Expression, biotinylation and purification of a biotin-domain peptide from the biotin carboxy carrier protein of *Escherichia coli* acetyl-CoA carboxylase

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A protein segment consisting of the C-terminal 87 residues of the biotin carboxy carrier protein from *Escherichia coli* acetyl-CoA carboxylase was overexpressed in *E. coli*. The expressed biotin-domain peptide can be fully biotinylated by coexpression with a plasmid that overproduces *E. coli* biotin ligase. The extent of biotinylation was limited *in vivo*, but could be taken to completion in cell lysates on addition of ATP and biotin. We used the co-expression of biotin ligase and acceptor protein to label the biotin-domain peptide *in vitro* with [^{3}H]biotin, which greatly facilitated development of a purification procedure. The apo (unbiotinylated) form of the protein was prepared by induction of biotin-domain expression in a strain lacking the biotin-ligase-overproduction plasmid. The apo domain could be separated from the biotinylated protein by ion-exchange chromatography or non-denaturing PAGE, and was converted into the biotinylated form of the peptide on addition of purified biotin ligase. The identity of the purified biotin-domain peptide was confirmed by N-terminal sequence analysis, amino acid analysis and m.s. The domain was readily produced and purified in sufficient quantities for n.m.r. structural analysis.

INTRODUCTION

The biotin carboxylases are a family of enzymes that catalyse the transfer of CO_2 between metabolites, using a covalently attached biotin moiety as a mobile carboxy carrier (Samols et al., 1988). The biotin group is attached post-translationally to a specific lysine residue of newly synthesized biotin carboxylases by the enzyme biotin ligase, which uses ATP to activate the carboxy group of biotin during the reaction (Shenoy and Wood, 1988). Biotinylation is a relatively rare modification, with the number of biotinylated proteins varying from one to five in different organisms (see Cronan, 1990), and a high degree of sequence homology is observed around the biotin-attachment site of all the biotinylated proteins for which sequence data are currently available (Lim et al., 1988; Samols et al., 1988; Kondo et al., 1991; Li and Cronan, 1992). This sequence homology is reflected in apparent structural conservation, as biotin ligase from various sources will recognize and biotinylate acceptor proteins from very different species both *in vivo* (Cronan, 1990) and *in vitro* (McAllister and Coon, 1966). Amino acid residues located 30–40 residues either side of the biotin-attachment site have been shown to be crucial for biotinylation (Murtif and Samols, 1987; Cronan, 1990; Reed and Cronan, 1991), whereas the residues adjacent to the biotinylated lysine are important for carboxy transfer, but can be altered without affecting biotinylation (Shenoy et al., 1992).

Work on the expression of fragments of the biotin-carrier subunit of transcarboxylase from *Propionibacterium shermannii* and the biotin carboxy carrier protein (BCCP) from *Escherichia coli* acetyl-CoA carboxylase (ACC) as fusion proteins with β-galactosidase (Cronan, 1990; Li and Cronan, 1992) has shown that the minimum size of the biotin domain necessary for biotinylation is about 75–80 amino acid residues. Biotin-domain peptides from yeast pyruvate carboxylase of a similar size are also biotinylated (A. Chapman-Smith, D. Val and J. C. Wallace, unpublished work), and the recognition of this portion of the domain by the biotin ligase indicates that it folds correctly in the absence of the rest of the protein. These observations are consistent with the view that the region around the biotin-attachment site exists as a relatively isolated and stable domain (Reed and Cronan, 1991).

The biotin domains also show considerable sequence similarity to the lipoyl-attachment regions of pyruvate dehydrogenase (Lim et al., 1988) which undergo an analogous post-translational modification in which the lipoyl cofactor is covalently attached to a specific lysine residue [for review see Perham (1991)]. The three-dimensional solution structure of the lipoyl domain of *Bacillus stearothermophilus* pyruvate dehydrogenase has recently been solved by n.m.r. (Dardel et al., 1993), and on the basis of the sequence homologies, has been used to predict the structure of the biotin domain of yeast pyruvate carboxylase (Brocklehurst and Perham, 1993).

We report here the production of a biotin-domain peptide from the BCCP of *E. coli* ACC which is suitable for n.m.r. structural analysis. The peptide is recognized by biotin ligase *in vivo* and *in vitro* and can be fully biotinylated by coexpression with *E. coli* biotin ligase.

MATERIALS AND METHODS

Bacterial strains

Strain BL21λDE3 was used as the host strain for production of the biotin-domain peptide by the bacteriophage T7 expression system. BL21λDE3 is an *E. coli* B strain (F^- *ompT* r_B^- m_B^-) lysogenized with λDE3, a prophage that expresses the T7 RNA polymerase downstream of the isopropyl β-D-thiogalacto-pyranoside (IPTG)-inducible *lac*UV5 promoter (Studier and Moffat, 1986).

Strain CY943 is a Δ*bio*C::cml *birA1* Tn*10*::*thi-39* derivative of strain MC1061 constructed to avoid the erratic and generally poor transformation of the Barker and Campbell (1981) *birA1* strains. First the Δ*bioC*::cml insertion of Oden et al. (1990) was transduced with phage P_1 into strain MC1061 with selection for chloramphenicol resistance to give strain CY914. The Tn*10*::*thi-*

Abbreviations used: ACC, acetyl-CoA carboxylase (EC 6.4.1.2); BCCP, biotin carboxy carrier protein; IPTG, isopropyl β-D-thiogalactopyranoside.
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39 insertion was transduced into strain BM4096 and tetracycline-resistant *birA1* recombinants were isolated, one of which (called CY912) was the source of a P_1 stock used to transduce strain CY914 to tetracycline resistance. These recombinants were then screened for the *birA1* phenotype to give strain CY918. Finally, a chloramphenicol-sensitive derivative of CY918 was isolated by ampicillin treatment in the presence of chloramphenicol (Faelen et al., 1978) to give strain CY943. Selection for and scoring of *birA* phenotypes was as described by Barker and Campbell (1981).

Nucleic acid manipulations

Transformation, isolation of plasmid DNA, restriction enzyme digestion and agarose-gel electrophoresis were carried out by conventional methods (Maniatis et al., 1982).

Bacterial growth, induction conditions and preparation of cell lysates

Bacterial cultures were grown at 37 °C in shake flasks in 2YT supplemented with 10 μM biotin and 0.5% arabinose where necessary, in the presence of 20 μg/ml chloramphenicol and 100 μg/ml ampicillin for plasmid maintenance. Overnight cultures were diluted 1:10 into fresh medium and grown to A_{600} 0.4–0.5 (usually 30–60 min) before the addition of IPTG to a final concentration of 0.1 mM. Induction was allowed to proceed for 2–4 h by which time the cells had reached an A_{600} of ~ 2.5. The cells were harvested, washed and resuspended in 30 ml of ligase buffer [40 mM sodium phosphate, pH 7.0, 1.5 mM $MgCl_2$, 5% glycerol, 0.2 mM dithioerythritol (Barker and Campbell, 1981)] per litre of culture medium. Cells were disrupted with a French press (82800–103500 kPa) and the cellular debris was removed by either centrifugation for 30 min at 30900 *g* or ultracentrifugation [1 h at 37000 *g* (60000 rev./min) in a Beckman Ti70 rotor]. Lysates were prepared specifically for SDS/PAGE analysis by adding 40 μl of 2% SDS containing 10% 2-mercaptoethanol per absorbance unit (600 nm) to the washed cell pellet.

Protein methods

PAGE was carried out on 12% polyacrylamide gels in the presence and absence of SDS in the Tris/tricine gel system, with detection of protein by Coomassie Blue staining, using the methods of Schagger and von Jagow (1987). Biotinylated proteins were detected by Western blotting as described by Lim et al. (1987) and incorporation of [^{3}H]biotin into trichloroacetate-precipitable material was determined as described by Cronan (1988). The nucleic acid content of protein samples was calculated by a modification of the method of Layne (1957). Unlike yeast enolase, the typical protein on which these calculations are based, BCCP and acyl carrier protein from *E. coli* contain no tryptophan residues and two and one tyrosine residues respectively, and can thus be considered similarly atypical. The acyl carrier protein has an $A_{280nm/260nm}$ ratio of 1.26 (Rock and Cronan, 1980) compared with 1.75 for enolase. The R_F values given by Layne were therefore modified by the ratio 1.26:1.75 to allow a better approximation of the nucleic acid content of protein preparations.

Protein concentrations were determined using the microbiuret assay of Munkres and Richards (1965). N-terminal sequence analysis was performed by automated Edman degradation in an Applied Biosystems 470A Sequenator. Molecular-mass determinations were carried out by electrospray ionization and matrix-assisted laser-desorption time-of-flight m.s. using VG Quattro and VG TofSpec mass spectrometers. Amino acid compositions were determined using the Hewlett–Packard AminoQuant system.

RESULTS AND DISCUSSION

Expression of an 87-residue biotin-domain peptide of BCCP (BCCP-87)

A 1.49 kb *Nco*I–*Sal*I restriction fragment from pTM4E was used as the source of the biotin-domain subgene. This fragment is identical with the corresponding fragment from pLS141 (Li and Cronan, 1992), encodes the C-terminal 87 residues of BCCP and covers the first 1.2 kb of the adjacent biotin carboxylase gene. The *Nco*I–*Sal*I fragment was ligated into the vector pET16b (Novagen), previously digested with *Nco*I and *Xho*I, to form pTM53, thus placing the BCCP-87 subgene under the control of the powerful T7 promoter and ribosome-binding sequences. Plasmids pTM53 and pBA11 [encoding the *E. coli* biotin ligase (Barker and Campbell, 1981)] were transformed into BL21λDE3 with selection for ampicillin and chloramphenicol resistance respectively to give strain TM205. This strain permitted IPTG-inducible expression of the BCCP-87 subgene from pTM53 as well as constitutive expression of the biotin ligase from pBA11. Initial experiments indicated production of a protein of around 10 kDa which was detectable on SDS/PAGE after induction of TM205 with IPTG (Figure 1, lanes 1 and 2). However, Western transfers showed that, after induction, the highly produced protein was not efficiently biotinylated by the ligase constitutively expressed from pBA11, and in fact the leaky production of the biotin-domain peptide observed in the absence of induction apparently saturated the capacity of the ligase *in vivo* (Figure 1, lanes 3 and 4). In order to increase the extent of biotinylation on

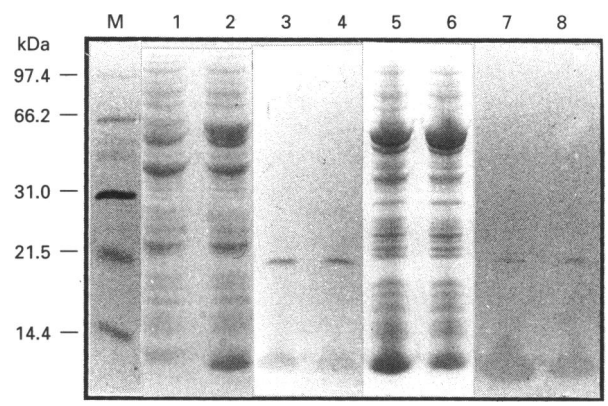

Figure 1 SDS/PAGE and Western-blot analyses of crude lysates

Strains were grown, induced with IPTG and lysates prepared as described in the Materials and methods section. Samples of the lysates (5 μl per lane containing 50 μg of total protein) were electrophoresed on SDS/Tris/tricine 12% polyacrylamide gels, with total protein and biotinylated proteins detected as described in the Materials and methods section. The lanes represent material from cultures grown in parallel and run at the same time on two identical gels, one of which was stained and the other transferred to nitrocellulose, and thus are directly comparable. Lanes 1 and 2, total proteins of strain TM205, containing plasmids pTM53 and pBA11, before and after IPTG induction for 2 h respectively; lanes 3 and 4, as for lanes 1 and 2, showing biotinylated proteins; lanes 5 and 6, total proteins of strain CY949 which contains plasmids pTM53 and pCY216. The strain was grown and induced with IPTG in the presence (lane 5) or absence (lane 6) of arabinose; lanes 7 and 8, biotinylated proteins of strain CY949 grown and induced with IPTG in the presence (lane 7) or absence (lane 8) of arabinose; lane M, biotinylated molecular-mass markers. The biotinylated protein running at ~ 20 kDa is endogenous *E. coli* BCCP.

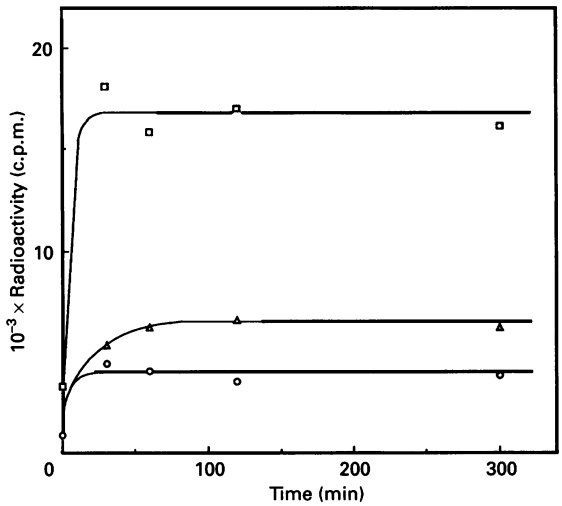

Figure 2 Incorporation of [^{3}H]biotin into trichloroacetic acid-precipitable material in crude lysates

Crude lysates in ligase buffer were prepared from strain CY949 after induction with IPTG for 3 h as described in the Materials and methods section. ATP (final concentration 1.5 mM) and [^{3}H]biotin (specific radioactivity 35 Ci/mmol) at 20 (○), 40 (△) or 100 nM (□) were added and the lysates incubated at 37 °C (Barker and Campbell, 1981). At the times indicated, samples were removed and incorporation of [^{3}H]biotin into trichloroacetate-precipitable material was determined as described in the Materials and methods section.

induction of the protein, we introduced pCY216, an inducible biotin-ligase-overexpression plasmid, in place of pBA11.

Construction of the biotin-ligase-overproduction plasmid pCY216

The source of the *E. coli birA* (biotin ligase) gene was pBA11 (Barker and Campbell, 1981). Plasmid pBA11 was digested with *Bsp*HI and *Bst*YI and the resulting 1.17 kbp fragment was ligated to pARA13 (Cagnon et al., 1991) previously digested with *Nco*I and *Bgl*II. The *Bsp*HI site is within the translational initiation methionine codon of *birA* whereas the *Bst*YI site is the *Bam*HI/*Bgl*II hybrid site formed during construction of pBA11 (Barker and Campbell, 1981). Ligation was followed by transformation of strain CY943 with selection for both ampicillin resistance and growth on 4 nM biotin. These manipulations resulted in plasmid pCY214 in which the *birA* gene was positioned downstream of the *ara* promoter and *araB* ribosome-binding site in the exact location normally occupied by *araB*. In order to construct a plasmid compatible with the pBR322-related replication origins utilized in most expression vectors, we substituted the origin of pACYC184 for that of pCY214. Plasmid pACYC184 was digested with *Bst*YI and ligated to pCY214 cut with *Sca*I and *Sac*I (all sticky ends were made blunt by treatment with T4 DNA polymerase plus the four dNTPs before ligation). The ligation mixture was transformed into strain CY943 with selection for both chloramphenicol resistance and growth on 4 nM biotin. One of these transformants was the source of plasmid pCY216. Plasmid pCY216 is chloramphenicol resistant, ampicillin sensitive and carries the *birA* gene under the control of the very active *araBAD* promoter and the *araC* gene that regulates the promoter.

Biotinylation of BCCP-87

Strain CY949, a BL21λDE3 derivative containing plasmids pCY216 and pTM53, was grown overnight in the presence of 0.5% arabinose to induce production of biotin ligase and then subcultured in the presence of arabinose and induced with IPTG for 3 h as described in the Materials and methods section. Arabinose was omitted from parallel control cultures. Production of the BCCP-87 protein was determined by SDS/PAGE (Figure 1, lanes 5 and 6), and an increase in the extent of biotinylation *in vivo* compared with cells producing ligase from plasmid pBA11 was apparent on Western-blot analysis of the lysates with avidin (Figure 1, lanes 7 and 8). In fact, leaky production of biotin ligase in the absence of arabinose gave higher yields of biotinylated protein than in TM205, with a further increase in the extent of biotinylation observed when the cells contained high levels of ligase before induction of the acceptor protein. However, the ability of lysates to incorporate [^{3}H]biotin into trichloroacetate-precipitable material on addition of ATP (Figure 2) indicated the presence of apoprotein in significant amounts. Incorporation of [^{3}H]biotin was linear with increasing biotin concentration over the range examined. It seems likely that *in vivo* the extreme efficiency of the T7 polymerase expression system results in limitation of the ATP available for the biotin ligase reaction. This means that the apoprotein produced even in the presence of large quantities of ligase can only be biotinylated on the addition of ATP *in vitro*. Indeed, Western-blot analysis indicated that the lysate continued to incorporate biotin up to a biotin concentration of at least 10 μM.

We used the coexpression of acceptor protein and biotin ligase to label the biotin domain with [^{3}H]biotin in crude cell lysates. Labelling provided a ready means to follow the biotinylated protein during purification and greatly facilitated development of a purification procedure. The straightforward and efficient production of labelled protein was also of advantage in subsequent experiments with purified material. The biotinylation procedure required growth and IPTG induction of strain CY949 in the presence of arabinose. Cells were grown, induced with IPTG, harvested and lysed in ligase buffer as described in the Materials and methods section. ATP and [^{3}H]biotin (35 Ci/mmol) were added to 2 ml samples of the resultant crude lysate to give final concentrations of 3 mM and 100 nM respectively, and the reaction mixture was incubated at 37 °C. After 1 h, the concentration of biotin was adjusted to 0.5 mM by addition of unlabelled biotin, and the reaction driven to completion by overnight incubation. Essentially all of the added [^{3}H]biotin could be incorporated into trichloroacetic acid-precipitable material in this way. The remainder of the lysate was incubated overnight at 37 °C with 0.5 mM unlabelled biotin and 3 mM ATP. This procedure resulted in complete biotinylation of the expressed protein, as determined subsequently by m.s. of the purified material (see below).

Purification of BCCP-87

The protocol we have developed is based on the purification of a similar small acidic protein, the acyl carrier protein from *E. coli* (Rock and Cronan, 1980). Crude lysate prepared from a 3-litre culture was incubated as described above to biotinylate expressed BCCP-87, and then extracted with an equal volume of Freon (1,1,2-trichlorotrifluoroethane) to remove lipids present in membrane fragments that clogged the chromatography columns. As this extraction resulted in some loss of BCCP-87, it was omitted in later purifications and the membrane fragments were removed by ultracentrifugation with improved yield. Propan-2-ol was added with mixing to the aqueous phase to a final concentration of 60%. After standing at room temperature for 1 h, the resultant precipitate was collected by centrifugation for 10 min at 12000 *g*. This step removed most of the nucleic acids and all but minor

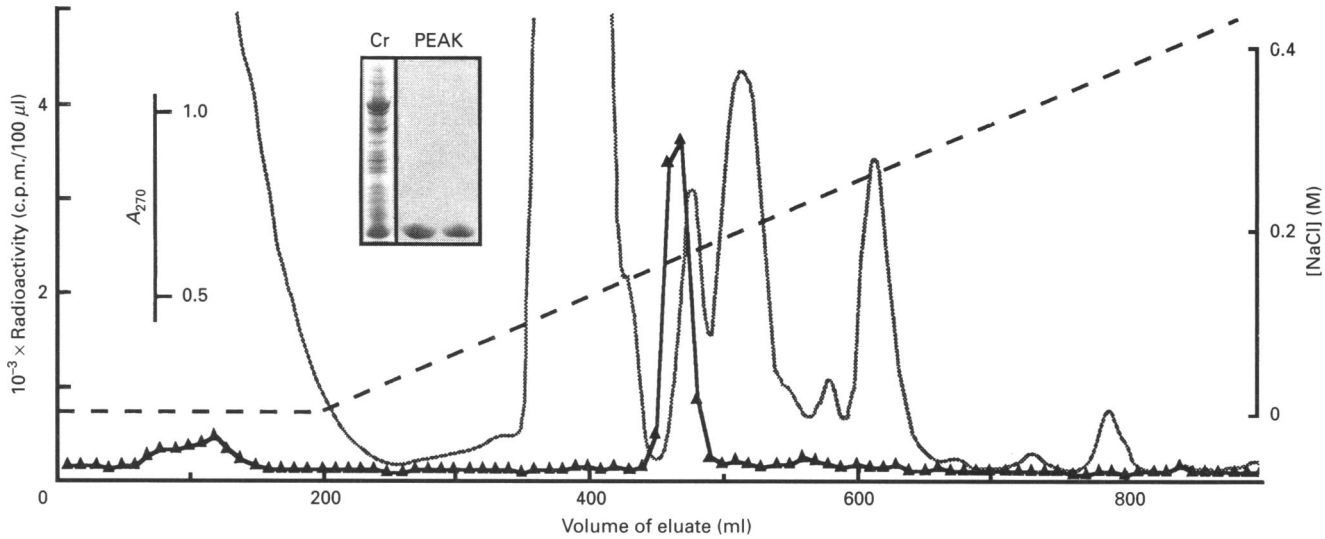

Figure 3 Purification of BCCP-87 by Q-Sepharose chromatography

The 60% propan-2-ol supernatant was chromatographed on Q-Sepharose as described in the text. ▲, Radioactivity; ——, A_{270}; – – – –, [NaCl]. The inset shows SDS/PAGE analysis of the major ^{3}H-containing fractions (PEAK), compared with the crude material before propan-2-ol precipitation (Cr).

amounts of the other cellular proteins. The propan-2-ol supernatant was fractionated by chromatography on a Q-Sepharose column (12 cm × 2.6 cm) with an 800 ml gradient (run at 2.5 ml/min) from 0 to 0.5 M NaCl in 50 mM Mops, pH 7.2, containing 0.1 mM EDTA. BCCP-87 which was eluted at 0.17 M NaCl was detected by the presence of ^{3}H radioactivity (Figure 3). SDS/PAGE showed that the labelled fractions contained a single protein species with a mobility identical with the induced band in the crude lysate (Figure 3, inset), and spectral analysis indicated the presence of nucleic acids coeluted with BCCP-87. Protein concentrations and nucleic acid content were determined in ^{3}H-containing fractions before and after dialysis against 2 mM ammonium acetate. The nucleic acid concentration was reduced from 5–8% to 1–4% during dialysis, indicating that the contaminants were low-molecular-mass nucleic acid species. Column fractions were pooled depending on nucleic acid content and lyophilized. The 3-litre cultures each yielded approximately 35–40 mg of purified material, 75% of which contained less than 2% nucleic acid.

Apoprotein was prepared from strain BL21λDE3 containing only plasmid pTM53. Cells were grown without arabinose, harvested and lysed as described above, except that the overnight ligase reaction was omitted. Purification of apo-BCCP-87 followed the same protocol as for biotinylated BCCP-87 and the protein eluted from Q-Sepharose was detected by the microbiuret assay. The unbiotinylated protein was eluted in the same fraction and contained a similar proportion of nucleic acid to that of the biotinylated protein, both before and after dialysis.

Analysis of purified BCCP-87

Sequence analysis indicated that the first 20 N-terminal residues of the purified BCCP-87 peptide corresponded to the amino acid sequence predicted from the DNA sequence (Li and Cronan, 1992), with the expected retention of the N-terminal methionine (Hirel et al., 1989). The N-terminal sequence of the apoprotein was identical with that of the biotinylated domain. The molecular mass determined by m.s. for the biotinylated form of the peptide was 9558.0 (Figure 4a), a value within 0.05% of the calculated molecular mass of 9560.09. Both these techniques showed that the purity of the sample was 98–99%. No apoprotein was detected in the biotinylated sample, indicating complete biotinylation of expressed acceptor peptide in the *in vitro* biotinylation reaction.

Electrospray m.s. of apoprotein prepared in the absence of the biotin-ligase-overproduction plasmid gave several peaks because of the presence of sodium adduct species (Figure 4b). The analysis indicated a molecular mass for the apoprotein of 9332.3, which is in good agreement with the calculated value of 9333.79. Other peaks revealed the presence of tightly bound Na^+ ions, giving additional molecular-mass species of 9354.4 and 9375.0, i.e. with one and two Na^+ ions bound respectively. The significance of these sodium adduct species is unknown. In addition, the amino acid compositions of both forms of the purified peptide closely matched the composition predicted from the DNA sequence. These data confirm the identity of the expressed and purified biotin-domain peptide as the C-terminal 87 residues of the BCCP subunit of *E. coli* ACC.

Separation of biotinylated and apo forms of BCCP-87

As the long-term aim of this work was to prepare BCCP-87 for n.m.r. structural analysis, we used the specificity of the biotin ligase reaction to demonstrate that the purification protocol produced a protein that remained in native form. In addition, the ability to separate the two forms of BCCP-87 allowed determination of the extent of biotinylation in preparations of the peptide without resorting to m.s.

Two approaches were used to determine conditions under which we could separate the biotinylated and unbiotinylated forms of the peptide, which differ by the single positive charge of the modified lysine. Purified biotinylated peptide and apopeptide were mixed and chromatographed on a Resource Q column (Pharmacia) under conditions similar to those used by Quinn et al. (1993) to separate the lipoylated and unlipoylated domains of human pyruvate dehydrogenase. Two protein peaks

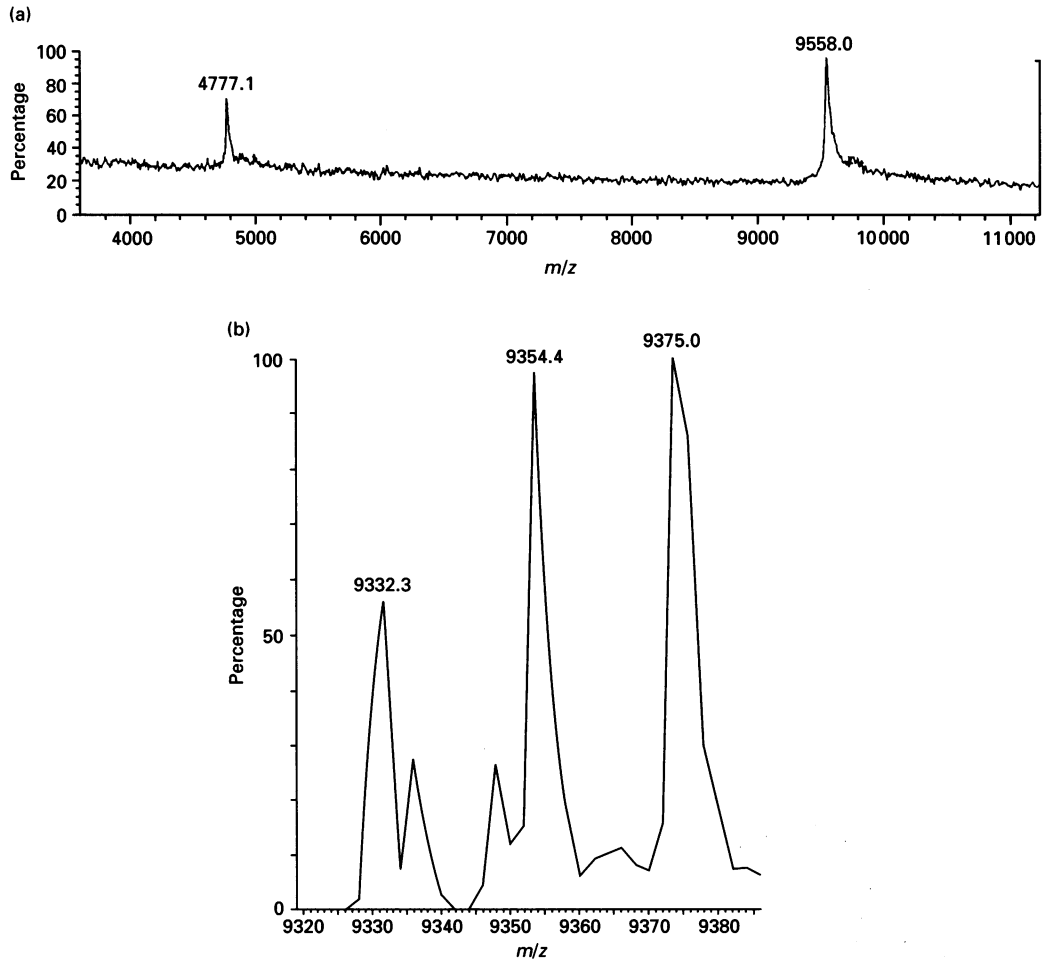

Figure 4 M.s. of purified BCCP-87

(**a**) The molecular mass of the biotinylated peptide (200 fmol), was determined by time-of-flight m.s. (see the Materials and methods section). The two peaks at 9558.0 Da and 4777.1 Da represent the single and double protonated mass forms respectively. (**b**) An enlargement of the relevant region of the electrospray m.s. profile of purified apo-BCCP, showing the apoprotein peak at a molecular mass of 9332.3 Da and sodium adduct species at 9354.4 Da and 9375.0 Da.

were resolved by the ammonium acetate gradient (Figure 5a). The first peak represented the apoprotein, as the microbiuret assay revealed the presence of protein not associated with ^3H radioactivity, whereas the second peak contained ^3H-labelled material and was identified as the biotinylated peptide. The first peak could be converted into the second peak on addition of purified biotin ligase and its substrates, ATP and biotin (Figure 5b), confirming that the two peaks corresponded to the biotinylated and unbiotinylated forms of the domain.

The two forms of the domain could also be clearly resolved when samples of purified apo- and biotinylated peptide were subjected to non-denaturing PAGE (Figure 6). Four bands were detected, two of which were biotinylated as identified by Western-blot analysis. The most negatively charged band was biotinylated, as would be expected from the predicted difference in charge resulting from this modification. The additional bands do not represent aggregated protein, as the purified protein gave a single peak on gel filtration (results not shown). The observation of two species which differ in charge in each form of the domain suggests the presence of deamidation products, because the more rapidly migrating band in each case tended to predominate in samples that had been subjected to heating or extensive handling. However, both forms of the biotinylated protein were clearly distinguishable from those of the apoprotein in this gel system. It seems unlikely that the additional bands are the result of denaturation, as the apoprotein appears to be extremely resistant to denaturation under the conditions used during purification, including 60% propan-2-ol, freeze–thawing and lyophilization, and retained 95% of its capacity to accept biotin after being heated at 65 °C for 1 h. The relative mobility of all four species was quite consistent in different preparations and no more than four bands has been observed. Given that BCCP-87 contains five amide residues (two asparagine and three glutamine), this suggests that the deamidation is specific. In addition, the pattern of mobility of the various species is consistent with deamidation of at least two residues, as the deamidated apoprotein is more negatively charged than the native biotinylated protein (Figure 6).

Prior incubation of apoprotein samples with purified biotin ligase, ATP and biotin resulted in a shift of the major protein species to the more rapidly migrating biotinylated band, and this was dependent on the presence of both ATP and biotin (Figure 6). The observation that both bands in the apoprotein sample could be biotinylated supports the suggestion that the additional

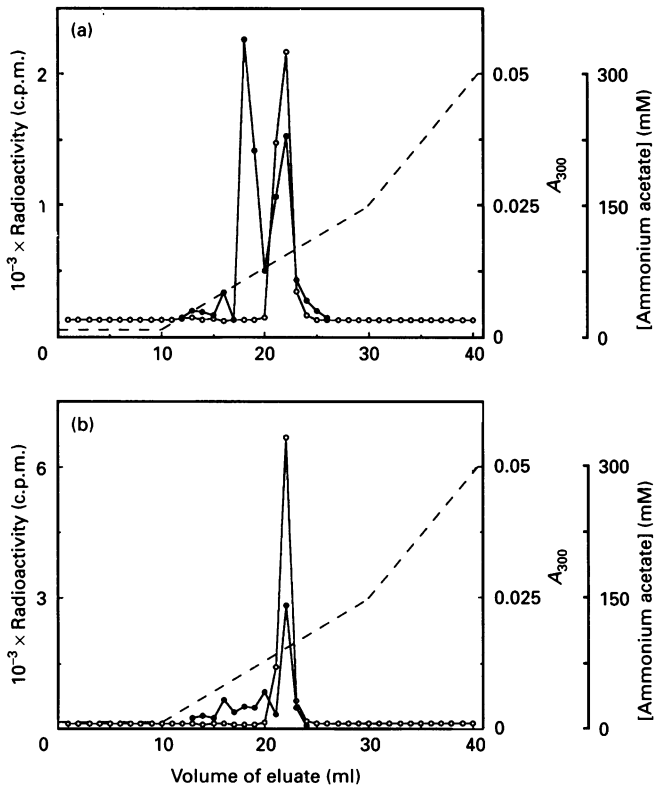

Figure 5 Resource Q anion-exchange chromatography of biotinylated and apo-BCCP-87 preparations

(**a**) Purified apo-BCCP-87 (0.65 mg) and biotinylated BCCP-87 (4.8×10^4 c.p.m.) were mixed and chromatographed on a 1 ml Resource Q anion-exchange column (Pharmacia) equilibrated with 10 mM ammonium acetate, pH 5.0. Elution was with an ammonium acetate gradient (10–300 mM, pH 5.0; – – – –) as indicated on the profile. Protein eluted in the 1 ml fractions collected (●) was determined using the microbiuret assay as described in the Materials and methods section, and the presence of [^{3}H]biotin (○) was detected by liquid-scintillation counting. (**b**) Material eluted in the first peak of the chromatogram shown in (**a**) (fractions 18 and 19) was incubated with purified *E. coli* biotin ligase (0.5 μM) (Abbott and Beckett, 1993) in a solution containing 40 mM sodium phosphate, pH 7.0, 1.5 mM $MgCl_2$, 1.5 mM ATP, 10 mM biotin (specific radioactivity 35 Ci/mmol), 0.2 mM dithiothreitol and 5% glycerol at 37 °C. After 15 min, the biotin concentration was adjusted to 0.4 mM by the addition of unlabelled biotin, and the reaction mixture incubated for a further 45 min (after Barker and Campbell, 1981). After dialysis against 10 mM ammonium acetate, the reaction mixture was rechromatographed on the Resource Q column under the same conditions, with detection of protein (●) and [^{3}H]biotin (○) as described in (**a**).

bands do not represent the denatured form. The two protein peaks eluted from the Resource Q column (Figure 5a) were also subjected to non-denaturing PAGE and the material from the first and second peaks co-migrated with purified apo- and biotinylated samples respectively, with some tailing of apoprotein into the second peak (Figure 6). After incubation of the material from the first peak with purified biotin ligase and the required substrates (Figure 5b), the protein eluted from the Resource Q column co-migrated on non-denaturing PAGE with the biotinylated protein (Figure 6).

Conclusions

The biotin domain from the BCCP of *E. coli* ACC can be isolated readily in amounts (20–30 mg) sufficient for n.m.r. spectroscopy. It is apparent that the apoprotein retains its native conformation during purification, and it is reasonable to suppose that the biotinylated form is similarly robust. This is the first biotin-

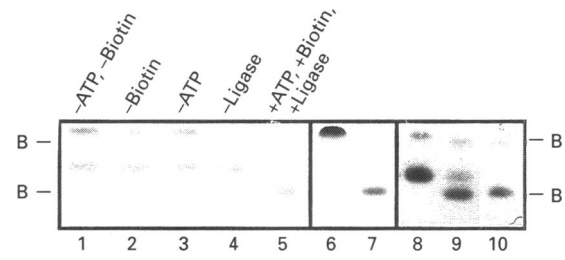

Figure 6 Non-denaturing PAGE of biotinylated and apo-BCCP-87

Purified biotinylated and apopeptide preparations and protein eluted from Resource Q (Figure 5) (3–5 μg per lane) were electrophoresed on non-denaturing 12% polyacrylamide Tris/tricine gels, pH 8.3, with detection of protein and biotinylated protein as described in the Materials and methods section. Mobility is towards the positive electrode. Lanes 1–5, purified apoprotein incubated with biotin ligase before electrophoresis (see legend to Figure 5), with reaction components omitted as indicated; lanes 6 and 7, purified apo- and biotinylated-BCCP-87 preparations respectively; lanes 8 and 9, peaks 1 and 2 eluted from Resource Q in Figure 5(a); lane 10, material eluted from Resource Q after incubation with biotin ligase (Figure 5b). **B**, Bands identified as being biotinylated by Western-blot analysis.

domain protein to be available for structural analysis and will provide important information about the three-dimensional structure of this highly conserved and metabolically significant class of enzymes.

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