Structure and selectivity in post-translational modification: attaching the biotinyl–lysine and lipoyl–lysine swinging arms in multifunctional enzymes

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The post-translational attachment of biotin and lipoic acid to specific lysine residues displayed in protruding β-turns in homologous biotinyl and lipoyl domains of their parent enzymes is catalysed by two different ligases. We have expressed in *Escherichia coli* a subgene encoding the biotinyl domain of *E.coli* acetyl-CoA carboxylase, and by a series of mutations converted the protein from the target for biotinylation to one for lipoylation, *in vivo* and *in vitro*. The biotinylating enzyme, biotinyl protein ligase (BPL), and the lipoylating enzyme, LplA, exhibited major differences in the recognition process. LplA accepted the highly conserved MKM motif that houses the target lysine residue in the biotinyl domain β-turn, but was responsive to structural cues in the flanking β-strands. BPL was much less sensitive to changes in these β-strands, but could not biotinylate a lysine residue placed in the DKA motif characteristic of the lipoyl domain β-turn. The presence of a further protruding thumb between the β2 and β3 strands in the wild-type biotinyl domain, which has no counterpart in the lipoyl domain, is sufficient to prevent aberrant lipoylation in *E.coli*. The structural basis of this discrimination contrasts with other forms of post-translational modification, where the sequence motif surrounding the target residue can be the principal determinant.

Keywords: biotin/biotinyl protein ligase/lipoic acid/lipoyl protein ligase/protein domains

Introduction

Biotin and lipoic acid are the covalently bound cofactors of several multicomponent enzyme complexes that catalyse key metabolic reactions. The lipoate and biotin moieties are attached in amide linkage through their carboxyl group and the N^6-amino group of a specific lysine residue in the relevant protein, thereby creating potential swinging arms capable of spanning some 3 nm. These mobile arms are required to shuttle catalytic intermediates between the successive active sites of the enzyme complexes. Biotin carries carboxy groups in the ATP-dependent carboxylases (Samols *et al.*, 1988; Knowles, 1989), whereas lipoic acid serves as an acyl group carrier in the ubiquitous 2-oxo acid dehydrogenase multienzyme complexes (Reed, 1974; Perham, 1991; Berg and de Kok, 1997) or as an aminomethyl group carrier in the glycine cleavage system (Fujiwara *et al.*, 1979; Kikuchi and Hiraga, 1982). In 2-oxo acid dehydrogenase complexes, the lipoyl group is attached to a lysine residue in one or more independently folded (lipoyl) domains of ~80 amino acid residues that constitute the N-terminal part of the dihydrolipoyl acyltransferase (E2) component (Reed and Hackert, 1990; Perham 1991; Berg and de Kok, 1997). The structure of the lipoyl domain from several different 2-oxo acid dehydrogenase complexes has been solved by nuclear magnetic resonance (NMR) spectroscopy (Dardel *et al.*, 1993; J.D.F.Green *et al.*, 1995; Berg *et al.*, 1996, 1997; Ricaud *et al.*, 1996; Howard *et al.*, 1998). It consists of a flattened 8-stranded β-barrel containing two 4-stranded anti-parallel β-sheets, with the N- and C-termini close together in one sheet and the lipoyl–lysine residue prominently displayed in a tight β-turn in the other (Figure 1). A similar structure occurs in the lipoylated H-protein (GCSH) of the glycine cleavage system (Brocklehurst and Perham, 1993; Pares *et al.*, 1994) but with the β7 strand missing, the exposed loop connecting the β1 and β2 strands of the lipoyl domain replaced by a helix, and an additional helix at the C-terminal end (Figure 1D).

Despite minimal conservation of amino acid sequence, biotinylated proteins also contain a domain similar in structure to the lipoyl domain (Brocklehurst and Perham, 1993). The structure of the biotinyl domain of the biotin carboxy carrier protein (BCCP) of acetyl-CoA carboxylase, the only biotinylated protein of *Escherichia coli*, has been established by X-ray crystallography (Athappilly and Hendrickson, 1995) and NMR spectroscopy (Roberts *et al.*, 1999). It is also a flattened β-barrel, comprising two 4-stranded antiparallel β-sheets, with the biotinyl–lysine residue located in the exposed β-turn between β-strands 4 and 5 (Figure 1A). Based on sequence alignments (Samols *et al.*, 1988), it is likely that all biotinylated proteins contain an homologous biotinyl domain, a view supported by NMR spectroscopy of the biotinyl domain of *Propionibacterium shermanii* transcarboxylase (Reddy *et al.*, 1998).

Biotinylation and lipoylation are both post-translational modifications. Biotin is attached to its target proteins by the action of biotinyl protein ligase (BPL), also known as biotin holoenzyme synthetase (Wood *et al.*, 1980). This enzyme catalyses the activation of biotin to biotinyl-5′-AMP at the expense of ATP and then transfers the biotinyl group to a specific lysine residue in the biotinyl domain (a reaction mechanism akin to that of an aminoacyl tRNA synthetase). In *E.coli*, the reaction is mediated by the BirA protein, which also acts as the repressor of the biotin operon (Barker and Campbell, 1981; Cronan, 1989). The *BirA* gene has been sequenced (Howard *et al.*, 1985) and

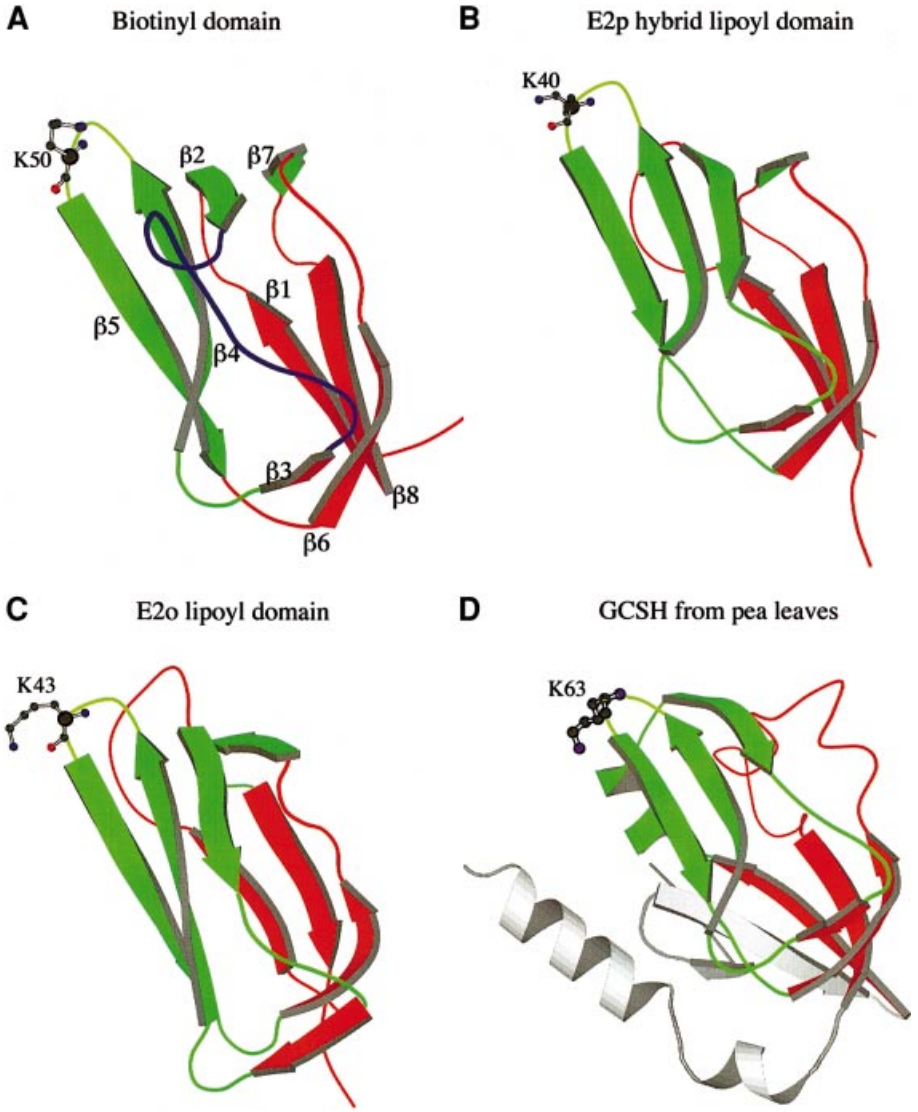

Fig. 1. Three-dimensional structures of biotinyl and lipoyl domains. (**A**) Biotinyl domain from the BCCP of *E.coli* acetyl-CoA carboxylase (Athappilly and Hendrickson, 1995); (**B**) hybrid lipoyl domain from *E.coli* E2p (J.D.F.Green *et al*., 1995); (**C**) lipoyl domain from *E.coli* E2o (Ricaud *et al*., 1996); and (**D**) H-protein from the glycine cleavage system of pea leaves (Pares *et al.*, 1994). The two β-sheets of the structures are shown in red and green, and the lysine residue that becomes biotinylated or lipoylated is indicated. The β-strands of the biotinyl domain are numbered from the N-terminus, and the loop (thumb) region between strands β2 and β3 is shown in blue. The figures were constructed using the programme MOLSCRIPT (Kraulis, 1991).

the crystal structure of the protein established (Wilson *et al.*, 1992). Lipoylation is similar but more complicated. In *E.coli*, the products of the *lplA* and *lipB* genes catalyse independent pathways of post-translational modification (Morris *et al.*, 1995). The lipoyl protein ligase LipB uses endogenous lipoyl–acyl carrier protein from the fatty acid biosynthesis pathway as the donor of the lipoyl group (Jordan and Cronan, 1997), whereas lipoyl protein ligase A (LplA) makes use of exogenous lipoic acid in a reaction formally identical to that of BPL and is responsible for most of the lipoylation under such conditions (Morris *et al.*, 1994, 1995). Its preferred substrate is D-lipoic acid although it can also use L-lipoic and octanoic acid (Morris *et al.*, 1994; D.E.Green *et al.*, 1995). It has no obvious similarity, apart from the chemistry of its mechanism, to BPL.

Biotinyl and lipoyl protein ligases do not require full length apo-proteins as substrates, being able to modify excised lipoyl and biotinyl domains (Ali and Guest, 1990; Cronan, 1990; Dardel *et al.*, 1990; Quinn *et al*., 1993, Berg *et al.*, 1994, 1995; Reche *et al*., 1998), but a folded domain appears to be required (Murtif and Samols, 1987; Cronan, 1990; Reed and Cronan, 1991). The means by which the enzymic machinery of the cell can distinguish between homologous lipoyl and biotinyl domains for correct post-translational modification is unclear. Accurate positioning of the target lysine residue in its tight β-turn in the structure is essential for both lipoylation (Wallis and Perham, 1994) and biotinylation (Reche *et al*., 1998), but the residues flanking the target lysine in the lipoyl domain are not crucial to the action of LplA. Replacing the DKA sequence at the tip of the β-turn in the *Bacillus stearothermophilus* lipoyl domain with the MKM sequence found in virtually all biotinylated proteins (Duval *et al.*, 1994) does not bring about biotinylation of the domain in

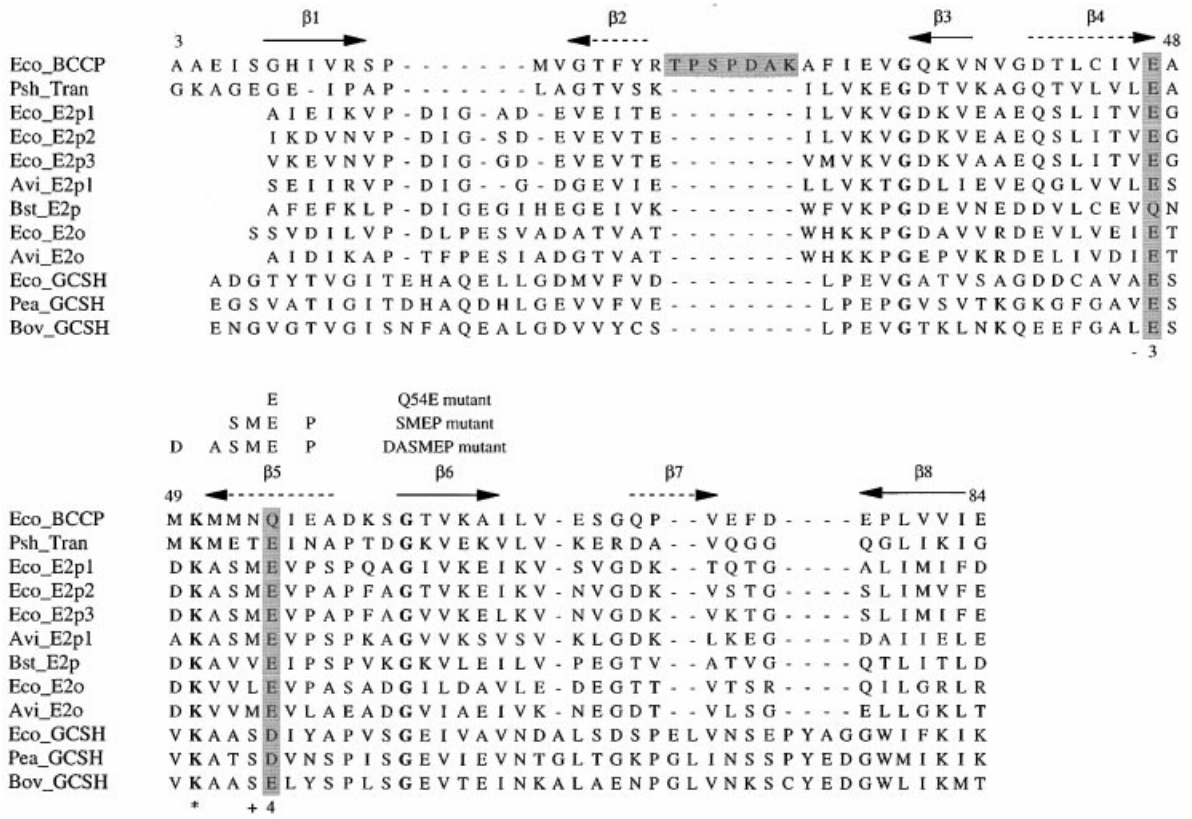

Fig. 2. Structure-based alignment of the amino acid sequences of the biotinyl domain from *E.coli* BCCP and lipoyl domains and H-proteins from diverse sources. Eco_BCCP, biotinyl domain of *E.coli* BCCP of acetyl-CoA carboxylase (AC P02905); Psh_Tran, biotinyl domain of *P.shermanii* methylmalonyl-CoA carboxyl transferase (1.3S subunit of transcarboxylase) (AC P02904); Eco_E2p1, Eco_E2p2 and Eco_E2p3, outer (N-terminal), middle and innerlipoyl domains , respectively, of *E.coli* E2p (AC P06959); Avi_E2p1, outer (N-terminal) lipoyl domain of *Azotobacter vinelandii* E2p (AC P10802); Bst_E2p, lipoyl domain of *B.stearothermophilus* E2p (AC P11961); Eco_E2o, lipoyl domain of *E.coli* E2o (AC P07016); Avi_E2o, lipoyl domain of *A.vinelandii* E2o (AC P20708); Eco_gcsh, lipoyl domain of *E.coli* H-protein (AC P23884); Pea_GCSH, lipoyl domain of pea leaf H-protein (AC P16048); Bov_GCSH, lipoyl domain of ox H-protein (AC P20821). Accession numbers are given for the SwissProt Data Base. The numbering is that of the excised *E.coli* BCCP biotinyl domain (as in Figure 6) and its β-strands are represented in extent and direction by numbered arrows above the amino acid sequence (solid arrows, red strands in Figure 1; broken arrows, green strands in Figure 1). Identical residues are shown in bold and the post-translationally modified lysine residue is marked with an asterisk. The amino acid substitutions created in the Q54E, SMEP and DASMEP mutant biotinyl domains are indicated. Residues located at position +4 and –3 with respect to the target lysine (K50 for the biotinyl domain) are shadowed, as are the seven residues deleted in the loop deletion (LD) mutant. The alignment was built using the known structures of the *E.coli* BCCP biotinyl domain (Athappilly and Hendrickson, 1995; Roberts *et al*., 1999), *B.stearothermophilus* E2p lipoyl domain (Dardel *et al.*, 1993); *E.coli* E2p hybrid lipoyl domain (J.D.F.Green *et al.*, 1995), *A.vinelandii* E2p lipoyl domain (Berg *et al.*, 1997); *E.coli* E2o lipoyl domain (Ricaud *et al.*, 1996) and *A.vinelandii* E2o lipoyl domain (Berg *et al.*, 1996) using the command MALIGN3D from the Modeller package (Sali and Blundell, 1993) and then optimized manually aided by visual inspection of the structures on a graphics terminal.

E.coli (Wallis and Perham, 1994), whereas introduction of the DKA motif into the BCCP biotinyl domain eliminates biotinylation and leads to low levels of lipoylation and aberrant octanoylation (Reche *et al.*, 1998).

We show here that the rational introduction of a set of amino acid replacements in the biotinyl domain of *E.coli* BCCP can switch its specificity as a substrate for post-translational modification, rendering it ineffective as a target for biotinylation but highly effective in provoking lipoylation. We further show that there exists a structural feature in the natural biotinyl domain, viz a surface loop between β-strands 2 and 3 that is absent from the homologous lipoyl domain, which acts to prevent the aberrant lipoylation of the biotinyl domain *in vivo* and *in vitro*. These results are in striking contrast with the specificity of other forms of post-translational modification, such as phosphorylation, where the sequence motif surrounding the target residue is often of paramount importance.

Results

Rationale for directed mutagenesis

The lysine residue targeted for post-translational modification is located in a β-turn in one of the two β-sheets that constitute both the biotinyl and lipoyl domains (Figure 1). It was reasonable to suppose that this region more generally might contain structural features that permit the ligases BPL and LplA to recognise their respective substrates. Therefore, we first identified key differences between the domains in the β-sheet that contains the swinging arm and then considered amino acid replacements in the *E.coli* biotinyl domain that would make it more closely resemble the lipoyl domain. In the Q54E mutant, the Gln residue (Q54) in the β5 strand at position +4 with respect to the target lysine (K50) of the biotinyl domain was replaced with Glu, since an exposed negatively charged side chain is found at this position in all lipoyl domains (Figure 2). The SMEP mutant biotinyl domain was designed to carry

Table I. Molecular masses of *E.coli* wild-type and mutant biotinyl domains determined by mass spectrometry

Domain	Apo-form (Da): measured (calculated)	Growth medium supplemented with biotin: modified form (Da)	Growth medium supplemented with lipoic acid: modified form (Da)
Wild-type	8978.3 ± 0.5 (8978.3)	9204 ± 1	9204.0 ± 0.3
Q54E	8979.4 ± 0.1 (8979.3)	9205.4 ± 0.2	9168 ± 1[a] 9206 ± 2[b]
SMEP	8920.6 ± 1.0 (8920.2)	9146.3 ± 0.5	9109 ± 1[a] 9146 ± 1[b]
DASMEP	8844.4 ± 0.3 (8844.0)	9032.6 ± 0.1	9033 ± 1
LD	8282.3 ± 0.1 (8281.6)	8507 ± 1	8470 ± 1

[a]More abundant modified component.
[b]Less abundant modified component.

the Q54E mutation plus three additional changes: M52S, N53M and E56P; these replace further residues in the β5 strand of the biotinyl domain with the corresponding residues of the three lipoyl domains of *E.coli* E2p [E2 component of the pyruvate dehydrogenase (PDH) complex] (Figure 2). The DASMEP mutant domain has the same mutations as the SMEP domain plus two more substitutions: M49D and M51A; M49 and M51 are the highly conserved methionine residues that flank the target lysine in virtually all biotinyl domains (Duval *et al*., 1994), whereas Asp and Ala are the corresponding residues in the E2p lipoyl domains (Figure 2).

The LD mutant biotinyl domain was designed on a different basis; it was generated by deleting 7 amino acid residues (TPSPDAK, residues 22–28) from the loop connecting the β2 and β3 strands of the biotinyl domain, residues which are not present in any of the lipoyl domains (Figure 2). These residues generate a protruding 'thumb' in the structure of the biotinyl domain, which is close in space to the β-turn carrying the biotinyl–lysine residue (Figure 1), and provide the principal sites of contact with the biotin that localize the swinging arm on the surface of the protein (Athappilly and Hendrickson, 1995; Roberts *et al*., 1999).

Post-translational modification of the wild-type and mutant biotinyl domains in vivo

The ability of the various biotinyl domains to undergo post-translational modification *in vivo* was checked by expressing the genes encoding them in *E.coli* cells grown in Luria–Bertani (LB) medium supplemented with either d-biotin (10 mg/l) or dl-lipoic acid (10 mg/l). The purified domains were analysed by means of non-denaturing 20% PAGE, in which the modified (holo-) form can be separated from the apo-form because it carries one fewer positive charge and therefore migrates more rapidly towards the anode. The molecular masses of the domains were also determined by means of electrospray mass spectrometry (ESMS). The nature and extent of the modification of the different domains (lower band in each lane in Figure 3) was found to vary with the growth medium.

The wild-type biotinyl domain was observed to be biotinylated in cells grown in medium supplemented with either biotin or lipoic acid, as judged by the molecular mass of the modified protein band; in both instances, the molecular mass was 226 Da more than that of the apo-domain (Table I), corresponding to addition of a biotinyl group (Krishna and Wold, 1993). However, the biotinylated product represented ~90% of the total domain when the gene was expressed in the presence of biotin (Figure 3, lane 1) but only 15% when the gene was expressed in the same medium supplemented with lipoic acid (Figure 3, lane 2). The Q54E mutant domain isolated from *E.coli* cells grown in a medium supplemented with biotin was also modifed to a similar extent (80%) as the wild type (Figure 3, lane 3), and the difference in molecular mass compared with the wild type (226 Da) was again indicative of biotinylation (Table I). In contrast, the same mutant domain obtained from cells grown in medium supplemented with lipoic acid was modified to a lesser extent, ~30% (Figure 3, lane 4), and the increase in mass of the modified form (188 Da; Table I) corresponded with that of a lipoyl group (Krishna and Wold, 1993). A minor component (<10% of the modified product, as judged from the mass spectrum) was also detected, with the molecular mass (9206 ± 2 Da) of the biotinylated form of the domain.

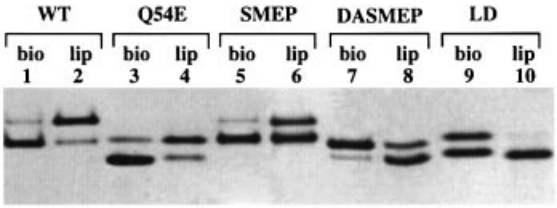

Fig. 3. Post-translational modification of the *E.coli* wild-type and mutant biotinyl domains *in vivo*. Wild-type and mutant biotinyl domains were isolated from *E.coli* BL21(DE3) cells transformed with the relevant plasmid and grown in the presence of either d-biotin (10 mg/l, bio) or DL-lipoic acid (10 mg/l, lip). Approximately 3 μg of each domain was subjected to non-denaturing PAGE (20% gels) and stained with Coomassie Brilliant Blue. Lanes 1 and 2, wild-type biotinyl domain (WT); lanes 3 and 4, Q54E mutant biotinyl domain (Q54E); lanes 5 and 6, SEMP mutant biotinyl domain (SMEP); lanes 7 and 8, DASMEP mutant biotinyl domain (DASMEP); lanes 9 and 10, LD mutant biotinyl domain (LD). In each lane, the upper band is the apo-form of the domain and the lower band is the post-translationally modified form.

The SMEP mutant domain isolated from cells grown expressed in the medium supplemented with biotin was 90% in the modified form (Figure 3, lane 5) and the molecular mass was that expected for the biotinylated domain. On the other hand, when isolated from cells grown in a medium supplemented with lipoic acid, 60% of the mutant domain was modified (Figure 3, lane 6) and the mass spectrum of the heavier component indicated a lipoylated product (Table I). A trace (5%) of the biotinylated SMEP domain was also identified from the mass spectrum (Table I). The DASMEP mutant domain isolated

from *E.coli* cells grown in a medium supplemented with biotin had become modified to a level of only 15% (Figure 3, lane 7) and the molecular mass of the modified protein corresponded with lipoylation, not biotinylation (Table I). The same domain was found to be modified to approximately the same extent (70%) as the SMEP mutant when purified from *E.coli* cells grown in LB medium supplemented with lipoic acid (Figure 3, lane 8), and ESMS gave only one molecular mass: that expected for the lipoylated protein (Table I).

The LD mutant domain differs from the previous mutants in that its only change is the deletion of the protruding thumb region between β-strands 2 and 3. When it was purified from *E.coli* cells grown in medium supplemented with biotin, it was found to be modified to a level of 55% (Figure 3, lane 9) and the mass spectrum corresponded with that of the biotinylated form of the domain. Likewise, >90% of the LD mutant domain obtained from cells grown in LB medium supplemented with lipoic was modified (Figure 3, lane 10), but ESMS gave a molecular mass that identified the modification as only lipoylation (Table I).

Post-translational modification of the wild-type and mutant biotinyl domains in vitro

The biotinylation and lipoylation of the various biotinyl domains *in vitro* were studied with the purified ligases, BPL and LplA. The incubation times were fixed at 1 h but the amount of ligase was varied and the products were separated by 20% non-denaturing PAGE. The extent of the modification, as judged by densitometry of the Coomassie Blue-stained gels, was plotted against the amount of either BPL or LplA. The slopes of these plots give a measure of the ability of the domains to undergo post-translational modification, expressed as percent modification per microgramme of ligase (see Materials and methods for details).

When tested as a substrate for LplA in the presence of lipoic acid, the wild-type biotinyl domain (Figure 4A) was observed to undergo lipoylation at a very slow rate, reaching only 40% modification in the presence of the maximum amount (10 μg) of ligase, a difference of 1000-fold in comparison with the wild-type E2p lipoyl domain (Figure 4F). The susceptibility to lipoylation was increased 10-fold by the introduction of the Q54E mutation (Figure 4B) and ~35-fold for both the SMEP and DASMEP mutant domains (Figure 4C and D). It increased substantially again (~340-fold) for the LD mutant biotinyl domain, coming close to the rate (a difference of no more than 3-fold) that we observed for the wild-type E2p lipoyl domain (Figure 4E). These results are summarized in Figure 4G.

In contrast, when tested as a substrate for biotinylation by BPL, the Q54E mutant domain was only slightly less effective than the wild-type biotinyl domain (Figure 5A and B). The biotinylation of the SMEP mutant domain was also comparable with that of the wild-type biotinyl domain (Figure 5C) whereas that of the DASMEP domain was badly affected, being scarcely detectable under the chosen conditions (Figure 5D). On the other hand, the LD mutant domain remained a good substrate for BPL, requiring only twice the amount of BPL to achieve the same modification as the wild-type domain (Figure 5E).

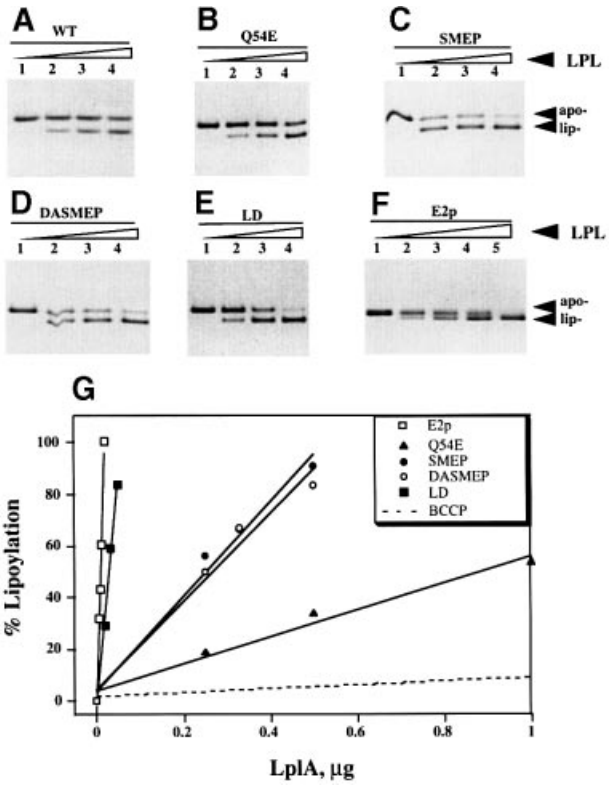

Fig. 4. Lipoylation of the *E.coli* biotinyl domains with LplA *in vitro*. Samples (2 μg) of purified apo-form of each biotinyl domain was incubated for 1 h with lipoic acid and differing amounts of LplA under the conditions described in Materials and methods and the products were analysed by means of non-denaturing PAGE (20% gels). The gels were stained with Coomassie Blue. (**A**) Wild-type biotinyl domain (WT) was incubated withouth LplA (lane 1) and with 4 μg LplA (lane 2), 6.6 μg LplA (lane 3) and 10 μg LplA (lane 4). (**B**)Q54E mutant biotinyl domain (Q54E) was incubated without LplA (lane 1), and with 0.25 μg LplA (lane 2), 0.5 μg LplA (lane 3) and 1.0 μg LplA (lane 4). (**C**) SMEP mutant biotinyl domain was incubated without LplA (lane 1) and with 0.25 μg LplA (lane 2), 0.33 μg LplA (lane 3) and 0.5 μg LplA (lane 4). (**D**) DASEMP mutant biotinyl domain was incubated without LplA (lane 1) and with 0.25 μg LplA (lane 2), 0.33 μg LplA (lane 3) and 0.5 μg LplA (lane 4). (**E**) LD mutant biotinyl domain was incubated without LplA (lane 1) and with 0.02 μg LplA (lane 2), 0.03 μg LplA (lane 3) and 0.05 μg LplA (lane 4). (**F**) Lipoyl domain (innermost) of *E.coli* E2p was incubated without LplA and with 0.008 μg LplA (lane 2), 0.01 μg LplA (lane 3), 0.013 μg LplA (lane 4) and 0.02 μg LplA (lane 5). The extent (%) of lipoylation was determined densitometrically and plotted against the quantity (μg) of LplA present in the reaction mixture. (**G**) Dotted line, wild-type biotinyl domain; ▲, Q54E mutant biotinyl domain; ●, SMEP mutant biotinyl domain; ○, DASMEP mutant biotinyl domain; ■, LD mutant biotinyl domain; □, E2p lipoyl domain.

These results are summarized in Figure 5F. Overall, the results of lipoylation and biotinylation with the purified ligases *in vitro* were fully consistent with those obtained for the post-translational modifications of the domains *in vivo*.

Discussion

Post-translational modification of specific lysine residues in the biotinyl and lipoyl domains of biotin- and lipoic acid-dependent enzymes is essential for their proper function. The biotinyl and lipoyl protein ligases have similar mechanisms but no obvious structural similarity. The biotinyl and lipoyl domains are closely related in three-

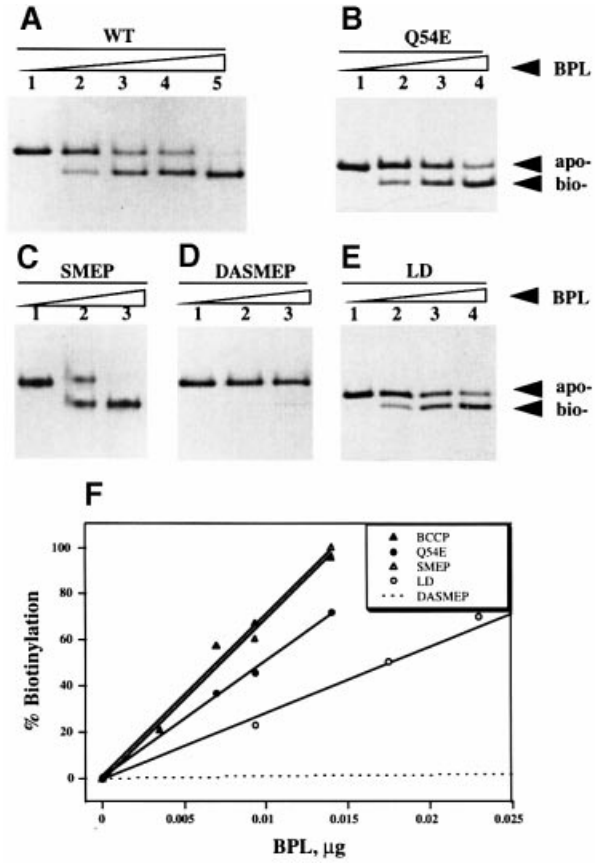

Fig. 5. Biotinylation of the *E.coli* biotinyl domains with BPL *in vitro*. Samples (2 µg) of purified apo-form of each biotinyl domain was incubated for 1 h with biotin and differing amounts of BPL under the conditions described in Materials and methods and the products were analysed by means of non-denaturing PAGE (20% gels). The gels were stained with Coomassie Blue. (**A**) Wild-type biotinyl domain (WT) was incubated without BPL (lane 1) and with 0.0035 µg BPL (lane 2), 0.007 µg BPL (lane 3), 0.0093 µg BPL (lane 4) and 0.014 µg BPL (lane 5). (**B**) Q54E mutant biotinyl domain (Q54E) was incubated with no BPL (lane 1) and with 0.007 µg BPL (lane 2), 0.0093 µg BPL (lane 3) and 0.014 µg BPL (lane 4). (**C**) SMEP mutant biotinyl domain was incubated without BPL (lane 1) and with 0.0093 µg BPL (lane 2) and 0.014 µg BPL (lane 3). (**D**) DASMEP mutant biotinyl domain was incubated without BPL (lane 1) and with 0.0093 µg BPL (lane 2) and 0.014 µg BPL (lane 3). (**E**) LD mutant biotinyl domain was incubated without BPL (lane 1) and with 0.0093 µg BPL (lane 2), 0.0175 µg BPL (lane 3) and 0.023 µg BPL (lane 4). The extent (%) of biotinylation was determined densitometrically and plotted against the quantity (µg) of BPL present in the reaction mixture. (**F**) Dotted line, DASMEP mutant biotinyl domain; ▲, wild-type biotinyl domain; ●, Q54E mutant biotinyl domain; △, SMEP mutant biotinyl domain; ○, LD mutant biotinyl domain.

dimensional structure, and there must therefore be one or more key differences between them to ensure their correct selection. In the *E.coli* cell there is only one biotinylated protein, the BCCP of acetyl-CoA carboxylase, but this exists alongside several lipoyl domains from the PDH and 2-oxoglutarate dehydrogenase (2OGDH) multienzyme complexes, and the GCSH. We are now in a position to understand how the necessary specificity of post-translational modification is achieved.

Thus, when a sub-gene expressing the biotinyl domain of the BCCP was overexpressed in *E.coli* cells growing in a medium supplemented with either biotin or lipoic acid, only the biotinylated form of the domain was obtained, although in the cells grown with lipoic acid the extent of modification was much lower (Figure 3). Likewise, with a large excess of purified *E.coli* LplA, the biotinyl domain could be lipoylated *in vitro* but compared very poorly with an *E.coli* E2p lipoyl domain (Figure 4). However, the introduction of the Q54E mutation in the β5 strand of the biotinyl domain (Figures 1 and 2) predominantly (25%) led to lipoylation of the domain *in vivo* in cells grown in lipoic acid-containing medium, accompanied by only 5% or less of biotinylation, although the domain was still extensively biotinylated if the growth medium were supplemented with biotin (Figure 3). Under these conditions, the lipoylation *in vivo* is likely to be due to LplA, since this enzyme utilizes free lipoic acid (Morris *et al*., 1995). Indeed, the Q54E mutation rendered the biotinyl domain a much better substrate for LplA *in vitro*, although it was still substantially poorer than the wild-type E2p lipoyl domain (Figure 4) and remained an effective substrate for BPL (Figure 5). A negatively charged Glu or Asp residue is found at this surface-exposed position in all lipoylated proteins (Figure 2) and our results make it clear that its replacement with Gln in the biotinyl domain of *E.coli* BCCP helps to prevent that protein from acting as a substrate for LplA *in vivo* or *in vitro*.

Lipoylation of the biotinyl domain was further improved by exchanging additional residues in the β5 strand for their counterparts in the *E.coli* E2p lipoyl domains (Figure 2). Lipoylation of the SMEP domain in cells grown in the presence of lipoic acid was doubled, with only a residual trace of biotinylation, but again without effect on the extent of biotinylation observed when the growth medium was supplemented with biotin (Figure 3). Corresponding effects were noted when the SMEP domain was tested as a substrate for LplA (Figure 4) or BPL (Figure 5) *in vitro*. The secondary importance of residues that neighbour Gln54 in the β5 strand for recognition by LplA is obvious. The hydrophobic residue, Ile55, adjacent to Gln54 is unlikely to be involved, since it participates in forming the hydrophobic core of the domain (Athappilly and Hendrickson, 1995; Roberts *et al*., 1999), as does the corresponding residue (always a hydrophobic side chain) in the lipoyl domain (Dardel *et al*., 1993; J.D.F.Green *et al*., 1995). Unlike LplA, BPL is evidently rather indifferent to the nature of the amino acid side chains in the β5 strand, though not of course to their contribution to the correct protein fold [in accordance with other evidence coming from a study of the interaction by means of NMR spectroscopy (P.A.Reche, M.J.Howard and R.N.Perham, unpublished work)].

In the DASMEP biotinyl domain, the two methionine residues that flank the target lysine residue in virtually all biotinyl domains (Duval *et al*., 1994) were replaced with the residues that occupy these positions in the β-turn of the *E.coli* lipoyl domains (Figure 2). The effect on biotinylation was substantial: the DASMEP domain was no longer biotinylated *in vivo*, even in *E.coli* cells grown in the presence of biotin (Figure 3), nor was it biotinylated by BPL *in vitro* (Figure 5). However, its ability to become lipoylated by LplA *in vitro* was unchanged (Figure 4). These results suggest that LplA has no particular preference for the residues that flank the target lysine residue, as inferred from studies of the lipoylation of the *B.stearothermophilus* E2p lipoyl domain in *E.coli* cells

in vivo (Wallis and Perham, 1994). They are also consistent with the observation (Reche *et al*., 1998) that the double mutation M49DM51A is itself sufficient to inhibit biotinylation of the *E.coli* BCCP biotinyl domain *in vivo* and *in vitro*. Replacement of the two methionine residues with other hydrophobic residues has been reported to have no major effect on the biotinylation of other biotinylated proteins tested in *E.coli* (Shenoy *et al*., 1988; Leon-Del-Rio and Gravel, 1994). Thus it would appear that BPL responds only to certain changes in residues flanking the target lysine and, given that the corresponding sequence in the lipoyl domain of the dihydrolipoyl succinyltransferase (E2o) of the 2OGDH complex is DKV, that it may be the negatively charged Asp side-chain that is of particular importance in preventing recognition by BPL.

The protruding thumb region between strands β2 and β3 of the *E.coli* BCCP biotinyl domain does not exist in the *E.coli* lipoyl domains (Figure 1). The LD mutant biotinyl domain, in which the seven residues (Thr22 to Lys28) that constitute this loop (Figure 2) were deleted, proved to be almost as efficient a substrate as the native E2p lipoyl domain for lipoylation *in vivo* (Figure 3) and by LplA *in vitro* (Figure 4). This is true notwithstanding the presence of Gln rather than Glu at position 54 in strand β5 (see above). At the same time, the LD domain retained significant ability to undergo biotinylation, *in vivo* (Figure 3) and *in vitro* (Figure 5). Thus, the protruding thumb between strands β2 and β3 is not critical for the interaction with BPL but its presence is sufficient to prevent the biotinyl domain from becoming lipoylated. This effect may be limited to *E.coli*, since the amino acids that constitute the thumb are not present in most other biotinyl domains (Reddy *et al*., 1998).

The biotinyl–lysine residue is immobilized in *E.coli* BCCP, both in the crystal (Athappilly and Hendrickson, 1995) and solution NMR (Roberts *et al*., 1999) structures, as a result of interactions between the biotin moiety and amino acid residues in the thumb region. In other biotinyl domains the protruding thumb is absent and, at least in the biotinyl domain from *P.shermanii* transcarboxylase (Reddy *et al*., 1997), NMR spectroscopy suggests that the biotinyl–lysine residue is free to move. Similarly, the lipoyl–lysine residue is essentially free to move on the surface of the lipoyl domain in the 2-oxo acid dehydrogenase complexes, as judged by NMR (Dardel *et al*., 1993) and other spectroscopic techniques (Ambrose and Perham, 1976; Grande *et al*., 1976). However, the lipoyl–lysine in the GCSH protein of pea leaves is localized by interactions with the protein in the oxidized form (Pares *et al*., 1994), and has moved to a different but again localized position with a different set of interactions in the charged (reductively aminomethylated) form (Cohen-Addad *et al*., 1995). The immobilization of the swinging arm in the GCSH protein appears to be associated with the protection of an unstable catalytic intermediate, exemplifying the 'hot potato' hypothesis (Perham and Reche, 1998). In the present context, it is of particular interest that the structural feature responsible for the immobilization of the biotinyl–lysine swinging arm in the *E.coli* BCCP is also sufficient to prevent aberrant post-translational modification of the target lysine residue.

There are hints of parallels in other organisms. In ox liver mitochondria, two isoforms of lipoyltransferase have been identified as catalysing the lipoylation of proteins using lipoyl-5′-AMP as the lipoyl group donor, but are unable to use lipoic acid and MgATP for lipoylation, suggesting that a second enzyme may be required to initiate the lipoylation reaction in ox liver (Fujiwara *et al*., 1994). An E63D mutation in ox GCSH does not affect the lipoylation of this protein by ox lipoyltransferase, whereas the mutation E63Q decreases it 4-fold (Fujiwara *et al*., 1991). Glu63 in ox GCSH corresponds to Gln54 in the *E.coli* biotinyl domain (Figure 2), suggesting that ox lipoyltransferase, like *E.coli* LplA, has a preference for a domain with a negatively charged side chain at position +4 with respect to the target lysine residue. Likewise, a Glu at position –3 is crucial for lipoylation mediated by ox lipoyltransferase (Fujiwara *et al*., 1996). Glu47 is present in the corresponding position of the *E.coli* lipoyl domains and BCCP biotinyl domain (Figure 2), but in the BCCP protein it is unlikely to be accessible to LplA because of shielding by the protruding thumb (Figure 1). When the thumb is deleted, Glu47 should become fully exposed, which may facilitate the lipoylation of the LD domain.

Structural cues are thus seen to dominate the recognition of the homologous biotinyl and lipoyl domains by two ligases, which are themselves structurally different; Bpl and LplA clearly recognise different structural features in separate locations on these closely similar proteins. Our results are in striking contrast to other forms of post-translational modification, where the amino acid sequence surrounding the target residue can be of crucial importance and unstructured synthetic peptides are adequate substrates.

Materials and methods

Materials

All chemicals used were of reagent grade or better. Bacterial growth media were purchased from Oxoid, Unipath Laboratories. *Escherichia coli* host strain SURE® was from Stratagene, and strains BL21(DE3) and BL21(DE3)plysS were from Novagen. dl-lipoic acid and d-biotin were purchased from Sigma. Restriction endonucleases and DNA modifying enzymes were from Pharmacia Biotech and New England Biolabs, Inc.; *Pfu* DNA polymerase was purchased from Stratagene Ltd. Oligonucleotide primers were synthesized by Mr M.Weldon in the Protein and Nucleic Acid Facility, Department of Biochemistry, University of Cambridge, UK.

Expression plasmids

Plasmid pTbpl carrying the gene encoding the BPL of *E.coli* was constructed by P.Reche and R.N.Perham (unpublished work). Plasmid TM202 (Morris *et al.*, 1994) carrying the gene encoding LplA was generously provided by Dr J.E.Cronan, Jr (University of Illinois, IL). Plasmid pET11cE2p expressing a subgene encoding the innermost lipoyl domain of the E2p chain of *E.coli* PDH complex (Packman *et al.*, 1984) was constructed by E.L.Roberts and R.N.Perham (unpublished work). Plasmid pGsthBCCP, carrying DNA encoding the biotinyl domain from the BCCP of *E.coli* acetyl-CoA carboxylase (P.Reche and R.N.Perham, unpublished work), generates a fusion protein with glutathione *S*-transferase (GST) at the N-terminus, followed by a thrombin cleavage site and the biotinyl domain. The DNA encoding the biotinyl domain in pGsthBCCP was engineered with several silent mutations that introduce three new unique restriction sites (*Hin*dIII, *Nsi*I and *Bsp*EI*)* to facilitate cassette mutagenesis. The DNA sequence of the modified sub-gene encoding the biotinyl domain is shown in Figure 6.

DNA manipulations

Standard protocols for molecular biology were used as described elsewhere (Sambrook *et al*., 1989). DNA fragments were purified after agarose gel electrophoresis using a Geneclean® kit (BIO101). A Qiagen

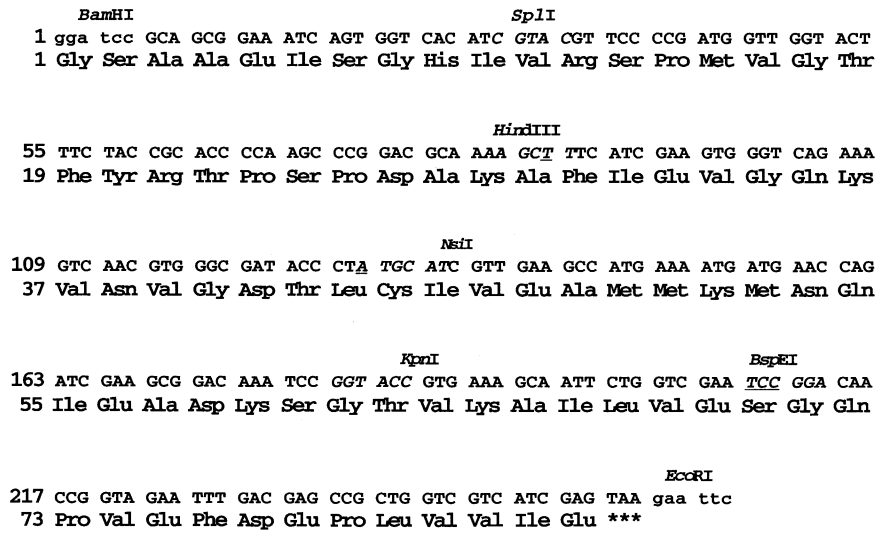

Fig. 6. DNA sequence of the modified sub-gene encoding the biotinyl domain in pGsthBCCP. The amino sequence of the biotinyl domain is given beneath the DNA sequence. Unique restriction sites are italicized, with the name of the enzyme above the DNA sequence. The mutations introduced in the sub-gene in order to generate the restriction sites for *Hin*dIII, *Nsi*I and *Bsp*EI are underlined. The biotinyl domain sub-gene was cloned into the *Bam*HI and *Eco*RI restriction sites (shown in lower case) of pGEX-2T. The amino acid sequence of the biotinyl domain starts at Ala3; Gly1 and Ser2 are derived from the construction of the expression plasmid and the thrombin cleavage (see text for details). The target residue for biotinylation is Lys50, in the sequence MKM.

Table II. Oligonucleotides used for the cassette mutagenesis

Cassette	Name	Sequence
smep	sen-smep	5′-TCGTTGAAGCCATGAAAATGTCGATGGAAATCCCGGCGGACAAATCCGGTAC
	ant-smep	5′-CGGATTTGTCCGCCGGGATTTCCATCGACATTTTCATGGCTTCAACGATGCA
dasmep	sen-dasmep	5′-TCGTTGAAGCCGACAAAGCATCGATGGAAATCCCGGCGGACAAATCCGGTAC
	ant-dasmep	5′-CGGATTTGTCCGCCGGGATTTCCATCGATGCTTTGTCGGCTTCAACGATGCA
ld	sen-ld	5′-GTACGTTCCCCGATGGTTGGTACTTTCTACCG
	ant-ld	5′-AGCTCGGTAGAAAGTACCAACCATCGGGGAAC

kit was used for plasmid isolation. Automated DNA sequence analysis was performed by Mr J.Lester and Mrs K.Pennock (Department of Biochemistry, University of Cambridge, UK). Polymerase chain reactions (PCRs) were carried out under mineral oil in reaction mixtures (100 μl) containing 200 μM dNTPs, 2 mM $MgCl_2$, 100 pmol each of sense and anti-sense primers, 10 ng of DNA template and 2.5 U of *Pfu* DNA polymerase. The conditions for PCR were: 5 min at 94°C (1 cycle); 1 min at 94°C, 1 min 30 s at 55°C, 2 min at 72°C (25 cycles); and 10 min at 72°C (1 cycle).

Mutations in the biotinyl domain

The Q54E mutant biotinyl domain was generated by overlap extension PCR (Ho *et al.*, 1989) using pGsthBCCP DNA as template. The overlapping primers used were senQ54E, 5′-ATGATGAAC**GAG**ATCG-AAGCGGAC, and antQ54E, 5′-GTCCGCTTCGAT**CTC**GTTCATCAT (the codon encoding the substitution is shown in bold), with primers pGD, 5′-CCAGCAAGTATATAGCATGGCCTTTGC, and pGR, 5′-AA-GCTGTGACCGTCTCCGGGAGC priming upstream and downstream, respectively, of the coding sequence. The final overlapping PCR fragment was purified after agarose gel electrophoresis, digested with *Bam*HI and *Eco*RI, and then cloned into pGEX-2T cut with the same enzymes, to give the plasmid pGq54esthBCCP. The SMEP, DASMEP and LD mutant biotinyl domains were generated by means of cassette mutagenesis. Thus, for the SMEP and DASMEP domains, the 52 bp *Nsi*I–*Kpn*I fragment in pGsthBCCP was replaced with the synthetic DNA cassettes, *smep* and *dasmep*, respectively (Table II). The *smep* and *dasmep* cassettes both encode the M52S, N53M, Q54E and E56P mutations, but the *dasmep* cassette encodes the additional mutations, M49D and M51A. The LD mutant biotinyl domain was generated by replacing the 53 bp *Spl*I (*Bsi*WI)–*Hin*dIII fragment in pGsthBCCP with a 32 bp synthetic DNA fragment (*ld*) (Table II) that creates a deletion of seven amino acid residues from Thr22 to Lys28, inclusive. The mutagenic cassettes were cloned into pGsthBCCP cut with the appropriate restriction enzymes to give the plasmids pGsmepsthBCCP, pGdasmepsthBCCP and pGldsthBCCP. All plasmids were propagated in *E.coli* SURE® cells and the DNA sequences of the mutated sub-genes were verified.

Purification of the biotinyl domains and of BPL and LplA

Biotinyl domains were isolated from *E.coli* BL21(DE3) cells transformed with the relevant plasmid and grown in LB medium (Sambrook *et al.*, 1989) containing 100 μg/ml of ampicillin and supplemented with either d-biotin (10 mg/l) or dl-lipoic acid (10 mg/l). The biotinyl domains were purified from the cell-free extract as described by Reche *et al.* (1998). Further purification of the biotinyl domains to separate the holo- and apo-forms was carried out by anion-exchange chromatography on a Pharmacia Resource™ Q HR 16/10 column, also as described by Reche *et al.* (1998). The E2p lipoyl domain was purified from *E.coli* BL21(DE3) cells transformed with pET11cE2p grown in LB medium containing 100 μg/ml ampicillin, as described by Dardel *et al.* (1990). BPL was purified from *E.coli* BL21(DE3)plysS cells transformed with the plasmid pTbpl, and LplA was purified from *E.coli* BL21(DE3) cells transformed with the plasmid TM102, both as described elsewhere (Reche *et al.*, 1998).

Kinetics of biotinylation and lipoylation

Biotinylation reactions were carried out in mixtures (15 μl) containing 50 mM Tris–HCl, pH 8.3, 40 mM $MgCl_2$, 2.6 mM ATP (pH 7.0), 200 μM d-biotin and 2 μg of protein substrate. Lipoylation reactions were carried out in mixtures (15 μl) containing 33 mM potassium phosphate buffer, pH 7.5, 1.0 mM ATP (pH 7.0), 2.0 mM $MgCl_2$, 200 μM dl-lipoic acid (pH 7.0) and 2 μg of protein substrate. Reactions were started by adding measured amounts of the relevant ligase (BPL or LplA) and incubation was at 30°C for 1 h. The extent of the modification was analysed by means of non-denaturing PAGE (20% acrylamide, 1% bis-acrylamide as separating gel; 5% acrylamide as stacking gel). The relative amounts of the modified and unmodified domains were determined densitometrically from scanned pictures of the Coomassie-stained gels (using the program Phoretix 1D Advanced,

version 3.0) and the percentage of modified protein (lipoylation or biotinylation) was plotted against the amount of ligase used in the particular reaction.

Protein chemical techniques

Protein purifications were carried out on a Pharmacia FPLC™ at 4°C and column fractions were analysed by means of SDS–PAGE (12.5 and 20% acrylamide) using the Pharmacia PhastSystem™. All gels were stained with Coomassie Brilliant Blue R-250. The concentrations of pure proteins were estimated by amino acid analysis (Packman *et al.*, 1988). Molecular masses of biotinyl domains were obtained by ESMS using a VG BioQ quadrapole mass spectrometer and myoglobin as the calibration standard. Protein samples for ESMS were dissolved in 50% acetonitrile, 1% formic acid to a final concentration of ~10 μM.

Acknowledgements

We thank Mr C.Fuller for skilled technical assistance. We thank the Federation of European Biochemical Societies for a Fellowship awarded to P.R. and the Biotechnology and Biological Sciences Research Council for a research grant awarded to R.N.P. The core facilities of the Cambridge Centre for Molecular Recognition are supported by the BBSRC and The Wellcome Trust.

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Received February 22, 1999; revised and accepted March 24, 1999