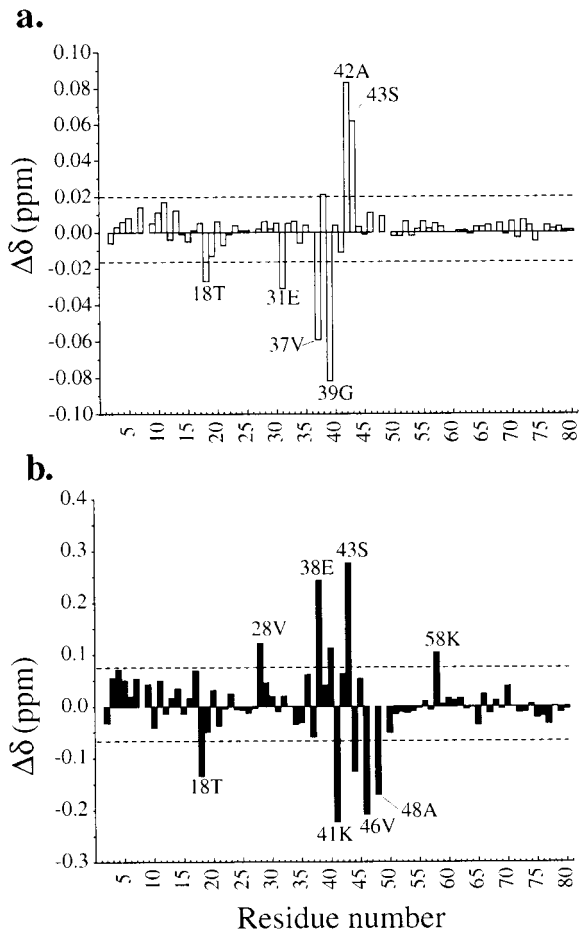

Figure 3. The effect of pyruvate on the interaction of E2plip^{apo} with E1p. The changes in backbone (a) $^{1}H^{N}$ and (b) ^{15}N chemical shift are shown. $\Delta\delta$ was calculated by subtracting the E2plip^{apo}:E1p chemical shift from the free E2plip^{apo} chemical shift. The broken lines represent the threshold values calculated as described in Materials and Methods. For further details, see the text.

The interaction of E2plip^{holo} with E1p

In the intact complex, the lipoyl domain has to be lipoylated to participate in catalysis and the natural substrate for reductive acetylation by E1p is the E2plip^{holo} domain. Lipoylation causes significant changes in chemical shift and the backbone dynamics of residues in the lipoyl-lysine β-turn, implying some potentially important interactions between the domain and the lipoyl-lysine sidechain (Jones *et al.*, 2000a).

In the presence of Elp, the changes observed in chemical shift were greater for the E2plip^{holo} (Figure 5) than for the E2plip^{apo} (Figure 1) domain. The largest changes were observed for residues in the lipoyl-lysine β-turn region, most notably Asp40, Lys41, Ala42, Ser43, Met44, Val46 and, to a lesser extent, Thr36 and Glu38. In addition, residues in the surface loop connecting β-strands 1 and 2 (Ile10, Gly11, Gly12 and Glu14) also underwent small yet significant changes in chemical shift. This is not entirely surprising, as the surface loop is close in space to the lipoyl-lysine β-turn and has been implicated as a contact region with Elp in previous studies (Wallis *et al.*, 1996; Jones *et al.*, 2000b). The chemical shifts of residues in and surrounding β-strand 7 (Gly62, Lys64 and Gly68) were also slightly perturbed. β-strand 7 lies parallel with β-strand 2 and the surface loop mentioned above. Also visible is the lipoyl-lysine amide proton (Lys41$^{\zeta}$; Lys-(H)^{15}N-C(O)-lip). The change in chemical shift was not as large as expected: 0.001 and 0.03 ppm for ^{1}H and ^{15}N, respectively, compared with 0.005 and 0.08 ppm for the Lys41 backbone amide.

The T_2 values for the E2plip^{holo} domain in the presence of E1p generally decreased in the presence of E1p (Figure 6). The overall decrease was greater than for the E2plip^{apo} domain, where the majority of T_2 ratios ranged between 1.2 and 1.4 (Figure 1), compared with 1.6 to 1.8 for E2plip^{holo}. This would indicate that the interaction with Elp is significantly stronger when the domain is lipoylated. Only six residues exhibited a change in T_2 above the calculated threshold: Glu16, Lys41, and to a greater extent, Gly39, Asp40, Ala42 and Ser43. All these residues lie in the lipoyl-lysine β-turn region, apart from Glu16 which is at the N-terminal end of β-strand 2, close in space to the lipoyl-lysine β-turn. Although the ratio suggests that the T_2 of Lys41$^{\zeta}$ has not changed significantly, the absolute change is quite high (376 ms for the free E2plip^{holo} and 226 ms in the presence of E1p i.e. $\Delta T_2 = 150$ ms).

The interaction of E2plip^{holo} with E1o and BSA

The lipoyl group is an essential part of a functional lipoyl domain. Contrary to what was observed for E2plip^{apo}, the E2plip^{holo} domain does appear to interact weakly with E1o. Thus, small but significant changes in chemical shift were observed in the presence of E1o (Figure 5), the most notable involving Ala42 and also Lys41$^{\zeta}$ (not shown). To confirm the significance of these changes, the same experiments were run with BSA replacing Elo; apart from the $\Delta\delta$ in the ^{15}N dimension for Ser43, all the other changes were judged to be significant.

There is a general drop in T_2 values, by a factor of ca 1.5, for the E2plip^{holo} domain in the presence of E1o (Figure 6). This is significantly greater than that observed for E2lip^{apo} in the presence of E1o (Figure 1(d)). No one residue appears to be affected to any extent greater than that of the global change, suggesting that the interaction with Elo may be restricted to the lipoyl group. The T_2 ratio of Lys41$^{\zeta}$ indicates that although it is the closest bonded amide to the dithiolane ring of the lipoyl group, it does not undergo any significant change. Nonetheless, the absolute change ($\Delta T_2 = 139$ ms) is quite high.

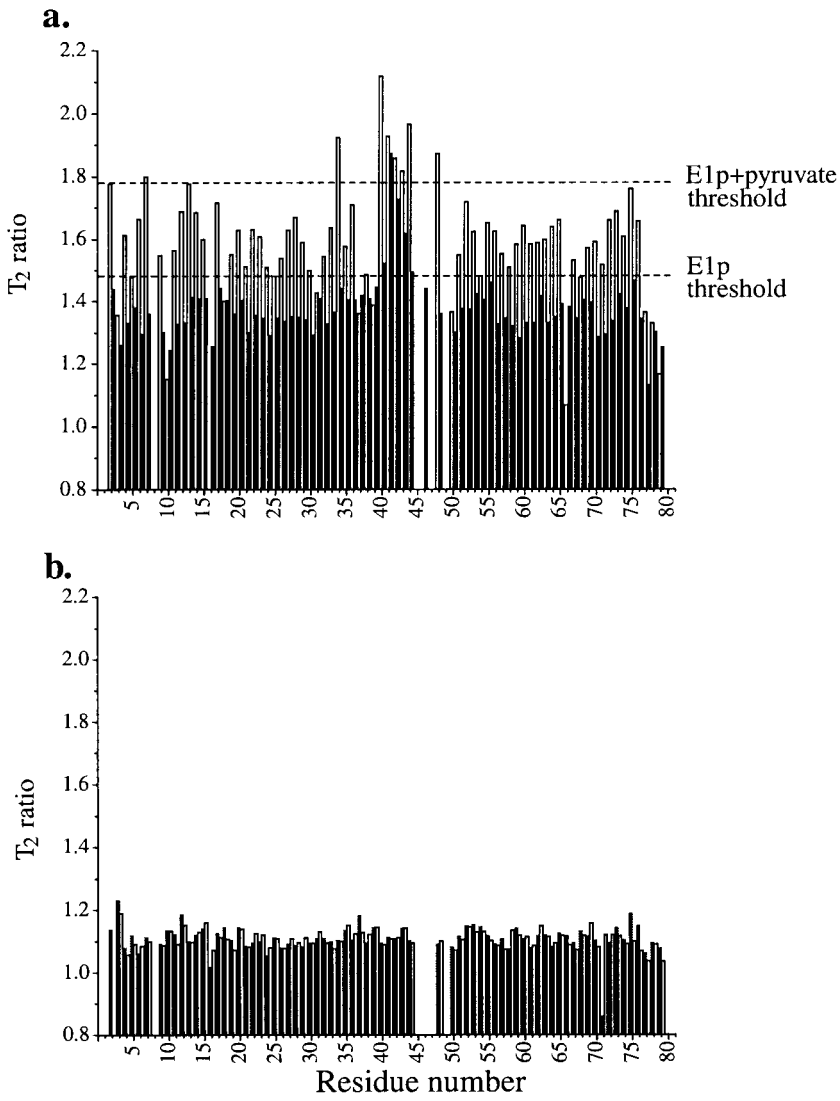

Figure 4. The effect of 2-oxo acid substrate on the T_2 times of E2plip^{apo} in the presence of Elp or Elo. (a) Comparison of T_2 ratios in the presence of E1p alone (filled) and E1p plus pyruvate (open). The broken lines are the threshold values calculated as described in Materials and Methods. (b) Comparison of the T_2 ratios of in the presence of E1o (open bars) or E1o plus 2-oxoglutarate (filled bars). For further details, see the text.

To test whether the T_2 data obtained for E2plip^{holo} in the presence of E1o were attributable to a specific interaction, BSA was again used in place of Elo. No significant interaction between E2lip^{holo} and BSA could be detected, as judged by the lack of change in the T_2 values.

Mapping the interaction sites on the surface of E2plip

For the E2plip^{apo} domain, both the chemical shift and T_2 data suggest that the main region undergoing an interaction with E1p is the lipoyl-lysine β-turn region and residues predominantly in the succeeding β-strand 5 (Figure 7). The other residue thought to be involved in the interaction is Gly11, which lies close in space to the lipoyl-lysine β-turn in the surface loop linking β-strands 1 and 2. Neither E1o nor BSA showed any evidence of an interaction with E2plip^{apo}.

In the presence of E1p and pyruvate, the interaction also appeared to be centred on the lipoyl-lysine region, with residues in both β-strands 4 and 5 of E2plip^{apo} now involved. The chemical shift data also identify a small patch of residues that may participate in the interaction: Ala48, Val28 and Glu31. All these residues lie close in space to each other (Figure 7). The chemical shift data also imply that Thr18, another nearby residue in β-sheet 2, and Lys58, on the opposite face of the protein, may interact with E1p. The T_2 data additionally indicate Asp13 in the surface loop as undergoing an interaction (Figure 7).

The interaction of the E2plip^{holo} domain with E1p is focused on the half of the protein containing the lipoyl-lysine residue (Figure 8). Residues in the lipoyl-lysine β-turn and its constituent β-strands 4 and 5, together with residues in the nearby surface loop, in β-strand 2 and, to a lesser extent, in and adjacent to β-strand 7, all undergo a change in chemical shift. The T_2 data mirror the chemical shifts, except that the interactions appear to be restricted to the lipoyl-lysine β-turn region and Glu16 in β-strand 2.

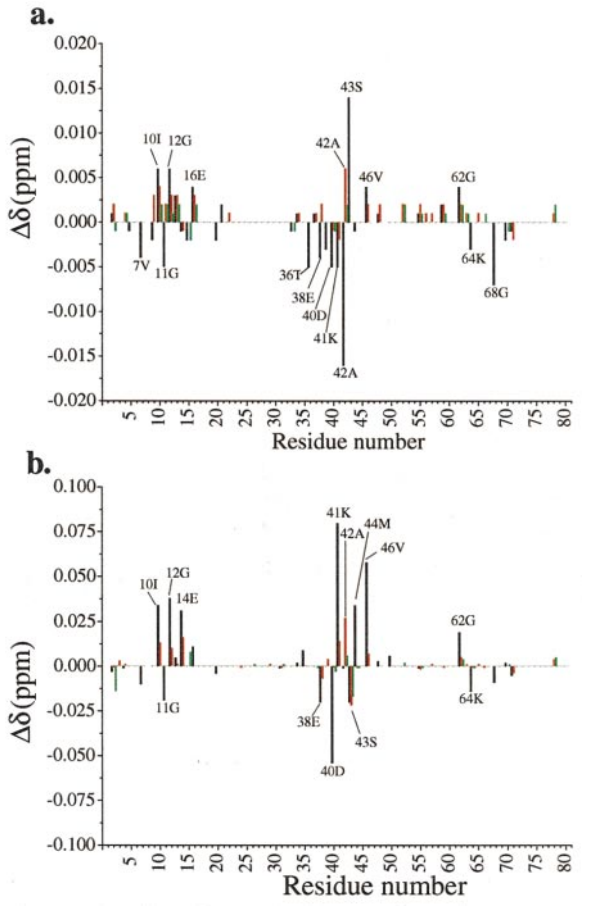

Figure 5. The effect of Elp, Elo and BSA on the chemical shifts of E2plip^{holo}. E1p (black), E1o (red), BSA (green). Shown above is the change in (a) ^{1}H and (b) ^{15}N chemical shift. $\Delta\delta$ was calculated by subtracting the E2plip^{holo}:E1p chemical shift from the free E2plip^{holo} chemical shift. For further details, see the text.

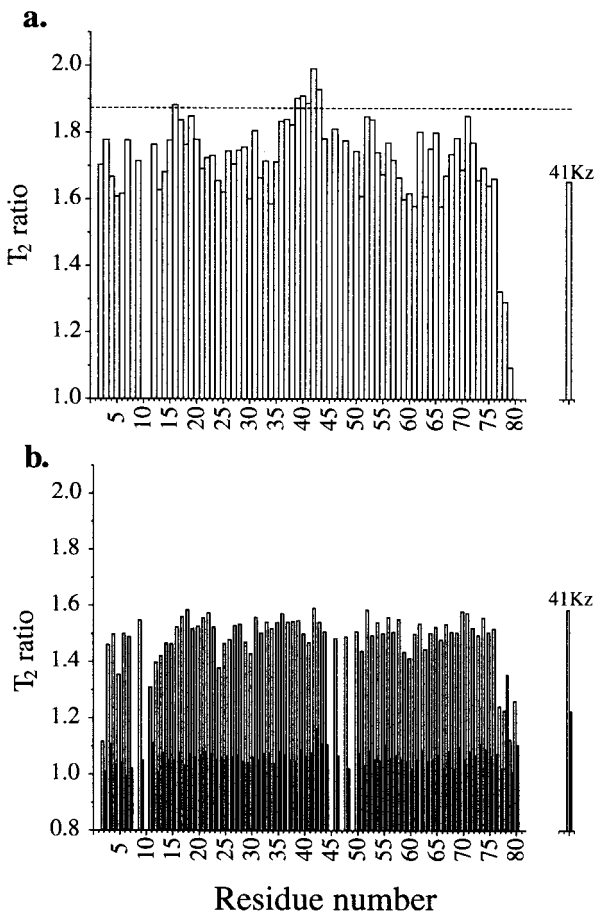

Figure 6. The effect of the interaction of E1p, E1o and BSA on the E2plip^{holo} T_2 times. (a) E1p; (b) Elo (unfilled) and BSA (black). The T_2 ratio was calculated (from equation (2)) as described in Materials and Methods. The broken line represents the threshold value calculated as described in Materials and Methods. For further details, see the text.

Discussion

Previous NMR studies to examine the interaction of the apo-lipoyl domain of *B. stearothermophilus* E2p with its cognate $\alpha_2\beta_2$ E1p indicated various regions of the domain as being involved (Wallis *et al.*, 1996; Howard *et al.*, 2000). The surface loop linking β-strands 1 and 2 (Figure 7) was thought to be of particular importance, but there was no indication that the lysine residue that would carry the lipoyl group (Lys41 in the *B. stearothermophilus* domain) underwent an interaction with E1p. These experiments did not involve the lipoylated form of the domain, the natural substrate of E1, as the lipoylated domain appeared to bind too tightly to the $\alpha_2\beta_2$ E1p, causing extensive line broadening (Wallis *et al.*, 1996).

Here, the *E. coli* E1p has the α_2 quaternary structure. In this instance, our results indicate that the lipoyl-lysine and adjacent residues in the lipoyl domain are consistently involved in the interaction with E1p. The prominent surface loop linking β-strands 1 and 2 also seems to have a role to play, as it does in other complexes (Wallis *et al.*, 1996; Berg *et al.*, 1998; Jones *et al.*, 2000b). However, in the *E. coli* E2plip^{apo} domain, only Gly11 in the loop undergoes what was classed as a significant change in chemical shift, and only the lipoyl-lysine region undergoes a T_2 change (Figure 1). Nonetheless, the overall backbone T_2 times drop from 147 ms to 102 ms in the presence of E1p, indicating that an E2plip^{apo}-E1p complex has transiently formed. From equation (1), the lifetime of the E2plip^{apo} spent in the uncomplexed state (t_{lip}) was estimated at about 375 ms. In contrast, in the presence of Elo or BSA, the average T_2 values for the structured region of the lipoyl domain remain close to the values for the free E2plip^{apo}, at about 132 ms and 140 ms, respectively.

The most notable effect on the E2plip^{apo}-E1p interaction was that observed on adding the substrate, pyruvate (Figure 2). Decarboxylation of

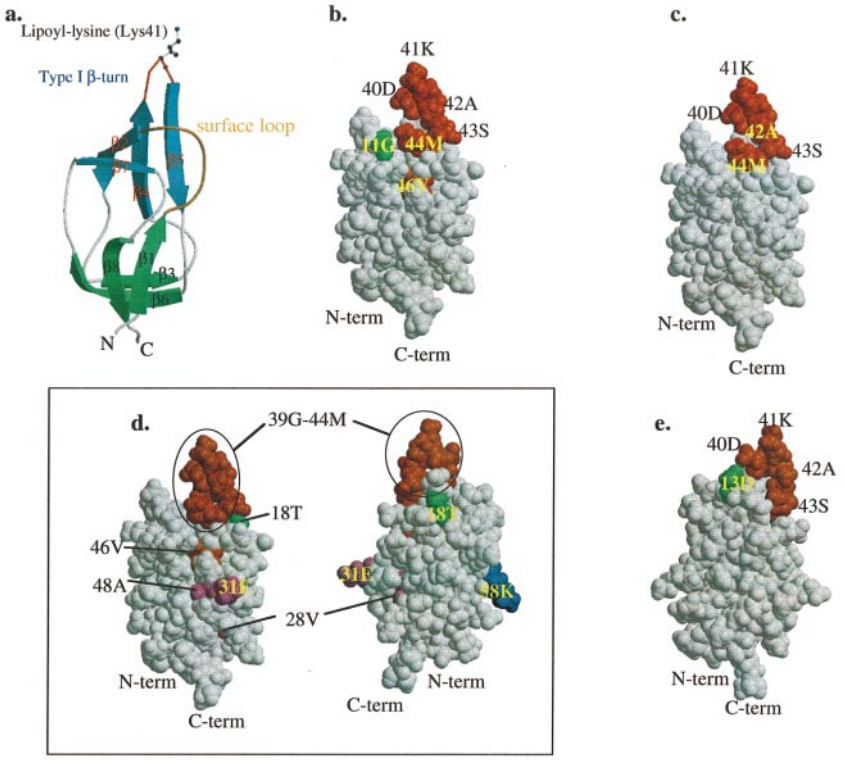

Figure 7. Points of contact between E2plip^{apo} and E1p. (a) Schematic representation of the E2plip^{apo} domain structure determined previously (Jones *et al.*, 2000a). Each β-strand is labelled, together with a prominent surface loop (orange) and the lipoyl-lysine (Lys41) β-turn (red). Residues exhibiting significant changes in (b) chemical shift and (c) T_2 values, in the presence of E1p. The effect of pyruvate on the (d) chemical shift and (e) T_2 values of E2plip^{apo} in the presence of E1p. The two orientations shown in (d) are related by a 90° rotation about the *z* axis. The diagram was produced using MOLSCRIPT (Kraulis, 1991).

pyruvate by E1p would lead to the formation of 2-(1-hydroxyethylidene)-ThDP and this, presumably through an effect on E1p structure, caused not only major changes in chemical shift for E2plip^{apo}, but also in the T_2 values (Figures 3 and 4). The crystal structure of an $\alpha_2\beta_2$ E1p from *P. putida* shows that the active (thiamin diphosphate-binding) site is well buried at the bottom of a 20 Å funnel-shaped cavity (Ævarsson *et al.*, 1999). Formation of 2-(1-hydroxyethylidene)-ThDP may induce a conformational change in Elp affecting access to this unusual active site.

In general, the measured T_2 values for the E2plip^{apo}-E1p interaction in the presence of pyruvate were lower than in the absence of pyruvate, the average T_2 over the backbone being 88 ms. The E2plip^{apo} domain is evidently binding more tightly and spending less time in the free state, with t_{lip} estimated at 237 ms. The analysis of chemical shifts implicated a small cluster of residues, Val28, Glu31

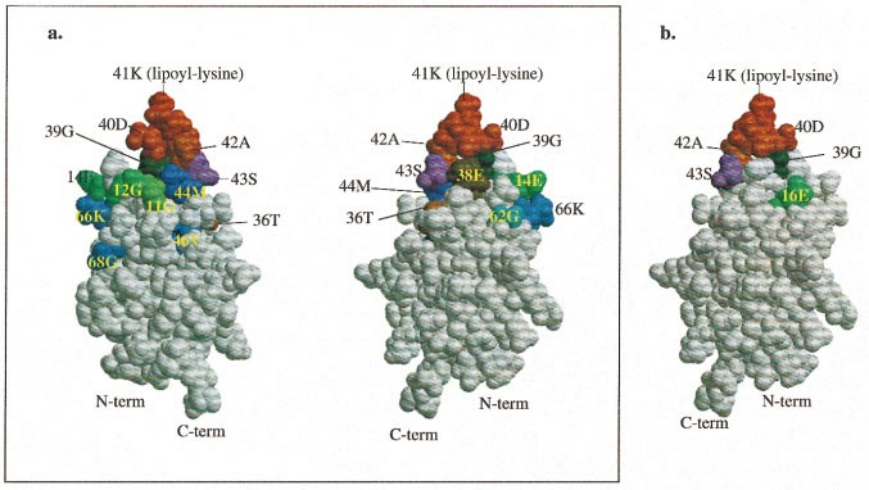

Figure 8. Points of contact between E2plip^{holo} and E1p. Residues exhibiting significant changes in (a) chemical shift and (b) T_2 values, in the presence of E1p. The two orientations shown in (a) are related by a 180° rotation about the *z* axis. The diagram was produced using MOLSCRIPT (Kraulis, 1991).

and Ala48, as being involved in the interaction with Elp in the presence of substrate, with the side-chain of Glu31 protruding out from the structure.

The problem with the chemical shift data, which in turn affects the calculation of the T_2 times, is the appearance of multiple cross-peaks on the addition of pyruvate (Figure 2). The reason for this is not known but multiple cross-peaks have also been observed when the lipoylated domain (E2plip^{holo}) is reductively acetylated (Jones *et al*., 2000a). One possible explanation is that the E2plip^{apo} domain undergoes a conformational change on binding to the substrate-loaded E1p and that this ''new'' conformation is in slow exchange with one or more other conformations. It is unlikely that one is observing the E1p-bound form of E2plip^{apo} in slow exchange with free E2plip^{apo}, as the size of the E2plip^{apo}-E1p complex would cause substantial line broadening. Another, more likely, explanation could be different conformations of the two subunits in E1p in the presence of ThDP and pyruvate (Hennig *et al*., 1997); if the two subunits of the Elp α_2 dimer have slightly different conformations, the multiple cross-peaks could be the result of E2plip^{apo} interacting with either one or other of the two active sites.

The number of residues undergoing a significant change in chemical shift in the presence of Elp was found to have increased for the E2plip^{holo} domain compared with E2plip^{apo}, and to be restricted to the lipoyl-lysine-containing half of the domain (Figures 7 and 8). An interesting feature of some of these residues, most notably Ile10, Gly12, Glu14, Thr36, Gly62 and Gly68, is that they were not affected by the presence or absence of pyruvate in the interaction of the E2plip^{apo} domain (Figures 1 and 3). What they have in common is that they also undergo a significant change in chemical shift upon lipoylation of the domain (Jones *et al*., 2000a). Thus, the chemical shift changes observed for these residues in the presence of E1p may simply reflect an interaction of the lipoyl group with E1p.

The lipoyl-lysine region is the only part of the E2p^{holo} domain that showed significant changes in both T_2 and chemical shift (Figure 8). The T_2 ratios for E2plip^{holo} had a larger background fluctuation, making interpretation difficult. Only six residues, Glu16, Gly39, Asp40, Lys41, Ala42 and Ser43, underwent a major change, although Lys41$^{\zeta}$ did undergo a large absolute change ($\Delta T_2 = 150$ ms). However, in the presence of E1p, the average T_2 values across the backbone were lower for E2plip^{holo} than E2plip^{apo} (82.0 ms and 102 ms, respectively). This implies that the E2plip-E1p interaction is tighter when the domain is lipoylated, as noted previously in studies of the *B. stearothermophilus* $\alpha_2\beta_2$ E1p-E2plip interaction (Wallis *et al*., 1996).

The results obtained for the interaction of E1o with E2plip^{holo} indicate that there is a small contribution to the recognition process from the lipoyl group itself. Thus, there were slight yet significant changes in chemical shift for E2plip^{holo} in the presence of E1o (Figure 5), involving residues mainly in the lipoyl-lysine β-turn region. The general decrease in the T_2 times, with an average of 94 ms, well below that observed (132 ms) for E2plip^{apo} in the presence of E1o (Figure 1) would imply the formation of a transient complex. The lifetime of the unbound state decreases from ca 2310 ms for E2plip^{apo} to ca 286 ms for E2plip^{holo}. However, given the lack of fluctuation in the background T_2 ratio (Figure 6), it would appear that the interaction is non-specific with respect to the protein. The most likely explanation is that the lipoyl-lysine residue but not the domain is recognised by E1o, though not in a catalytically productive way such that reductive acetylation can follow (Graham *et al*., 1989; Jones *et al*., 2000b). It is unfortunate that experiments with Elp in the presence of pyruvate could not be performed as catalysis would occur, resulting in reductive acetylation of E2plip^{holo}.

In general, it is now clear that the El components of 2-oxo acid dehydrogenase complexes can recognize the lipoyl group as such, but that specific recognition of the lipoyl domain to which it is attached is required for reductive acylation to ensue. The surface loop on the domain between β-strands 1 and 2, close in space to the lipoyl-lysine β-turn, is an important transient contact point between El and the lipoyl domain. However, a mosaic of other contacts distributed chiefly over the half of the domain that contains the lipoyl-lysine residue is required to ensure a productive encounter. The structural subtleties involved can now perhaps be further defined by reference to the structure of the El component (Ævarsson *et al*., 1999) and the pattern of interaction sites on the lipoyl domain disclosed by the NMR experiments, as above.

Materials and Methods

Sample preparation

The ^{15}N-labelled E2plip^{apo} domain was purified from over-expressing *E. coli* cells as described elsewhere (Dardel *et al*., 1991; Ricaud *et al*., 1996; Jones *et al*., 2000a). The E2plip^{holo} domain was generated *in vitro* using LplA, ATP-Mg and R-lipoate (Asta Pharmaceuticals) and its identity confirmed by positive-ion electrospray mass spectrometry, all as described elsewhere (Jones *et al*., 2000b). The domains were exchanged into 20 mM sodium phosphate buffer (pH 6.85).

Batches of 1 l of LB medium containing 100 μg/ml ampicillin were inoculated with a single colony of *E. coli* BL21(DE3) cells freshly transformed with the plasmid pETE1p, which carries a gene encoding the pyruvate decarboxylase (E1p) of the PDH complex from *E. coli* (P.A.R. & R.N.P., unpublished work). The cells were grown at 37 °C until an A_{600} of 0.6-0.8 was reached, and protein expression was induced with IPTG (final concentration of 0.6 mM) at 25 °C for 16 hours. Cells were harvested by centrifugation at 5000 *g* for ten minutes, resuspended (10 ml/g of wet cell pellet) in buffer A (20 mM sodium phosphate, 0.5 mM EDTA, 0.1 mM DTT, pH 7.0) containing 1 mM PMSF, disrupted in a French press (SLM-AMINCO), and the cell debris

removed by centrifugation (15,000 ***g*** for one hour at 4 °C). Recombinant E1p was fractionally precipitated from the cell-free extract with ammonium sulphate (20-60 % saturation), the precipitate was dissolved in buffer B (20 mM sodium phosphate, 0.5 mM EDTA, 0.1 mM DTT, 1 M $(NH_4)_2SO_4$, pH 7.0) (3 ml per mg of precipitated protein) and applied to a HiLoad™ 16/10 Phenyl Sepharose column HP (Pharmacia), previously equilibrated with buffer B. Recombinant E1p was eluted from the column by applying a linear gradient from buffer B to buffer A (200 ml) at a flow rate of 2.5 ml/min. Fractions containing E1p were pooled, dialysed against buffer C (20 mM Tris-HCl, 0.5 mM EDTA, 0.1 mM DTT, pH 7.0) and concentrated to 15 ml using a 50 kDa cut-off Ultrafree®-15 concentrator (Millipore) before being loaded onto a HiLoad™Q HR 16/10 column, previously equilibrated with buffer C. E1p was then eluted from the column by applying a linear gradient (120 ml) of 0-0.5 M NaCl in buffer C at a flow rate of 2.5 ml/minute. Fractions of interest were pooled, dialysed against 20 mM sodium phosphate buffer (pH 6.0), concentrated and stored at −80 °C.

Interaction studies

All samples contained the ^{15}N-labelled lipoyl domain at a concentration of 0.6 mM, ThDP-Mg at a molar ratio (E1:ThDP-Mg) of 1:2, 0.02 % (w/v) sodium azide, 10 % ^2H_2O and 18 mM sodium phosphate (pH 6.85). For the studies of the E2plip^{apo} interaction, E1p was present at a concentration of 0.1 mM or 0.2 mM, E1o at a concentration of 0.1 mM, and bovine serum albumin (BSA) at a concentration of 0.1 mM, as appropriate. Where applicable, the 2-oxo acid was added at a molar ratio (2-oxo acid:E1) of 2:1 and left at room temperature (circa 22 °C) for 30 minutes to allow oxidative decarboxylation to occur. For the E2plip^{holo} domain, E1p, E1o or BSA were present at a concentration of 0.1 mM. No 2-oxo acid was added. The total volume for all samples was 600 µl and the same buffer was used throughout.

NMR spectroscopy

Chemical shift analysis

Heteronuclear single quantum correlation (HSQC) spectra were recorded using 16 scans for each t_1 point and 256 increments at 298 K, with spectral widths of 6009.625 Hz (^1H) and 927.29 Hz (^{15}N). The assignment of the spectra has been described (Jones *et al*., 2000a). The chemical shifts were assigned using the program ANSIG3.3 (Kraulis, 1989).

T_2 measurements

The ^{15}N T_2 values were obtained as described by Jones *et al*. (2000a); ^{15}N T_2 data were obtained using delay values of 7.92, 300.96, 47.52, 142.56, 79.2, 205.92 and 15.84 ms, recorded in that order. The spectral width in the ^1H and ^{15}N dimensions were 10000 Hz and 1672.17 Hz, respectively. T_2 times were calculated as described by Jones *et al*. (2000a).

The threshold levels set for a change in chemical shift or in ^{15}N T_2 value due to secondary processes is the mean of all the chemical shift changes ($\Delta\delta$) or T_2 ratios plus one standard deviation (Howard *et al*., 2000).

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